Alterations in mRNA and Protein profiles in eIF4E-transfected human lung carcinoma cells

A thesis submitted for the degree of Ph D

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By

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The research work described in this thesis was performed under the supervision of

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2006

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of ..... PhD ..... (insert title of degree for which registered) is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Date: 19/05/06

Acknowledgements

To begin, I'd like to thank Prof Martin Clynes for allowing me the opportunity to conduct my PhD studies in the NICB in DCU He has been very supportive throughout this time and it is very much appreciated

I would like to thank Dr Paula Meleady for supervising my project, helping all-round, and also being supportive

I'd like to give big thanks to Dr Andrew Dowd for all the sterling work he did on the proteomics section of my thesis I couldn't have done it without ya, you know who to call if you're stuck for a drummer Thanks also to Paul, John, Mick and everyone else in the proteomics lab I'll come back with a bigger poster when Liverpool win the Champions League again

Thanks to Finbar for doing confocal work with me, and Joanne for doing thesis corrections

I'd like to thank Eoin Ryan for making some sense of my array data, bioinformatics genius that he is

Also thanks for everybody who helped at various stages with reagents, equipment, advice etc , it all helped

The guys from the differentiation lab, Finbar, Will, Derek, Jason

Everyone in Molecular Biology

All the laydeez down in the Diabetes lab

All the Tox Crew and Immunology too

Special Thanks to my mam coffee buddies Finbar and Paddy, ye helped keep me sane (no small task) Sorry for kicking you in the unmentionables Pad, it was Wills tequila what done it Finbar I'll get you next time

Thanks to all our Indian researchers for the wonderful show they put on and all the delicious munchables provided

Huge thanks to Carol and Yvonne in the office, hope the printer doesn't drive ye insane Maired for getting the orders out Also Joe for keeping the prep room going, much appreciated

Thanks to Niall for being a top class office buddy, and was always very tolerant of me in the dark days when the end was not in sight

All the lads, Don, Rob, Enda, Ado, Al, Ken, Bren, Justin etc , thanks for being around to help me let off steam and not giving me too much abuse about still being a student

Of course none of this would be possible without my parents Michael and Deirdre who have given me so much and to whom I owe just about everything, lots of love My brothers and sisters Declan, Mike, Fiona and Aoife, thanks for putting up with me I'd also like to thank my Grandmother Noeleen who has always been there for me

This thesis is dedicated to Dudley O'Donnell and Michelle Keating

ABBREVIATIONS

ATCC	-	American Tissue Culture Collection
AMP	-	Ampicillin
BrdU	-	Bromodeoxyuridine
cDNA	-	complementary Deoxyribonucleic acid
CCSP	-	Clara Cell Secretory Protein
DEPC	-	Diethyl Pyrocarbonate
DMEM	-	Dulbeccos Modified Eagle Medium
DMSO	-	Dimethyl sulphoxide
ECM	-	Extracellular Matrix
eIF	-	eukaryotic Initiation Factor
4E-BP	-	4E-Binding Protein
FAK	-	Focal Adhesion Kinase
FCS	-	Foetal Calf Serum
GAPDH	-	Glyceraldehyde-3-phosphate dehydrogenase
kDa	-	kiloDaltons
PBS A	-	Phosphate Buffered Saline A
NSCLC	-	Non-Small Cell Lung Carcinoma
SCLC	-	Small Cell Lung Carcinoma
NE	-	Neuroendocrine
RT-PCR	-	Reverse Transcriptase Polymerase Chain Reaction
mTOR	-	mammalian Target of Rapamycin
MMP	-	Matrix Metalloproteinase
SFM	-	Serum-Free Medium
TE	-	Tris EDTA
TBS	-	Tris Buffered Saline
tRNA	-	transfer RNA
ODC	-	Ornithine Decarboxylase
SDS	-	Sodium Dodecyl Sulphate
Sp	-	Surfactant protein
PABP	-	poly(A)-binding protein
PAGE	-	Polyacrylamide Gel Electrophoresis

TGFβ	-	Transforming Growth Factor Beta
RNase	-	Ribonuclease
mRNA	-	Messenger RNA
HSM	-	Hormone Supplemented Media
FGF	-	Fibroblast Growth Factor
RT	-	Room Temperature
UHP	-	Ultra high pure water
UTR	-	Untranslated Region
v/v	-	volume/volume
w/v	-	weight/volume
rpm	-	revolutions per minute

Abstract

Previous work has shown that treatment of the lung cell carcinoma cell line DLKP with the differentiation modulating agent bromodeoxyuridine (BrdU) causes post-transcriptionally regulated changes in the expression of growth and differentiation related genes These changes in gene expression were found to coincide with an increase in the level of expression and phosphorylation of the translation initiation factor eIF-4E In this study we have overexpressed eIF4E in DLKP cells to determine its role in mediating the changes seen in BrdU treatment and what effects it may have on the growth of lung cancer cells in general as studies have shown eIF4E to play a role in regulating gene expression in carcinogenesis We also analysed the overexpression of Ser209 mutated non-phosphorylatable eIF4E in DLKP cells to determine the role of the eIF4E Ser209 (S209) phosphorylation site in regulating translational changes in gene expression and functional changes in DLKP cells The exact role of eIF4E Ser209 phosphorylation in translation initiation is currently unknown and conflicting views have emerged as to whether it is necessary for regulation of translation by eIF4E

Stable transfections were carried out using wild type (4E), S209 mutant (4E-S209) and HA (hemagluttinin) epitope-tagged human eIF4E constructs Stable transfections were also carried using empty pcDNA plasmid vector as a negative control Western blot analysis showed that transfected HA-tagged eIF4E protein was effectively overexpressed in DLKP cells The transfected cells were cloned out by limiting dilution and clones were chosen for further analysis Two clones expressing wild type HA tagged eIF4E, two clones expressing HA-tagged 4E-S209 phosphorylation site mutant and two pcDNA vector transfected control clones have been analysed in this study

An eIF4E overexpressing clone which expresses a high level of transfected protein showed increased keratin 8 expression These cells also showed an increase in β1 integrin expression which was not seen in other eIF4E overexpressing clones indicating high levels of 4E overexpression may induce expression of this protein Immunocytochemical analysis of alpha integrin subunits showed increased expression of alpha 3 integrin in eIF4E overexpressing cells

Invasion assays were performed on eIF4E overexpressing cells as increased 4E expression has been detected in certain cancers The eIF4E overexpressing clone which expresses a high level of transfected protein displayed a large increase in invasiveness compared to control transfected cells whereas other eIF4E transfected clones did not display increased invasiveness eIF4E-S209 mutant transfected cells showed similar levels of invasiveness compared to controls

Large scale analysis of the effects of eIF4E overexpression on protein expression levels was undertaken using the novel 2D-DIGE (2 dimensional-differential in gel analysis) two dimensional electrophoresis technique Differentially expressed proteins were identified using mass-spectrometry based techniques Among the proteins identified were proteins involved in mRNA processing, protein degradation and cytoskeletal regulation Of particular interest, were a number of proteins involved in regulating cytoskeletal dynamics whose expression was down regulated in eIF4E-

S209 mutant overexpressing cells A common regulatory element in the mRNA of these proteins was identified which led to the development of a hypothesis for localised translation of these proteins The possible involvement of eIF4E in regulation of localised translation of these proteins represents a novel aspect of translational regulation by eIF4E which may contribute to its role in oncogenesis Changes seen in the expression of proteins involved in mRNA processing and protein degradation indicate that other post-transcriptional processes apart from translational regulation may play an important role in regulating gene expression in these cells Oligonucleotide microarray analysis of the mRNA expression levels of genes in eIF4E overexpressing cells has also been conducted to determine the effects of eIF4E overexpression on transcriptional regulation downstream of its effects on translation regulation Microarray analysis showed that there are changes in the expression of a large number of genes with diverse cellular functions This indicates changes in transcriptional regulation occur as a result of eIF4E overexpression mRNA expression levels of a large number of genes involved in cytoskeletal regulation were found to be altered in eIF4E and eIF4E-S209 mutant overexpressing cells

Microarray analysis showed that the integrin signalling gene FAK (focal adhesion kinase) was downregulated in both 4E and 4E S209 overexpesssing cells Western blot analysis showed this gene was also downregulated at the protein level Immuno-fluorescent staining of FAK showed the localisation of this protein was altered in eIF4E and eIF4E-S209 mutant overexpressing cells and may affect the growth and invasiveness of these cells

A large number of genes and proteins found to be altered in proteomic and microarray anlysis were involved in regulating actin cytoskeletal dynamics Cells were therefore analysed for expression of actin cytoskeletal structures Major changes were detected in actin cytoskeletal structures in an eIF4E overexpressing clone which expresses a high level of transfected protein

We have therefore conducted an in depth analysis into the regulation of growth and differentiation related gene expression by eIF4E

1.0 Introduction

1.0 Introduction

1.1 Translation

Translation has come to be recognised as an important site of regulation of gene expression, with the initiation stage as the most commonly observed target for physiological control Modulation of initiation can influence both the overall global rate of protein synthesis and also the relative rates of synthesis of different proteins, frequently control at these two levels is superimposed Control of the overall rate of protein synthesis is potentially important in achieving cell growth during the G1 phase of the cell cycle, while the concentrations of an increasing number of specific proteins involved in the control of cell proliferation or differentiation are now thought to be modulated at least in part at the translational level (Pain, 1996)

Two particular steps of the initiation pathway appear to be hot spots for physiological regulation, the binding of Met-tRNA to the 40S ribosomal subunit, mediated by eIF2, and the initial binding of the 43S preinitiation complex to the 5' end of the mRNA, mediated by eIF4E and associated factors The first of these, which precedes mRNA involvement, is mainly, but not exclusively, relevant to regulation of the overall global rate of protein synthesis, whereas the mRNA binding step can, in addition, exert preferential effects on the translation of different mRNAs (Pain, 1996) Two themes which repeatedly surface during investigation of translational regulation are the phosphorylation of initiation factors and also the influence of structural features in the 5' and 3' untranslated regions of mRNA molecules The influence of these two themes may be combined where features of an mRNA molecule render its translation especially sensitive to modulation of the activity of particular initiation factors Several of the initiation factors are phosphoproteins but the clearest links between phosphorylation and the regulation of translation concern the factors eIF2 and eIF4E Regulatory features in mRNA molecules include structures that may act directly (e g by impeding 40S subunit binding or scanning) or indirectly, by providing a binding site for a *trans*-acting protein (Pain, 1996)

1.1.1 Mechanism of translation initiation

Initiation of protein synthesis involves the sequential binding of first the 40S and then the 60S ribosomal subunit to a messenger RNA molecule The process in eukaryotes can be divided into three stages (1) association of initiator tRNA (Met- tRNA) and several initiation factors with the 40S ribosomal subunit to form the 43S preinitiation complex, (2) the binding of this complex to mRNA followed by its migration to the correct AUG initiation codon, and (3) the addition of the 60S ribosomal subunit to assemble an 80S ribosome at the initiation codon, ready to commence translation of the coding sequence This last step requires the prior release of the initiation factors bound to the 40S ribosomal subunit during the earlier stages, these factors are then recycled to catalyse further initiation events (Pain, 1996)

1.1.2 eIF4E

All known RNA polymerase II transcripts are modified cotranscriptionally by addition of an inverted 7-methylguanosine, linked by a 5'-5' triphosphate bridge to the first transcribed residue This group known as the 'cap', functions in splicing, polyadenylation, nuclear export, stability and recognition of mRNA for translation (Lewis and Izaurralde, 1997) In 1978, a 24 kDa cytoplasmic protein was found to cross link specifically to a cap analogue, and was designated the 24 K cap binding protein, later renamed eIF4E eIF4E was able to reverse the inhibitory effect of cap analogue on mRNA translation and so was shown to be directly involved in the initiation stage of translation (McKendrick *et al* , 1999)

The cap plays a critical role in protein synthesis by demarcating the 5' terminus of mRNA, this 'marker' interacts with the initiation factor eIF4E, which, through its interaction with other translation initiation factors recruits the translational machinery to the 5' end of mRNAs

1 1.2 1 eIF4E Structure

The 3D structure of eIF4E resembles a cupped hand or glove concave structure and consists of eight antiparallel β strands, three α helices and ten loop structures The eight antiparallel β strands form a curved β sheet with the three α helices located at the back of this structure providing it with support (Marcotrigiano *et al*, 1997, Tomoo *et al*, 2003) The concave backbone structure provides a scaffold for the mRNA cap recognition pocket consisting of three receiving parts for the 5'-terminal m^7G base, the triphosphate and the second nucleotide The C- and N- terminal regions of the protein are flexible and are important for regulation of eIF4E function The C-terminal flexible region has been shown to function as a receiving pocket for the second nucleotide and also contains the Ser209 regulatory phosphorylation site (Tomoo *et al*, 2003) The N- terminal flexible region is involved in the binding of the 4E-BP repressor proteins and the eIF4G scaffold protein (Tomoo *et al*, 2003, Gross *et al*, 2003)

eIF4E functions in translation initiation as part of a trimeric complex known as eIF4F eIF4F consists of the cap binding protein eIF4E, eIF4A (an RNA helicase), and eIF4G, which serves as a scaffold protein for the assembly of eIF4E and eIF4A The N-terminal third of eIF4G interacts with eIF4E while the C-terminal two thirds contains two separate binding sites for eIF4A and one binding site for eIF3 It is thought that through its interaction with eIF4E, eIF4G functions by bringing the eIF4A helicase activity to the mRNA 5' end to facilitate ribosome binding by unwinding mRNA 5' end secondary structure eIF4G also contains binding sites for the proteins PABP (poly A binding protein) and the Mnk eIF4E kinases PABP is a protein that simultaneously binds to the poly(A) structure at the 3' terminus of an mRNA and also the eIF4G scaffold protein which brings about circularisation of the mRNA and may contribute to increased translational efficiency (Fig 1)
eIF4E is the least abundant of all initiation factors and under most circumstances the availability of eIF4E is considered to be the rate limiting factor in the binding of ribosomes to the mRNA Consequently eIF4E is a major target for regulation

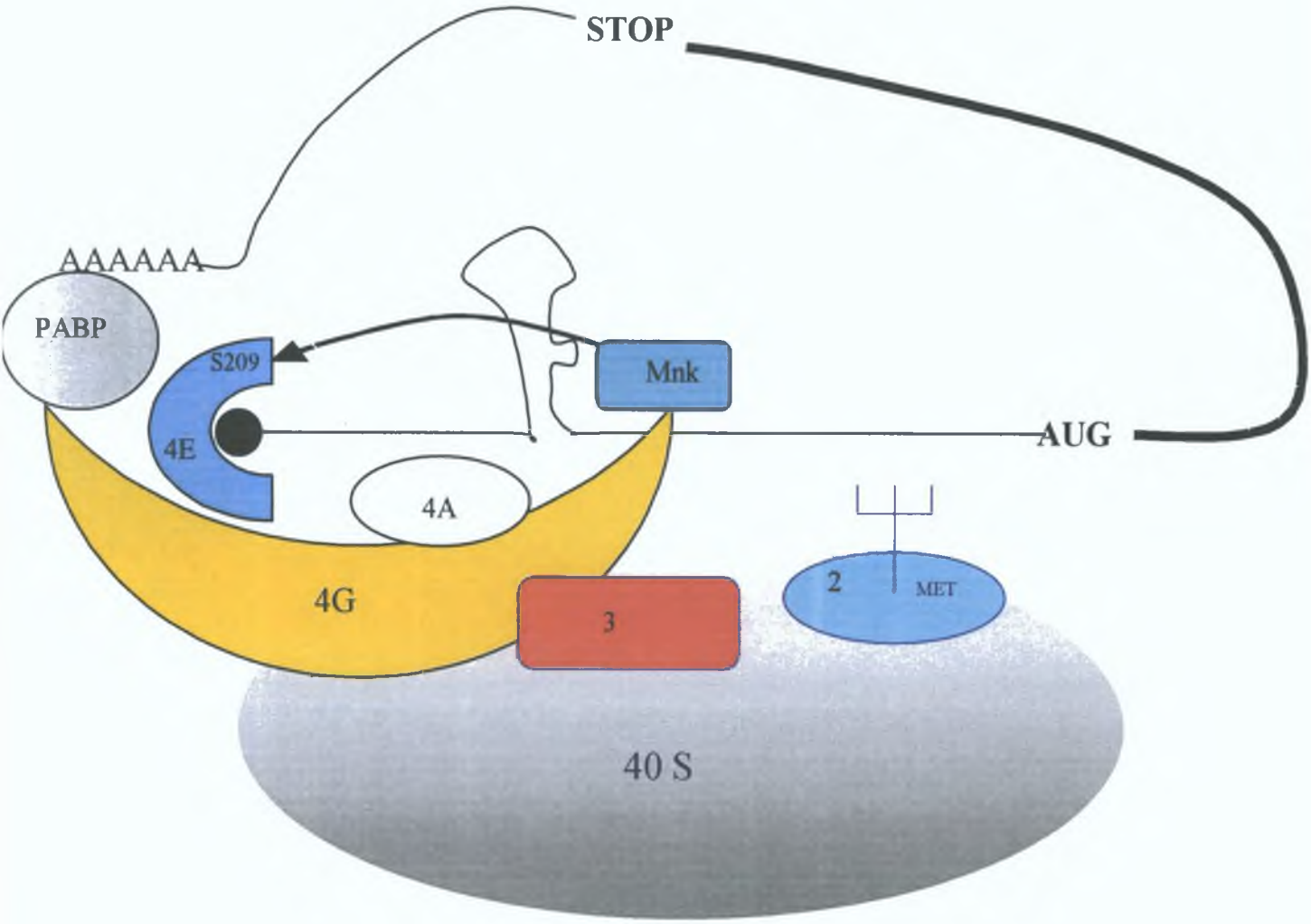


Figure 1. Recruitment of initiation complexes to the 5'-cap structure.
eIF4E binds to the 5'-5' m^7GpppG cap structure (represented by a black dot) at the 5'-end of the messenger RNA. Binding of the scaffold protein eIF4G to the dorsal site of eIF4E allows recruitment of several other factors to the mRNA, e.g., eIF4A, the poly(A)-binding protein (PABP), which binds to the N-terminus of eIF4G, and the Mnks which bind to the C-terminus of eIF4G. A central domain in eIF4G binds eIF3, which brings in the 40S small ribosomal subunit and consequently eIF2 with the initiator methionyl tRNA (Met-$tRNA_i^{Met}$). The helicase activity of eIF4A is thought to be required for unwinding of secondary structures (represented by loop structure above) in the 5'UTR region, allowing subsequent movement of the whole complex along the 5'-UTR, until the initiation codon (AUG) of the open reading frame is recognised by the anticodon of the Met-tRNA. The interaction of the mRNA with PABP through its poly(A)-tail and the binding of PABP to eIF4G circularises the mRNA, a process that is thought to be important for re-initiation of translation or may be required for verification that the mRNA is full length. The open reading frame of the mRNA is shown as a thick line. Initiation factors are abbreviated. The arrow indicates the phosphorylation of eIF4E at Ser209 by the Mnks. The trident structure represents the initiator $tRNA_i^{Met}$.

1 1 2 2 eIF4E Regulation

Recruitment of the translational machinery to mRNA is responsive to a variety of extracellular stimuli, including exposure to hormones, growth factors and cytokines, nutrient availability and various types of cellular stress
eIF4E activity is regulated at multiple levels, (1) via modulation of its transcription, (2) by phosphorylation of the eIF4E protein and (3) through its interaction with a family of translational repressor proteins

1 1 2 2 1 Transcriptional Regulation

Although the mechanism regulating transcription of the eIF4E gene is not completely understood, the eIF4E promoter was shown to contain two *bona fide* myc binding sites, both of which are required for expression of a heterologous reporter gene (Jones *et al*, 1996) Consistent with this observation eIF4E mRNA expression is upregulated in cells overexpressing C-myc and transcription of the eIF4E gene is responsive to activation of a myc-estrogen fusion protein (Rosenwald *et al*, 1993)
Studies have shown that myc protein is an important regulator of cell proliferation and growth (Adhikary and Eilers, 2005) Since eIF4E itself plays a key role in cell growth and proliferation, it is possible that eIF4E is an important downstream target of myc

1 1 2 2 2 eIF4E Phosphorylation Regulation

Another mechanism for regulation of eIF4E is by phosphorylation at its major physiological phosphorylation site, Ser 209 Phosphorylation of eIF4E on Ser 209 is increased following treatment of cells with growth factors, hormones and mitogens (Pyronet *et al*, 1999) These effects appear to be mediated via the MEK/Erk pathway, as they are blocked by inhibitors of MEK (Fig 2) (Flynn and Proud, 1996, Wang *et al*, 1998, Waskiewicz *et al*, 1999) Certain cytokines and stressful conditions also increase the phosphorylation of eIF4E and these effects appear to involve the p38 MAP kinase pathway (Wang *et al*, 1998, Morley and McKendrick, 1997) Although certain other stresses (e g heat shock oxidative stress or osmotic stress) also activate the p38 MAP kinase pathway, they do not increase phosphorylation of eIF4E (Wang *et al*, 1998) This is probably due to the fact that

they cause loss of eIF4F complexes, as a result of dephosphorylation of 4E-BP1, which then sequesters eIF4E (Wang *et al*, 1998, Patel *et al*, 2002), separating it from the Mnk eIF4E kinases bound to eIF4G

Initial reports on the effects of eIF4E phosphorylation claimed phosphorylated eIF4E had a higher binding affinity for the cap structure and this contributed to increased translation initiation (Bu *et al*, 1993, Minich *et al*, 1994)
Analysis of the co-crystal X-ray structure of eIF4E bound to m^7GDP led to a model to account for the reported increased cap affinity engendered by Ser 209 phosphorylation (Marcotrigiano *et al*, 1997) According to the crystal structure it was considered possible that Lys 159 could form a salt bridge with phosphorylated Ser 209, creating a retractable clamp over the mRNA, thus stabilising the interaction between the mRNA 5' end and eIF4E This original crystallographic analysis conducted by Marcotrigiano *et al* (1997) involved analysing eIF4E bound to the cap analog m^7GDP rather than the complete cap structure or a capped oligonucleotide More recent crystallographic studies by Tomoo *et al* (2002, 2003) and Niedzwiecka *et al* (2002) did use larger ligands (m^7GpppA and m^7GpppG) and have higher resolution These studies show that formation of a salt bridge between Lys 159 and phosphorylated Ser 209 is not possible as the distance between these residues is too large Molecular dynamics simulations conducted by Tomoo *et al* (2003) of cap-bound eIF4E led to the suggestion that Ser209 phosphorylation may affect the size of the entrance to the cap binding site and this may be responsible for the effects of eIF4E phosphorylation

The study by Minich *et al* (1994) claiming phosphorylated eIF4E has a higher affinity for the cap structure was conducted before the discovery of the Mnk eIF4E kinases and used chromatography on RNA-Sepharose to separate phosphorylated from unphosphorylated eIF4E The fraction of eIF4E that was not retained on this resin was found to consist only of the phosphorylated form, while the bound material was unphosphorylated Using fluorescence methods, it was found that the fraction containing the phosphorylated eIF4E showed a three to four times higher affinity for cap analogs and for capped (globin) RNA Two important factors which call these results into question are, (a) the basis of the resolution of the phosphorylated and non-

phosphorylated eIF4E forms on RNA-Sepharose is unclear and, (b) it is possible that one or other fraction was contaminated with other proteins that influence the affinity of eIF4E for RNA For example, the 4E-BPs, which greatly increase the binding of eIF4E to cap (Ptushkina *et al* , 1999) were not known at this time and would not have been detected by the methods used in this study

Recent studies using more advanced techniques have reported that phosphorylation of Ser 209 actually decreases the affinity of eIF4E for the cap structure (Scheper *et al* , 2002, Zuberek *et al* , 2003) Scheper *et al* (2002) used highly active preparations of the eIF4E kinase, Mnk2, to produce stoichiometrically phophorylated eIF4E Using fluorescence spectroscopy and surface plasmon resonance techniques, they showed that phosphorylation of eIF4E markedly reduces its affinity for capped RNA, primarily due to an increased rate of dissociation Zuberek *et al* (2003) applied a unique protein engineering technique, Intein mediated protein ligation, to synthesize eIF4E which is selectively phosphorylated at Ser 209 Using synthetic cap analogs, they compared quantitatively the cap affinity for phosphorylated and unphosphorylated eIF4E A 1 5-fold to 4 5-fold reduction in cap affinity for phosphorylated eIF4E was observed The series of the cap analogs used in the study included those with increasing negative charge as a result of phosphate chain elongation and one with increasing number of nucleotides in the RNA chain The decrease in affinity was found to be dependent on the negative charge of the 5'-to-5' phosphate chains as well as the presence of a longer tetraribonucleotide strand

Studies analysing eIF4E phosphorylation in various systems have produced results which would suggest that phosphorylation of eIF4E may not be necessary for translation (McKendrick *et al* , 2001, Morley and Naegele, 2002) and may even have a negative effect on translation (Knauf *et al* , 2001) Lachance *et al* (2002) have analysed the role of eIF4E phosphorylation in *Drosophila* by mutating the equivalent of Ser209 (Ser251) to Alanine, thereby preventing phosphorylation of the protein This mutation caused a retardation of development and reduced size of the adult animals This study strongly indicates a role for phosphorylation of eIF4E in cell and organismal physiology In contrast, a recent study by Ueda *et al* (2004) showed that

in mice lacking Mnk1 and Mnk2, eIF4E phosphorylation is not necessary for normal cell growth and development

The recent developments that have arisen from the study of eIF4E phosphorylation, i e the reduced cap affinity of phosphorylated eIF4E, requires new models to explain the function of eIF4E phosphorylation in the initiation process The accuracy of these models depend on what point in the sequence of events involved in the initiation process eIF4E phosphorylation actually occurs

One possible explanation for the role of eIF4E phosphorylation in the translation initiation process is that eIF4E phosphorylation may occur after the 40S ribosomal subunit has bound to eIF4F through the eIF3- eIF4G interaction Phosphorylation of eIF4E at this point could then cause the release of the 40S ribosomal subunit and associated translation initiation factors from the 5' end of the mRNA They would then scan towards the AUG intitiation codon where upon binding of the 60S ribosomal subunit, they would be released and free to start new rounds of translation initiation

1 1 2 2 2 1 Mnk eIF4E Kinases

The Mnks were identified simultaneously by the work of two independent groups using screens for substrates and binding partners of Erk and p38 MAP kinases (Fukunaga and Hunter, 1997, Waskiewicz *et al*, 1997) Each group identified two related kinases, now termed Mnk1 and Mnk2 They share substantial similarity (88%) in their catalytic domains and their N- and C- termini also share high levels of similarity (respectively 77% and 65%) Both Mnk species interact with eIF4F complexes *in vivo* (Waskiewicz *et al*, 1999, Pyrronet *et al*, 1999, Scheper *et al*, 2001) The Mnk kinases bind to the eIF4F scaffold protein eIF4G to phosphorylate eIF4E (Fig 1) This is consistent with studies that show that 4E is more highly phosphorylated when in the eIF4F complex than when free (Waskiewicz *et al*, 1999, Pyrronet *et al*, 1999)

Mnk1 and Murine Mnk2 can be activated by phosphorylation *in vitro* by Erk or by p38 MAP kinase (Fukunaga and Hunter, 1997, Scheper and Proud, 2002, Waskiewicz *et al*, 1997) although there are important differences in their *in vivo* activities *In vivo*,

Mnk1 displays a low level of activity, which is greatly enhanced by treatment of cells with agents that activate either the Erk or p38 MAP kinase α/β pathway (Fukunaga and Hunter, 1997, Waskiewicz *et al*, 1997, Wang *et al*, 1998) The effects of these treatments are blocked by inhibitors of these pathways (Fig 2) In contrast to Mnk1, Mnk2 has high basal activity which is not further enhanced by agents that activate ERK/p38 MAP kinase (Scheper *et al*, 2001) This high basal activity can be reduced by inhibitors of these pathways, it seems the low basal activity of these pathways in unstimulated cells is sufficient to activate Mnk2 This suggests that Mnk2 may be unusually readily phosphorylated and activated by Erk/p38 MAP kinase and experiments performed *in vitro* bear this out (Scheper *et al*, 2001) The differences in basal activity or regulation of Mnk2 as compared to Mnk1 have important implications for the control of eIF4E phosphorylation In cells that mainly contain Mnk1, the level of eIF4E phosphorylation will be determined by two factors The first is the state of activation of the ERK or p38 MAP kinase pathways, which regulate the activity of Mnk1 The second is the level of eIF4F complexes which bring together eIF4E and the Mnks through their common binding partner, eIF4G The level of eIF4F complexes is determined by factors such as amino acid availability and other stimuli including growth factors and insulin which affect the phosphorylation of the 4E-BP repressor proteins

The high basal activity of Mnk2 is likely to have two important consequences for cellular levels of eIF4E phosphorylation Firstly, this is likely to be relatively high in cells possessing significant levels of these kinases (provided eIF4E is not sequestered from eIF4F complexes by 4E-BPs) Secondly, the primary determinant of eIF4E phosphorylation in cells mainly expressing a Mnk2 isoform will be the level of eIF4F complexes rather than increases in Erk/p38 MAP kinase activity

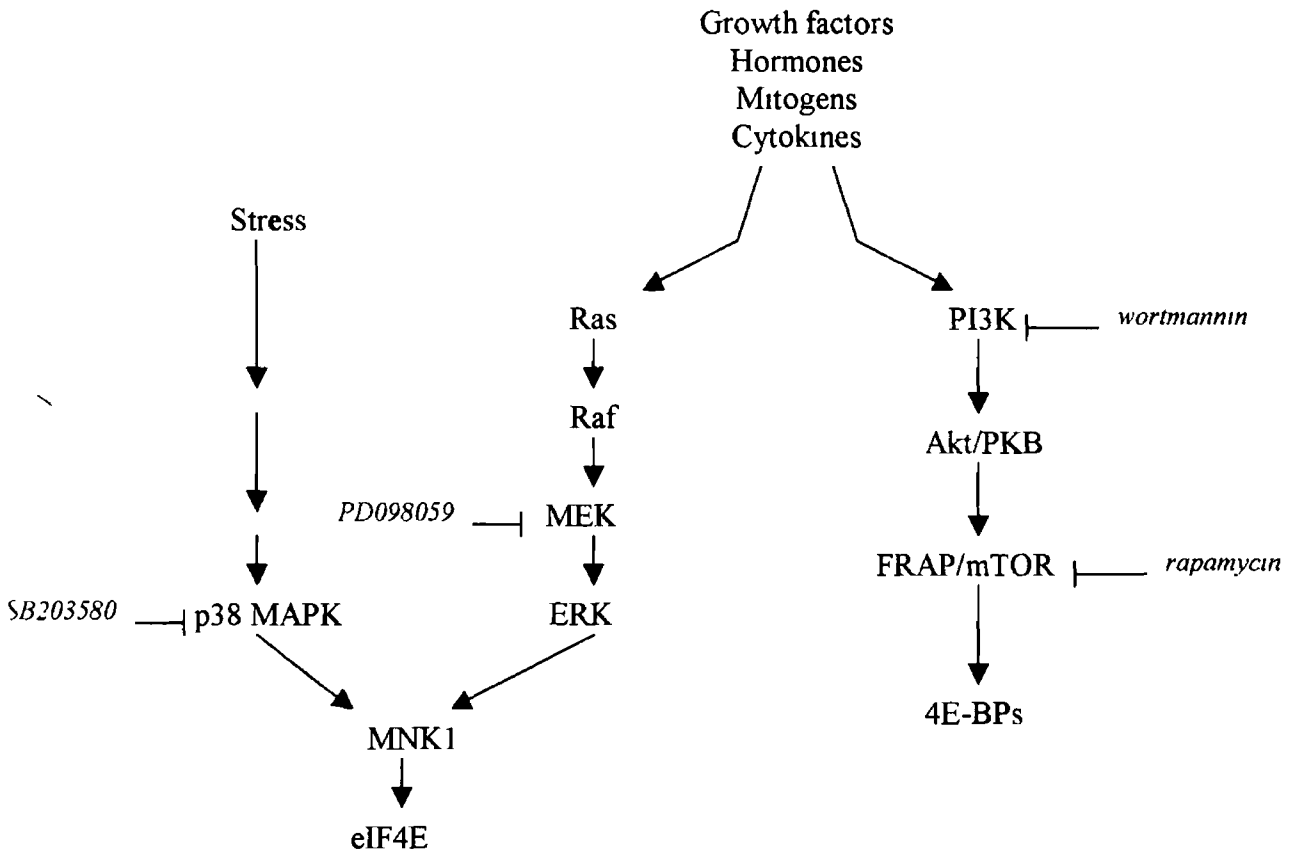


Figure 2 Intracellular signalling pathways involved in the phosphorylation of the translation initiation factors eIF4E and the 4E-BPs The RAS pathway leading to eIF4E phosphorylation and the PI3K pathway leading to 4E-BP phosphorylation are depicted Also shown are the targets of several pharmacalogical inhibitors (italics) utilised in the studies delineating these pathways

1.1 2 2 3 4E Binding Proteins

Using the far Western hybridisation technique, Pause *et al* (1994) isolated cDNAs encoding two small (~12 kDa) proteins which interact with eIF4E and which share 56% overall identity at the ammo acid level These proteins termed 4E-BP1 and 4E-BP2 were demonstrated to inhibit cap dependent translation both in an *in vitro* cell-free translation assay and *in vivo* (Pause *et al*, 1994) Binding of the 4E-BPs to eIF4E prevents the association between eIF4G and eIF4E and thus, the assembly of a functional eIF4F complex

Both eIF4G and the 4E-BPs share a small amino acid motif YXXXXLΦ (where X is any amino acid and Φ is an aliphatic residue), responsible for interaction with eIF4E (Mader *et al*, 1995) Deletion of this sequence or mutation of either the tyrosine or the LΦ residue to alanine abolishes eIF4E binding

1 1 2 2 3 1 Regulation of 4E-BP phosphorylation

The phosphorylation state of specific serine/threonine residues in the 4E-BPs regulates the affinity of these proteins for eIF4E (Fig 3) Hypophosphorylated 4E-BPs bind efficiently to eIF4E but phosphorylation of a critical number of residues in these proteins abrogates this binding Phosphorylation of 4E-BP1 has been shown to occur in an ordered hierarchical manner (Gingras *et al*, 2001)

Hormones (insulin, angiotensin), growth factors, cytokines, mitogens, G coupled receptor ligands and Adenovirus infection have all been reported to induce phosphorylation of 4E-BP1 accompanied by a resultant decrease in its ability to interact with eIF4E Conversely heat shock (in certain cell types) and infection with poliovirus or EMCV have been reported to decrease 4E-BP1 phosphorylation (Raught and Gingras, 1999)

Phosphorylation of 4E-BPs was initially thought to be mediated through MAPK pathways (Pause *et al*, 1994) Subsequent studies have shown that inhibitors which are without effect on MAPK activity (rapamycin, wortmannin and SQ2006) completely prevent 4E-BP1 phosphorylation (Fig 2) (Raught and Gingras, 1999)

The intracellular signalling cascade leading to 4E-BP1 phosphorylation shares many similarities with the pathway leading to p70s6k activation (p70s6k is the protein kinase responsible for the phosphorylation of ribosomal protein S6) Phosphorylation of 4E-BP1 is wortmannin and rapamycin sensitive, is induced by the same agents as p70s6k and its phosphorylation in response to mitogen treatment occurs with similar kinetics 4E-BP1 lies on a pathway containing the phosphotidylinositol 3-OH kinase (PI3K) and its downstream effector the serine/threonine Akt/PKB (Gingras *et al*, 1998) 4E-BP1 phosphorylation is also dependent on the FKBP-rapamycin associated

protein/ mammalian target of rapamycin (FRAP/mTOR) kinase (Fig 2) (Gingras et al, 1998, Brunn *et al*, 1997)

The PI3Ks are a family of lipid kinases responsible for the phosphorylation of the hydroxyl group at position three of the inositol ring of phosphatidylinositols PI3Ks have been implicated in the regulation of many cellular processes, including resistance to apoptosis, cell motility, differentiation and proliferation (Vanhaesebrook *et al*, 1997) Wortmannm inhibits PI3K signalling by binding to its catalytic subunit Overexpression of the PI3Kα subunit (p110α) induces phosphorylation of 4E-BP1 in a wortmannin sensitive manner (Gingras *et al*, 1998)

The Akt/PKB Ser/Thr protein kinases were first identified in 1991 independently by three different groups Two groups identified the approximately 60 kDa kinase as a result of its homology with both protein kinase C (PKC) and protein kinase A (PKA), this gave rise to the name PKB At the same time this kinase was identified as the product of the oncogene v-akt of the acutely transforming retrovirus AKT8 found in a rodent T-cell lymphoma (Marte and Downward, 1997)

Akt is activated by PI3K-generated lipid products, which bind to its pleckstrin homology (PH) domain and target Akt to the plasma membrane The binding of these lipid products (PtdIns(3,4,5)P3) and translocation to the plasma membrane enables the subsequent phosphorylation of T308 (located in the kinase domain of Akt) by the kinase PDK1 PDK1 is a constitutively active kinase that is neither stimulated by insulin nor inhibited by PI3K inhibitors (Downward, 1998) To fully activate Akt, phosphorylation of S473, mediated by PDK2 is also necessary PDK2 is also activated by the lipid products of PI3 kinase After its phosphorylation at both sites, Akt can detach from the membrane and phosphorylate its targets within the cell

A study by Gingras *et al* (1998) has shown that a dominant negative mutant of Akt blocks insulin mediated phosphorylation of 4E-BP1, indicating that Akt is required for *in vivo* phosphorylation of 4E-BP1 This study also showed that an activated Akt induces phosphorylation of 4E-BP1 on the same sites that are phosphorylated upon serum stimulation (Gingras *et al* 1998) Phosphorylation of 4E-BP1 by the activated

form of Akt was wortmannin insensitive but was sensitive to the inhibitor rapamycin suggesting that a rapamycin sensitive kinase(s) acts downstream of Akt to induce phosphorylation of 4E-BP1

FRAP/mTOR is the mammalian homologue of the yeast TOR proteins and the target of the FKBP12-rapamycin complex (an immunophilin-immunosuppressant interaction) This very large (289 kDa) protein is a member of a newly emerging family of kinases termed the PIKs (phosphatidyl inositol kinase-related kinases) (Keith and Schreiber, 1995), some members of this family appear to function instead as protein kinases The role of FRAP/mTOR in mammalian translation initiation was confirmed when it was demonstrated that expression of a rapamycin resistant FRAP/mTOR protein confers rapamycin resistance to 4E-BP1 phosphorylation (Brunn *et al*, 1997)

Initially it was not known whether FRAP/mTOR protein was directly regulated by Akt/PKB or if it lied on a parallel signalling pathway Studies have now shown mTOR to be directly phosphorylated by Akt (Nave *et al*, 1999) Akt was shown to phosphorylate mTOR at Ser2448 *in vitro* These increases closely paralleled previously described increases in activity of mTOR by insulin and PKB (Nave *et al*, 1999)

Studies have shown that mTOR plays a critical role in the phosphorylation of 4E-BP1 Inhibition of mTOR activity in insulin treated cells through treatment with rapamycin attenuates 4E-BP1 phosphorylation Overexpression of mTOR increases 4E-BP1 phosphorylation in cells (Raught and Gingras, 1999) mTOR is also known to mediate 4E-BP1 phosphorylation in response to levels of nutrients in cells (Proud, 2002)

The exact mechanism by which mTOR acts on 4E-BP1 is unclear Direct phosphorylation of 4E-BP1 by mTOR or indirect regulation of phosphorylation by an mTOR regulated kinase or phosphatase has been suggested (Gingras *et al*, 2001) New insights have emerged regarding the biochemical mechanism by which mTOR signaling is regulated The regulatory associated protein of mTOR, raptor, has been identified as novel mTOR interacting protein and regulator (Kim *et al*, 2002, Hara *et al*, 2002) Raptor association with mTOR is required for efficient phosphorylation of

mTOR (Kim *et al*, 2002, Hara *et al*, 2002) and has been suggested to function as a scaffold protein that brings mTOR in close proximity to it's substrates (Hara *et al*, 2002) In addition, raptor has also been suggested to function as a bidirectional regulator of mTOR, inhibiting mTOR under nutrient starvation conditions and activating it when adequate supplies of amino acid and carbohydrates are available (Kim *et al*, 2002) A TOR signalling (TOS) motif present in the extreme C terminus of 4E-BP1 has been found to be essential for phosphorylation at mTOR regulated sites (Schalm *et al*, 2003) This same motif is also necessary for binding of raptor, indicating it's importance for regulating 4E-BP1 (Choi *et al*, 2003, Schalm *et al*, 2003, Beugnet *et al*, 2003) Another motif at the N terminus of 4E-BP1 known as the RAIP motif has also been found to play an important role in phosphorylation of 4E-BP1 by mTOR but this is considered to be independent of the 4E-BP1/raptor interaction and has led to the suggestion that other as yet unidentified co-factors are involved in 4E-BP phosphorylation (Beugnet *et al*, 2003)

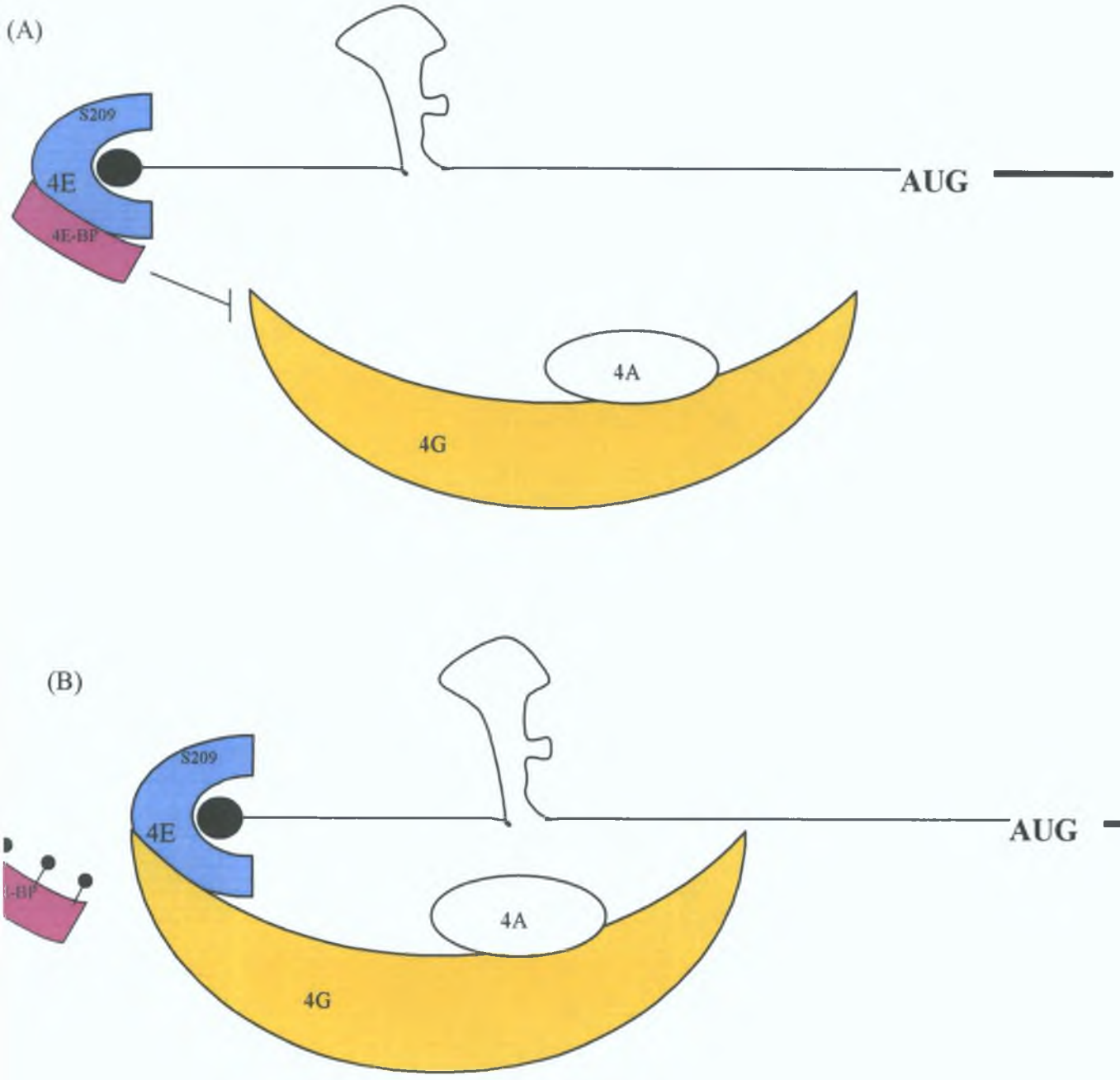


Figure 3. (A) 4E-BP bound to eIF4E prevents eIF4E binding to eIF4G and eIF4F complex formation, **(B)** Phosphorylation of 4E-BP causes release of 4E-BP from eIF4E allowing eIF4F complex formation

1.1.3 Eukaryotic initiation factor eIF2

eIF2 plays a central role in the maintenance of what is generally considered a rate limiting step in mRNA translation

eIF2 is a multimeric protein consisting of three dissimilar subunits termed α, β and γ, in order of increasing molecular mass The eIF2α amino acid sequence is unremarkable with the exception of Ser51, which is a phosphate acceptor for three protein kinases haem regulated inhibitor (HRI), double stranded RNA activated protein kinase (PKR), and the nutrient-regulated protein kinase (GCN2) Yeast eIF2α contains additional kinase sites which are not conserved in the mammalian protein The eIF2β peptide contains the binding sites for eIF5 and eIF2B eIF2γ is thought to be the primary guanine nucleotide binding site

1 1 3 1 Function and regulation

The most well defined function of eIF2 is to recruit the initiator tRNA and conduct it as a Met-tRNA-eIF2-GTP ternary complex to the 43S ribosomal subunit Following the association of mRNA with the 40S subunit and location of the subunit at the AUG start codon, eIF5 binds to eIF2 and stimulates the hydrolysis of eIF2-bound GTP It has been proposed that either eIF5 or the β- or γ-subunits of eIF2 contains the GTPase actvity responsible for GTP hydrolysis

Following GTP hydrolysis the eIF2-GDP complex is released from the ribosome Although the release mechanism has not been delineated, eIF2 has been found associated with the 60S ribosomal subunit and it has been suggested that transfer to the 60S subunit is important in eIF2 recycling (Chakrabarti and Maitra, 1992)

Prior to binding Met-tRNA , the GDP bound to eIF2 must be exchanged for GTP, a reaction mediated by eIF2B (Webb and Proud, 1997) The best characterised mechanism for regulating eIF2B activity involves phosphorylation of eIF2α (Pain, 1996) Phosphorylation of eIF2α on Ser51 converts eIF2 from a substrate to a competitive inhibitor of eIF2B, the phosphorylated form of eIF2α having a much

higher affinity (about 150-fold) than the unphosphorylated form Although eIF2B is able to recycle GDP for GTP when eIF2α is phosphorylated, eIF2B is not released after the exchange Because eIF2B is present in limiting amounts compared with eIF2, it is potentially a major regulator of overall rates of translation initiation (Kleijn *et al* , 1998)

Three protein kinases that specifically phosphorylate eIF2α at Ser51 have been cloned and sequenced

(A) The haem controlled repressor or haem-regulated inhibitor (HCR or HRI), which co-ordinates globin synthesis to haem avilability in reticulocytes

(B) In mammalian cells, the double stranded RNA-activated kinase PKR, is important in the defence of mammalian cell populations against viral invasion It is markedly induced by transcriptional activation in response to interferons α or β released by neighbouring cells Upon subsequent viral infection, the kinase is activated and severely inhibits translation by increasing eIF2α phosphorylation This prevents the utilisation of the translational apparatus for the production of viral proteins and hence restricts viral replication within cells

The primary structure of human PKR deduced from its cDNA sequence shows a 551-amino acid protein consisting of an amino-terminal regulatory domain and a carboxy terminal catalytic domain Binding of double stranded RNA activates PKR which undergoes a conformational change, leading to autophosphorylation and the formation of homodimers

Although PKR plays an important role in mediating the antiviral effects of interferons, PKR is also implicated in regulating cell proliferation in uninfected cells and may have a tumour suppressor function under normal conditions Studies of human malignancies and tumour cell lines suggest that, in general, patients bearing tumours with a higher PKR content have a more favorable prognosis (Pain, 1996, Jagus *et al* , 1999)

(C)The third kinase, GCN2, present in the yeast *Saccharomyces cerevisiae*, regulates GCN4 transcription factor, mRNA translation

1 1 3 1 1 eIF2B

eIF2B is a complex multimeric protein consisting of five dissimilar subunits named α, β, γ, δ and ε, in order of increasing molecular mass This feature sets eIF2B apart from the vast number of guanine nucleotide exchange factors (GEFs), which are mostly monomeric proteins

Genetic studies in yeast suggest a model where eIF2B can be functionally divided into catalytic (ε,γ) and regulatory (α, β, δ) subdomains The ε-subunit has been shown to contain the GEF activity although full activity requires the other subunits The ε-subunit also interacts with the β subunit of eIF2 and is important for the formation of the eIF2B holoprotein The regulatory domain responds to eIF2α phosphorylation

The ε-subunit has also been shown to be phosphorylated by GSK-3, which causes inhibition of eIF2B activity GSK-3 is inactivated by insulin in a PI 3- kinase dependent manner

1.1.4 Mechanisms of Translational Control

As mentioned earlier, translation is now recognised as an important site of regulation of gene expression Regulation at the translational level enables cells to respond rapidly to external stimuli Mitogenic stimulation of many cell types causes a 2-fold to 3-fold increase in the general rate of protein synthesis as well as an additional selective increase in the translation of a subset of translationally repressed mRNAs (Willis, 1999) Included in this group of mRNAs are many of those involved in the control of cell growth, which often encode growth factors and proto-oncogenes The 5'UTRs of approximately 90% of vertebrate mRNAs examined to date are between 10 and 200 bases long, yet two thirds of the mRNAs known to encode protoncogenes or factors related to cell proliferation contain atypical 5'UTRs which are more than 200 bases long and/or contain more than one AUG codon (Fig 4)

Theories have now developed stating that the size of an mRNAs 5'UTR and the presence of certain features within the 5'UTR have the ability to cause an mRNA to be poorly translated or "weak" under normal conditions The expression of these weak mRNAs can be significantly increased by upregulation of translation A large proportion of these weak mRNAs appear to code for proteins involved in cellular proliferation and differentiation

Features of mRNAs that are poorly translated under normal conditions (e g cells in the resting state) and whose translation may be upregulated under other conditions (e g after growth induction) comprise one or more of the following (i) long and highly structured 5'UTRs, (ii) additional upstream initiation codons, (iii) upstream open reading frames (uORFs) or (iv) internal ribosome entry sites (IRESs) (Clemems and Bommer, 1999)

(i) Long and highly structured 5'UTRs

The 5'UTRs of the majority of cellular mRNAs are up to 100 nucleotides long However, there is a small group of mRNAs which bear a considerably longer 5'UTR, and the majority of mRNAs coding for growth related proteins belong to this group (Kozak 1987, 1991) These 5'UTRs are often GC rich (70-90%) which is indicative of a high degree of secondary structure and in many cases secondary structure predictions reveal the potential to form extended stem-loop structures either within

the 5'UTRs or larger parts of the molecule The secondary structure seems to be inhibitory for the scanning of the mRNA from the cap to the AUG initiation codon, and such mRNAs are particularly dependent for their translation on the activity of the cap dependent unwinding machinery They are therefore good candidates for mRNAs for which translation is specifically upregulated through activation of components of initiation factor eIF4F

(ii) Additional initiation codons within the 5'UTR

Apart from extended areas of secondary structure, mRNAs with long leader sequences may contain additional upstream initiation codons Examples are predominantly mRNAs coding for growth factors and proto-oncogene products, e g FGF-2 or c-myc The additional initiation codons are often non-AUG codons, with CUG being the most common one and they may give rise to slightly larger protein products with specific cellular functions or localisation The usage of the upstream codon versus the normal AUG codon varies considerably between different mRNAs and depends on the cellular conditions (Touriol *et al* , 2003)

(iii) Upstream open reading frames

Some mRNAs with long leader sequences contain one or more upstream open reading frames within their 5'UTRs, and these uORFs are often inhibitory for the translation of the downstream coding region (Willis, 1999)

(iv) Internal ribosome entry sites

Another feature of mRNAs containing long 5'UTRs is the presence of internal ribosome entry sites (IRESes) Internal initiation was originally identified in the case of picornavirus RNAs, which bear extremely long and structured 5'UTRs, where it seemed unlikely that the scanning and unwinding machinery works all the way from the 5'-end to the AUG start codon IRESes usually comprise highly structured areas within the 5'UTRs and often feature a polypyrimidine tract near the 3' terminal end The mRNAs of certain cellular growth promoting genes have been found to contain IRESes, for example FGF-2, PDGF and c-myc mRNAs The IRESes of cellular mRNAs seem to differ from those of picornaviral RNAs (Clemems and Bommer, 1999)

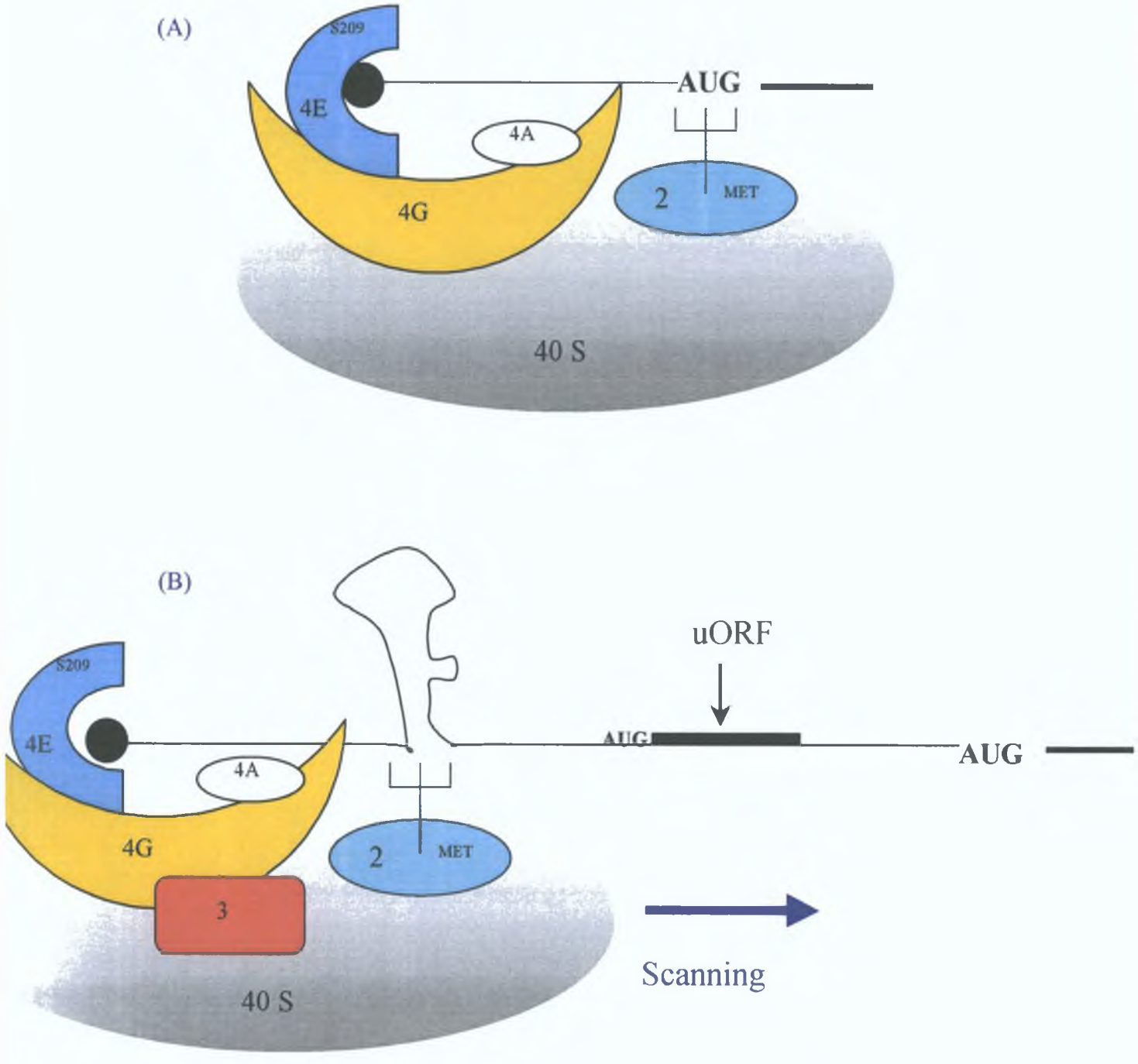


Figure 4. Translation intitation on (A) Short non structured 5'UTR mRNA and (B) Long structured 5'UTR mRNA containing an upstream open reading frame (uORF).

1.1.5 Translational control of growth and differentiation

There is now a growing body of evidence to suggest that translational control of protein expression plays an important role in various cellular processes such as transformation, growth and differentiation The major site of translational regulation is initiation and translation initiation factors, mainly eIF4E, have been shown to be involved in these processes

1 1 5 1 eIF4E and Cancer

eIF4E overexpression studies in cell lines have shown that overexpression of eIF4E has the ability to cause transformation and growth deregulation of cells (De Beneddetti and Rhoads, 1990, De Beneddetti *et al*, 1994) It has also been shown that reducing the level of eIF4E with antisense RNA inhibits the oncogenic and metastatic properties of several oncogenic cell lines (Rinker-Schaeffer *et al*, 1993, Graffe *et al*, 1995)

Analysis of protein synthesis in CHO cells overexpressing eIF4E revealed that whereas the synthesis of most proteins increased only moderately (from 0 to 10%) the synthesis of several polypeptides was increased greatly (De Benedetti, 1994) This is in agreement with the theory of mRNA competition for translation that would predict a small increase in the translation of "strong" mRNAs, but a much greater effect on the translation of "weak" and less abundant transcripts Overexpression of eIF4E in CHO cells and also a rat cell line (CREF-4E) in this study showed a greater than average increase in the expression of the proto-oncogene c-Myc

eIF4E has been shown to regulate other mRNAs important for malignant transformation including, Ornithine Decarboxylase (ODC), a key enzyme in polyamine synthesis and Cyclin D1, a protein kinase regulator involved in the control of the G1 to S-phase transition

In the case of ODC, it has been shown that ODC levels are drastically increased in eIF4E transformed cells ODC overexpression itself results in a transformed phenotype and its expression can be suppressed in RAS transformed cells by depleting eIF4E (Graffe *et al*, 1997) The transformed phenotype of eIF4E overexpressing cells can be suppressed by expressing a dominant negative mutant of ODC or by treatment with an inhibitor of ODC (Shantz and Pegg, 1999)

Translational repression of cyclin D1 was shown to be relieved in NIH-3T3 cells overexpressing eIF4E This effect is now known to be caused by increased nucleo-cytoplasmic transport of the cyclin D1 mRNA which is regulated by eIF4E (Strudwick and Borden, 2002)

Also upregulated by overexpression of eIF4E are FGF-2 and VEGF, two important regulators of angiogenesis in cancer Expression of FGF-2 and VEGF were found to be elevated in breast cancer, which correlates with elevated eIF4E levels (Clemens and Bommer, 1999, DeBenedetti and Harris, 1999)

1 1 5 2 Translation Factors in Lung Cancer

Although translation factors have been shown to be upregulated in a wide range of cancers and the overexpression of translation factors in cells has been shown to induce cellular transformation (Clemens and Bommer, 1999, De Benedetti and Harris, 1999, Watkins and Norbury, 2002), the role of translational factors in lung cancers is relatively unknown

A study of the expression of a large number of genes at the protein and mRNA level in lung adenocarcinomas showed a poor correlation between mRNA and protein levels for the majority of genes analysed (Chen *et al*, 2002) This shows that post-transcriptional regulation can play a major role in gene expression in lung cancer although translational regulation may only partially account for the poor correlation between mRNA and protein levels in this study A study of growth related gene expression in proliferating and non-proliferating rat lung epithelial cells in primary culture showed evidence for growth-dependent translational control of certain genes (Clement *et al*, 1990)

eIF4E expression has been shown to be elevated in adenocarcinomas of the human peripheral lung and in bronchioalveolar carcinoma where higher eIF4E levels were found to correlate with more invasive subtypes (Seki *et al*, 2002) Levels of eIF4E and eIF2α have been shown to be increased frequently in bronchioalveolar but not in squamous cell carcinoma of the lung (Rosenwald *et al*, 2001)

eIF4G is overexpressed in squamous cell lung carcinoma (Bauer *et al*, 2001, Bauer *et al*, 2002) It is possible eIF4G overexpression may function to increase the amount of

the translation initiation complex eIF4F, which in turn may result in the translational activation of the same target mRNAs as seen in eIF4E overexpressing cells.

Other translation factors showing increased expression in lung cancers include the p170 subunit of eIF3 which was shown to be upregulated in lung cancer tissue (Pincheira *et al.*, 2001) and also eIF5A expression in lung adenocarcinomas (Chen *et al.*, 2003). The roles of these proteins in translation are not very well known but it is likely their overexpression may affect translational control.

1.1.5.3 eIF4E control of Differentiation

A role for eIF4E in embryogenesis and differentiation was suggested by experiments that showed injection of eIF4E into *Xenopus* embryos leads to the induction of mesoderm in ectodermal explants. eIF4E injection also induced mesodermal differentiation in explants normally differentiating towards ectodermal tissues. eIF4E injection in *Xenopus* oocytes resulted in preferential translation of *Xenopus* activin, a mesoderm inducing member of the TGF-β superfamily, whereas total protein synthesis was unaffected (van der Welden and Thomas, 1999).

1.1.5.3.1 Translational Control in Lung Differentiation

Although there are few direct reports of translational regulation in lung development, proteins which have been reported to be translationally regulated such as TGFβ1 and FGF-2 are known to play roles in lung development and differentiation (Warburton *et al.*, 2000). The transcription factors C/EBPα and C/EBPβ are also known to be translationally regulated and have been shown to be involved in the regulation of differentiation specific genes (e.g. Surfactant proteins, CCSP) in lung cells (Calkhoven *et al.*, 2000; Cassel and Nord, 2003).
There is now strong evidence to show translational regulation plays a role in the proliferation and differentiation of cells. Translational regulation in lung development and differentiation should prove to be an interesting research area for the future.

1.2 Lung epithelial development and organisation

Lung development in mammals occurs as an outgrowth of the embryonic gut In humans, it originates from the ventral wall of the primitive oesophagus between 4 and 5 weeks of gestation (Bishop, 2004) From then on, the endoderm/epithelium undergoes dichotomous branching into the surrounding mesenchyme This highly ordered process of repeated bud outgrowth and division of terminal units is known as branching morphogenesis and gives rise to the pulmonary tree and defines the proximal-distal axis of the lung The development of mammalian lung is divided into four phases and in humans the timing of these phases are embryonic 0-5 weeks, glandular 5-16 weeks, canalicular 16-26 weeks and saccular 26 weeks to term The primordial lining that forms in the embryonic stage develops into pseudostratified epithelium during the early glandular phase and as branching progresses, columnar epithelium forms During the glandular and into the canalicular phase, the initial thick layer of stratified epithelial cells starts to thin and shows gradation, becoming thinner along the length of the tree Submucosal glands first appear at around 10 weeks in the trachea but not until 16 weeks in the bronchi Bronchioles appear during the canalicular stage, marking the start of the formation of gas exchange units A lumen is present at this stage and the epithelium starts to look more cuboidal, an appearance that remains into the final saccular phase where alveolar ducts and air sacs begin to open The final formation of alveoli takes place post-natally Thus the mature lung has distinct anatomical regions lined by different types of epithelial cells In the mature lung, the trachea and major bronchi are lined by pseudostratified epithelium The major phenotypes in the proximal airways are ciliated and mucous secretory (or goblet) cells, with the more infrequent neuroendocrine cells and the less well differentiated basal cell lying in a basal position The bronchioles are also lined by ciliated cells, but possess a separate phenotype known as the Clara cells that are non ciliated The alveoli are lined by flattened squamous (type I pneumocytes) and cuboidal (type II pneomocytes) cells (Bishop, 2004)

Neuroendocrine cells first appear in the lung at around 8 weeks of gestation They contain biogenis amines, commonly serotonin and/or peptides, including bombesin and calcitonin gene related peptide They are relatively frequent in the developing

lung where they play a major role in airway growth and development but form >1% of epithelial cells in adult lung, where they are seen as scattered elements in the epithelium or in innervated epithelial corpuscles, so called neouroepithelial bodies (Bishop, 2004)

1 2 1 Lung Stem Cells

The average normal adult lung contains approximately $70m^2$ of gas diffusion surface The lining in many areas consists of only a monolayer of cells and can be as thin as 0 1μm in the case of alveolar type I pneumocytes (Bishop, 2004) The epithelial surface of the lung is constantly open to potential injury and therefore, in order to maintain the protective lining, rapid response mechanisms are in place that lead to epithelial renewal Stem cell and progenitor cells play a key role in mediating these response mechanisms Although there is considerable debate over the exact nature of stem cells, a generally accepted definition is that they are clonogenic cells that are capable of self renewal and multi lineage differentiation This stable population of undifferentiated cells gives rise to progenitors that have little or no capacity to self renew and that show signs of differentiation In lung, there are various types of stem cells, differing according their position within the pulmonary tree, and that they often form pools, ready to proliferate in response to injury and effect local repair However, recent studies have indicated that blood-borne cells acquired via the circulation may play a role in lung epithelial repair in response to injury (Bishop, 2004, Fine, 2004)

1 2 1 1 Stem Cell Niches

1 2 1 1 1 Proximal airways

In the trachea and bronchi, basal cells are widely believed to be stem cells The basal cells and the parabasal cells that he just above them, form a pluripotential reserve that, unlike the surrounding epithelium usually survives injury The basal cells appear at around 10 weeks of gestation in the human trachea, are roughly triangular and lie under the columnar epithelial layer, with one edge anchoring the epithelium to the basement membrane Recent studies involving labelling of tracheal epithelial basal cells in mice have shown these cells have the ability to form large colonies in *in-vitro*

assays, can engage in multipotent differentiation and have the capacity to fully restore a fully differentiated epithelium following lung injury (Hong *et al*, 2004, Schoch *et al*, 2004)

1.2 1 1 2 Bronchioles

Recent research has shown that subsets of Clara cells fulfil the criteria of adult niche specific stem cells Pools of stem cells have been discovered that express Clara Cell Specific Protein (CCSP) but are not typical Clara cells as they are resistant to airway pollutants such as naphthalene (Giangreco *et al*, 2002, Hong *et al*, 2001) Generally, Clara cells are enriched with cytochrome P-450 enzymes that would make them vulnerable to naphthalene In addition, these variant CCSP-expressing (or vCE) cells show multipotent differentiation The vCE cells are located in neuroepithelial bodies and at the broncho-alveolar duct junction (Giangreco *et al*, 2002, Hong *et al*, 2001) Neuroedocrine cells are not considered themselves to be stem cells but are thought to closely interact with vCE progenitor cells in neuroepithelial bodies and play a role in epithelial regeneration (Reynolds *et al*, 2000) Cells have been identified within neuroepithelial bodies that express markers of both CCSP and the neuroendocrine cell marker CGRP (Reynolds *et al*, 2000) indicating these cells are closely related and may derive from a common progenitor

1.2 1 1 3 Alveoli

Type II pneumocytes have been shown to restore the alveolar epithelium following generalized damage by oxidants e g oxygen and nitrogen dioxide, by giving rise to either new type II cells or the squamous type I pneumocyte, the latter being destroyed by most types of lung injury (Bishop, 2004)

1.3 Integrins

The integrin superfamily consists of a major class of transmembrane glycoproteins that mediate cell-matrix and cell-cell adhesion Extracellular matrix molecules serve as ligands for the integrins and are crucial for the orderly development of tissues during morphogenesis, maintenance of adult tissue, wound healing and oncogenesis (Mizejewski, 1999)

The integrins, composed of α- and β- chain heterodimeric complexes, serve as integral cell membrane receptors that form focal adhesion contacts with various ECM ligands (i e fibronectin, laminin, vitronectin, the collagens, thrombospondin, entactin, fibrinogen, intercellular adhesion molecule (ICAM) and the vascular cell adhesion molecule) (Mizejewski, 1999) Investigations have also further linked integrin interactions with cytoplasmic cytoskeletal filament-associated proteins such as actin, vinculin, talin, α-actinin, paxillin and divalent cation-dependent proteins such as calreticulin

Integrins also function as cellular signalling receptors Integrins themselves do not contain any catalytic activity and are thus unable to independently initiate signalling cascades Rather, the short cytoplasmic domains of α- and β-subunits serve as scaffolds for the assembly of multiprotein signalling complexes (Giancotti and Ruoslahti, 1999, Sheppard, 2003)

Each integrin generally consists of a noncovalently linked α- and β- subunit, with each subunit having a large extracellular domain, a single membrane spanning domain and a short, non-catalytic cytoplasmic tail

Eighteen human α-subunits and 8 β-subunits have been identified that can form a total of 24 integrin heterodimers (Sheppard, 2003), with each heterodimer pairing being specific for a unique set of ligands For example, integrin αvβ3 binds a wide range of ECM molecules, including fibronectin, fibrinogen, von Willebrands factor, vitronectin and proteolysed forms of collagen and laminin, whereas integrin α5β1 selectively binds fibronectin (Hood and Cheresh, 2002)

1.3.1 Integrin Signalling

The cytoplasmic tails of integrins are generally short and always devoid of any enzymatic features. Hence, integrins transduce signals by associating with adapter proteins that connect the integrin to the cytoskeleton, cytoplasmic kinases and transmembrane growth factor receptors.

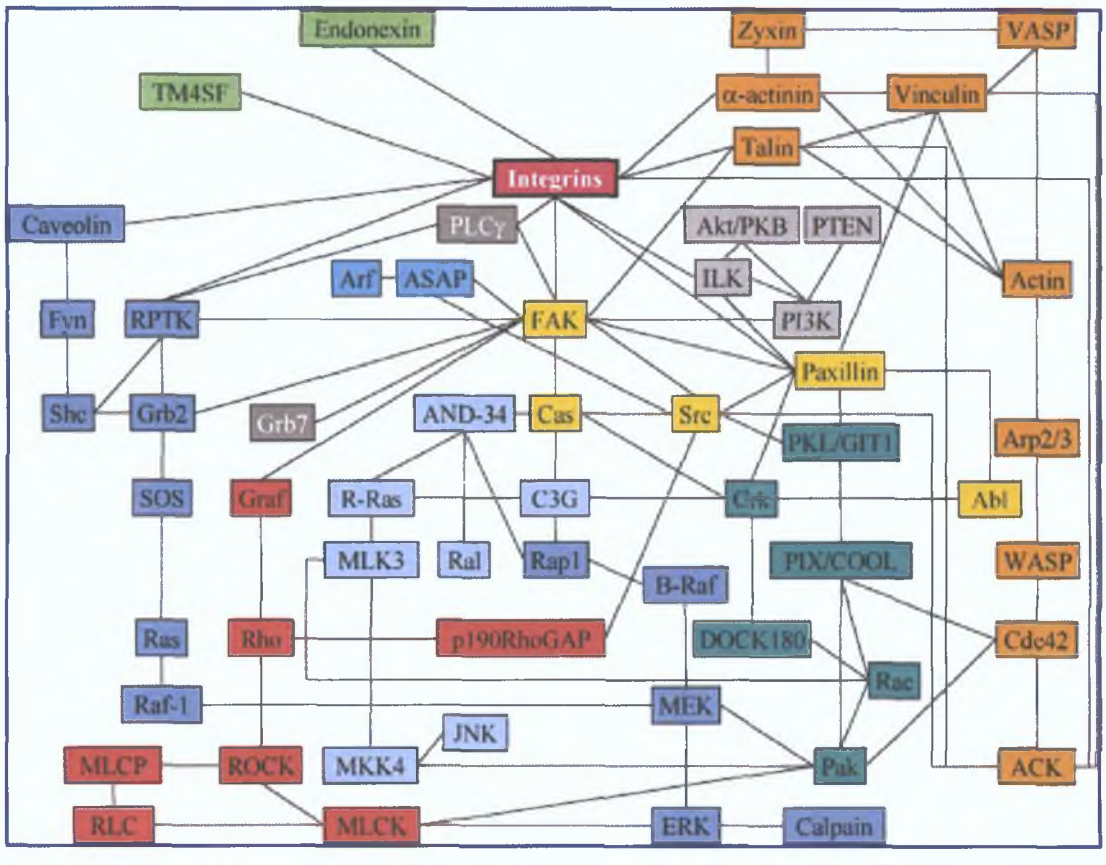


Figure 5. Integrin signalling network. Different classes of signalling protein are colour coded, e.g. focal adhesion proteins are yellow, actin cystskeleton regulatory proteins are orange, PI3 kinase signalling in grey.

Integrin signalling and assembly of the cytoskeleton are intimately linked. As integrins bind to the ECM, they become clustered in the plane of the cell membrane and associate with a cytoskeletal and signalling complex that promotes the assembly of actin filaments. The reorganisation of actin filaments into larger stress fibers, in turn, causes more integrin clustering, thus enhancing the matrix binding and organisation by integrins in a positive feedback system. As a result, ECM proteins

integrins and cytoskeletal proteins assemble into aggregates on each side of the membrane Well developed aggregates can be detected by immunoflourescence microscopy and are known as focal adhesions and ECM contacts In this manner, integrins serve as integrators of the ECM and cytoskeleton, the property for which they are named (Giancotti and Ruoslahti, 1999)

The β1 integrin cytoplasmic domain interacts directly and indirectly with a large number of cytoskeletal and signalling proteins (Fig 5) The β1 integrin subunit was first shown to colocalise with extracellular fibronectin and several intracellular cytoskeletal components including actin, α-actinin, vinculin and talin It was subsequently demonstrated that two actin binding proteins, talin and α-actmin directly associate with the cytoplasmic tail of β1 mtegrin (Miranti and Brugge, 2002) The observation that talin and α-actinin bind other cytoskeletal proteins such as zyxin, paxillin and vinculin, which in turn bind tensin, led to the proposal that the complex of integrin-linked cytoskeletal proteins in focal adhesions are important for maintaining strong cell-substrate adhesions and promoting cell spreading (Miranti and Brugge, 2002)

As connections between integrins and cytoskeletal proteins were being characterised, evidence began to accumulate that integrins could not only regulate actin cytoskeletal rearrangements but also modulate gene expression and cell differentiation Attachment to the ECM was found to either induce gene expression in the absence of other factors or to be a critical requirement for the induction of genes involved in differentiated cell functions In addition, antibodies to β1 were shown to block the differentiation of myoblasts and fibronectin inhibited the differentiation of keratinocytes (Miranti and Brugge, 2002)

The involvement of integrins in regulating gene expression and cell differentiation motivated investigators to identify the cytoplasmic proteins involved in transducing the signals required for these events The evidence that v-Src, an oncogenic tyrosine kinase, localises to focal adhesions and that focal adhesions, could be immunostained with antibodies to phosphotyrosine, provided the first hints of a connection between integrins and tyrosine phosphorylation

The first direct evidence for mtegrin-mediated regulation of tyrosine kinases came from studies in platelets, where activation by agonists results in a rapid and strong

induction of tyrosine phosphorylation that is predominantly induced by fibrinogen binding to specific integrin receptors Because tyrosine kinases were first identified in association with oncogene products, such as v-Src and v-Abl and subsequently with several growth factor receptors, they had been associated with the specific regulation of cell proliferation pathways The finding that platelet activation and integrin receptors trigger the activation of tyrosine kinases broadened the functions of these kinases to include events triggered by adhesion receptors in differentiated, post-mitotic cell functions Integrins were also found to regulate sodium-proton antiporters, and protein kinase C (PKC) was shown to associate with integrin containing focal adhesions (Miranti and Brugge, 2002)

A major breakthrough in integrin-mediated intracellular signalling came from the identification and cloning of the protein tyrosine kinase FAK (focal adhesion kinase) (Miranti and Brugge, 2002) FAK, originally identified as a v-Src substrate (Kanner *et al*, 1989), was found to localise to focal adhesions and to be inducibly tyrosine phosphorylated after the attachment of cells to ECM proteins, or of platelets to fibrinogen (Guan *et al*, 1991, Hanks *et al*, 1992, Kornberg *et al*, 1989, Lipfert *et al*, 1992, Schaller *et al*, 1992) FAK was also found to be activated by growth factors and other agonists, in addition to integrins These findings supported the concept that integrins are signalling receptors, as well as mediators of cell adhesion (Miranti and Brugge, 2002)

The signalling events that occur upon integrin binding to ligands in the extracellular matrix involve the complex interaction of a large number of cytoskeletal proteins, tyrosine kinases and other signalling proteins Only a small proportion of these proteins bind directly to integrin cytoplasmic domains but the proteins that become localised at integrin containing focal adhesions often bind numerous proteins and therefore play a role as scaffolding proteins for the organisation of signalling complexes Through this complex network of protein interactions, integrins can transmit signals from its extracellular interactions to a wide variety of internal signalling pathways within the cell which affect cellular processes such as proliferation, apoptosis, migration, morphogenesis and invasion This is the underlying reason that integrins are such important molecules in regulation of normal cellular function in growth and development and in dysregulation of cellular function in disease states such as cancer

The timing and sequencing of protein interaction in focal adhesion complexes are important in regulating their functions Focal adhesions are dynamic complexes that constantly form, disintegrate and then reform This dynamic regulation is important for cellular processes associated with focal adhesions, e g migration

1 3 1 1 Integrin Signalling Mechanism

On binding to ECM molecules, integrins are thought to undergo a conformational change which allows the intracellular domain of their β-subunit to interact with focal adhesion proteins such as talin and α-actinin The detailed sequence of events following ECM binding to integrin is not known, but the β-subunit cytoplasmic domain interacts directly with talin and talin in turn interacts with both vinculin and paxillin FAK appears to localise to nascent focal adhesions because it binds to paxillin Upon activation, FAK combines with Src family kinases which then phosphorylate paxillin and p130cas (Fig 6) Both of these molecules serve as scaffolds for the recruitment of various adaptors and signalling intermediates (Giancotti, 1999)

1 3 1 1 1 FAK Activation

Clustering of integrin results in rapid phosphorylation of FAK at Tyr397 as well as several additional sites within the kinase and c-terminal domains Phosphorylation at Tyr397 correlates with increased catalytic activity of FAK and appears to be important for tyrosine phosphorylation of focal adhesion associated proteins as well as phosphorylation at Tyr576 and Tyr577, two highly conserved residues positioned within the 'catalytic loop' of the kinase domain Phosphorylation of these residues is important for the maximal adhesion induced activation of FAK and signalling to downstream effectors (Parsons, 2003)

Phosphorylation of FAK in response to integrin engagement leads to the formation of phosphotyrosine docking sites for several classes of signalling molecules and may be important for the conformation-induced binding of proteins to other structural motifs within the N- and C-terminal non-catalytic regions Phosphorylation on Tyr397 creates a high-affinity binding site for the SH2 domain of Src family kinases and leads to the recruitment and activation of Src through the formation of a bipartite kinase complex Tyr397-dependent activation of FAK and the recruitment of Src have been

implicated in the efficient tyrosine phosphorylation of additional sites on FAK as well as the FAK binding proteins Cas and paxillin Phosphorylation of Tyr397 also appears to be important for the recruitment of other SH2 containing proteins, including the 85kDa subunit of phosphoinositide 3-kinase (PI3-kinase), phospholipase c (PLC)-γ and the adapter protein Grb7 The phosphorylation of Tyr397 as well as Tyr925, creates a binding site for the Grb2-SOS complex (Parsons 2003)

FAK contains four sites of serine phosphoryaltion within the C-terminal domain (Ser722, Ser843, Ser 846 and Ser 910) The role of serine phosphorylation in the regulation of FAK function is poorly understood, however, the proximity of several of these phosphorylated serine residues to sites of protein-protein interaction suggests a role for serine phosphorylation in modulating binding/stability of downstream signalling proteins (Parsons, 2003)

The C-terminal harbors multiple protein-protein interaction sites In addition to the paxillin-binding site in the FAT domains, two additional sites contain proline-rich recognition sites for SH3-domain-containing proteins (Pro2 and Pro3, Fig 6) Pro2 provides the major binding motif recognised by the SH3 domain of Cas, a multi-functional adapter protein Upon integrin clustering, Cas is localised to adhesion complexes and is tyrosine phosphorylated FAK mutants that lack the binding site for Cas exhibit compromised signalling to downstream effectors The Pro3 motif binds the SH3 domains of two regulators of small GTPases GRAF, a GAP (GTPase-activating protein) for Rho and ASAP1, a GAP for Arf1 and Arf6 The binding of ASAP and GRAF to FAK appears important to link adhesion complex signalling with the concerted regulation of small GTP-binding proteins in the Rho and Arf families, proteins that play an important function in cytoskeletal reorganisation

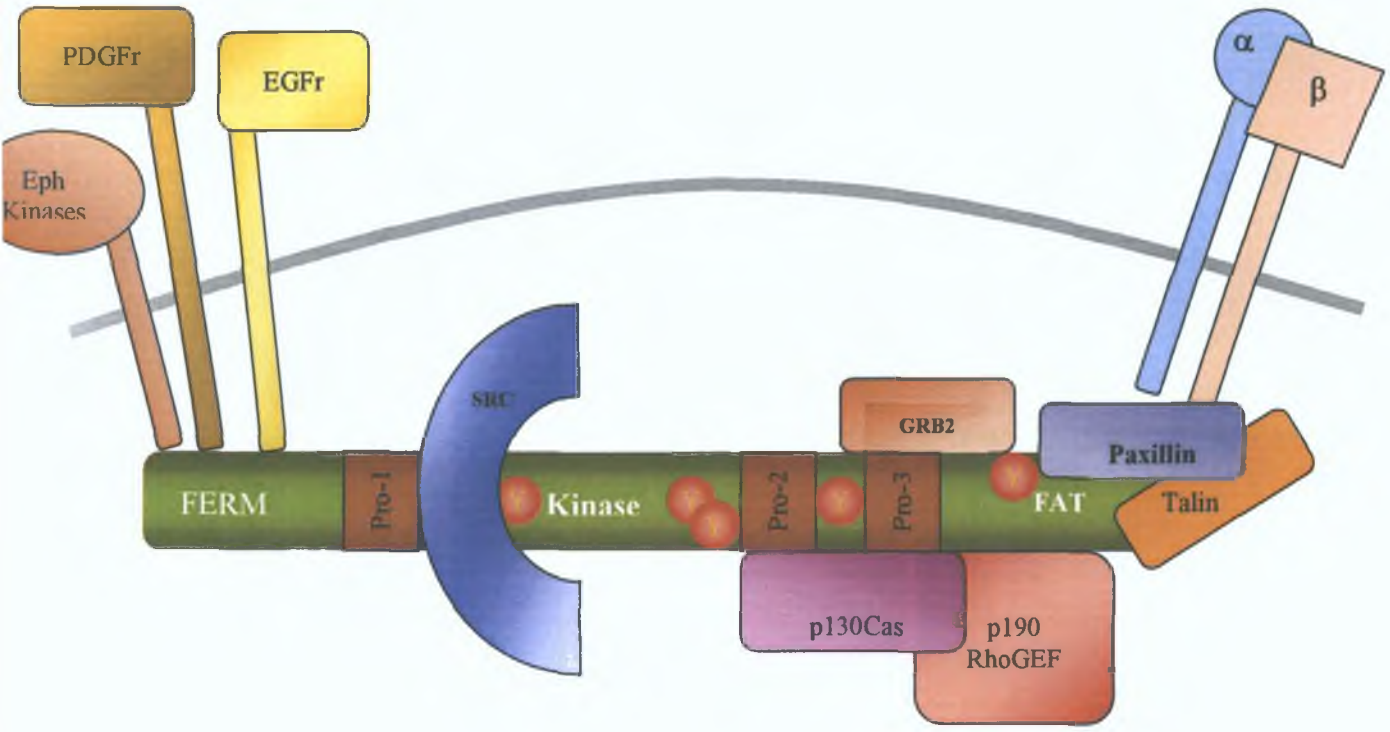


Figure 6. FAK protein interactions. α and β represent integrin receptors.

1.3.1.1.2 FAK regulates cell motility and invasion

In cell motility, FAK signalling controls the formation and turnover of focal contact sites and for cell invasion, FAK signalling alters MMP expression and promotes generation of an invasive phenotype (Schlaepfer *et al.*, 2004).

FAK consists of a central catalytic domain and amino and carboxyl terminal non-catalytic domains. A focal adhesion targeting sequence within the carboxyl terminus is required for localisation to focal adhesions. Inhibition of FAK activity by

expression of its carboxyl terminus decreases cell motility and cells from FAK deficient mice also show reduced migration (Schlaepfer *et al* , 2004)
Transformation of FAK deficient fibroblasts with the v-Src oncogene promotes cellular motility equal to that of FAK re-expression However, these cells are not invasive and required intact FAK expression to produce this phenotype (Schlaepfer *et al* , 2004)
The importance of FAK in regulating invasiveness caused by v-Src overexpression in NIH-3T3 cells was analysed by the expression of the FAK dominant negative inhibitor FRNK (Hauck *et al* , 2002) FRNK consists of the C-terminal domain of FAK and functions as a potent negative inhibitor of FAK by competitively preventing FAK localisation to focal contacts Stable expression of FRNK in v-Src-transformed NIH 3T3 fibroblasts inhibited cell invasion through Matrigel and blocked experimental metastases in nude mice without effects on cell motility The reduced invasiveness of FRNK expressing cells correlated with reduced expression of MMP-2 gene expression and MMP-2 secretion Overexpression of MMP-2 rescued FRNK blockage to cell invasion (Hauck *et al* , 2002)

1 3 1 2 Integrin signalling to actin

After ligand binding, integrins activate signalling cascades that affect formation, turnover and linkage of actin filaments The stimulation of Rho-GTPases is of special importance in this respect These molecules are essential for the organisation of the actin cytoskeleton and promote specialised actin structures such as stress fibres (RhoA), lammelipodia (Rac1) and filipodia (Cdc42) (Brakebusch and Fassler, 2003) In addition, Rho-GTPases are involved in cell proliferation, survival, polarity, vesicle transport and various other activities Integrins can stimulate Rho-GTPases via different pathways, of which those via FAK and Src-like kinases seem to be most important (Brakebusch and Fassler, 2003)
Rho-GTPases can be activated by FAK through several mechanisms A p130Cas-Crk-DOCK180 complex can activate Rac1, which promotes lammelipodia formation Second, PI3-K can stimulate Rho-GTPase-activating GEF molecules via PIP3 production, which in turn stimulates Rho-GTPases FAK can directly or indirectly interact via paxillin with the adapter GIT1 and with GEFs of the Cool/PIX family, which activate Rac1 and Cdc42 Finally, Src-like kinases can activate GEFs through phopsphorylation In short, integrin activation triggers the formation of various

phosphoprotein complexes that can modify the actin cytoskeleton particularly by activating Rho-GTPases (Brakebusch and Fassler, 2003)

1 3 2 β1 integrin alternative splicing

Alternative splicing of mRNA leads to additional complexity of the integrin family Variants of both the extracellular and cytoplasmic domains have been reported (Melker and Sonnenberg, 1999) Alternative extracellular domains may account for different ligand-binding affinities or variations in the state of activation, while variants of the cytoplasmic domain may modulate integrin activity, cytoskeletal associations and/or signalling events (Flier and Sonnenberg, 2001)

Cytoplasmic variants of several β- subunits have been described The four cytoplasmic variants of the β1-subunit, the most abundantly expressed family of the integrin subunits, are the best described $\beta1_A$, $\beta1_B$, $\beta1_C$ and $\beta1_D$

$\beta1_A$ is present in all tissues except cardiac and skeletal muscle, which instead express the highly homologous $\beta1_D$ variant The two variants share the first 24 amino acids of their cytoplasmic domains, and the two NPXY focal adhesion localisation sequences (cyto-2 and –3 domains) in the C-terminus are also conserved In non-muscle cells, both transfected $\beta1_A$ and $\beta1_D$ localised in focal contacts and activate focal adhesion kinase (FAK), MAP kinase and RhoA However $\beta1_A$ and $\beta1_D$ are not functionally equivalent in embryonic development The replacement of $\beta1_A$ by $\beta1_D$ results in embryonic lethality in mice, which might be due to impaired migration of neuroepithelial cells, whereas replacement of $\beta1_D$ by $\beta1_A$ does not lead to severe abnormalities in striated muscle *in vivo* (Flier and Sonnenberg, 2001)

The $\beta1_B$ and $\beta1_C$ variants are minor forms and are present in man but not in mouse Both variants behave as inactive integrins, which is probably due to their failure to become localised at focal adhesions Expression of $\beta1_B$ or $\beta1_C$ in cells decreases the ability of cells to adhere and to migrate on extracellular matrix components Similarly expression of $\beta1_B$ or $\beta1_C$ inhibits DNA synthesis and cell proliferation, whereas $\beta1_A$ does not inflict such inhibition (Flier and Sonnenberg, 2001)

1 3 3 Integrin expression in cancer

Malignant transformation is characterised by disruption of cytoskeletal organisation, decreased adhesion and altered adhesion dependent responses Studies of integrin expression in transformed cells suggest that various integrin subunits may contribute either positively or negatively to the transformed cell phenotype For the various types of cancers, different changes in integrin expression are associated with tumor growth and metastasis Tumor progression leading to metastasis appears to involve equipping cancer cells with the appropriate adhesive (integrin) phenotype for interaction with the ECM A consistent finding is the lack of spatial organisation of integrin expression in epithelial tumors In carcinomas, the spatial arrangement of integrins becomes quite disordered, with a diffuse and less abundant cellular distribution The integrin expression pattern also changes during cancer progression from transformation, to primary tumor growth and progression, to cell invasion and metastasis (Mizejewski, 1999)

1 3 4 Integrin expression in the lung

Normal lung development involves major changes in the expression of ECM molecules and integrin receptors (Coraux *et al*, 1998) At least seven different integrins ($\alpha2\beta1$, $\alpha3\beta1$, $\alpha6\beta4$, $\alpha9\beta1$, $\alpha v\beta5$, $\alpha v\beta6$ and $\alpha v\beta8$) are expressed on airway epithelial cells of healthy adults The integrin $\alpha5\beta1$ is generally not seen in healthy adult airway epithelium *in vivo* but is rapidly induced in airway epithelia in response to injury (Sheppard, 2003) The epithelial cells that line conducting airways utilize input from integrins for airway branching during morphogenesis (Kreidberg *et al*, 1996) to establish polarity and remain attached to their basement membrane (Sheppard, 2003) Integrins are also induced in response to injury and inflammation and it is likely that lung epithelial cells utilise these integrins to detect, co-ordinate and spatially organise complex responses to airway and lung injury

1.4 The Actin Cytoskeleton

The integrity of the actin cytoskeleton is essential for cells to form and maintain their shape and structure Remodelling of the actin cytoskeleton in dynamic cellular processes produces changes in shape and motility in response to external stimuli, and is therefore involved in signal transduction These features of the actin cytoskeleton are regulated by a large number of actin binding proteins, which were initially considered to be structural components that organise a stable actin cytoskeleton, but are now known to be regulators of cellular dynamics and key components of signalling processes

Actin dynamics are required for a number of physiological processes including the absorptive function of the intestinal epithelium, for mechanosensing in the inner ear, for oriented nerve and capillary growth and during defence processes that involve phagocytosis, migration and the activation of immunologically competent cells (Revenu et al , 2004) The actin cytoskeleton is essential for cell-motility events that are required for normal organogenesis and also for pathophysiological processes such as wound repair or tumor cell-migration (metastasis) (Revenu *et al* , 2004)

1 4 1 Cell motility and actin dynamics

To migrate, cells use dynamic rearrangements of the actin cytoskeleton for the formation of protrusive structures and for generation of intracellular forces that lead to net cell translocation This is initiated by a transition from a non-polarised to a polarised state, most often induced by cues from the extracellular environment Polarised motile cells extend distinct protrusive regions in the direction of translocation Two cellular actin rich structures are typically associated with cell migration lammelipodia are large veil like sheets, which contain highly branched and cross-linked actin filaments, filopodia are long thin structures that often project beyond the edge of the lammelipodium and have parallel bundles of actin filaments For a cell to translocate (i e to move from one place to another), the cell needs to attach at a new site and retract its rear The new attachment sites or focal contacts that constitute a link between the substrate and the actin cytoskeleton are formed behind the leading edge While they mature into focal adhesions they pull the cell forward

Subsequently attachment sites at the rear of the cell are released, allowing the cell to pull its rear towards the direction of movement (Lambrechts *et al*, 2004)
As mentioned above, lammelipodia and filipodia are composed of actin filaments These are formed by the polymerisation of globular monomeric actin (G-actin), an ATP binding protein, into double stranded helical filaments (F-actin) These are structurally and kinetically polarised, possessing a faster growing (+) end and a slower growing (-) end Once incorporated at the (+) end, ATP-G-actin hydrolyses its bound ATP After release of inorganic phosphate, ADP-actin molecules are left in the filament and finally dissociate at the (-) end ADP-actin monomers are subsequently reloaded with ATP and shuttle back to the barbed (+) ends for a new round of polymerisation This process results in what is known as treadmilling-a net flow of actin subunits throughout the filament (Lambrechts *et al*, 2004, Revenu *et al*, 2004)
Many proteins bind to actin and influence its dynamics or state These proteins are referred to as actin binding proteins (ABPs) Among ABPs, some link actin filaments in a loose network (crosslinking proteins) or in a tight bundle (bundling proteins), or anchor filaments to membranes Others bind to the barbed (+) end of the filament and prevent further elongation (capping proteins), whereas some cause fragmentation of filaments (severing proteins) or might favour the depolymerisation of pointed (-) ends
A general model for actin based cell motility can be summarised as a four stage continuous cycle of (1) polarisation, (2) protrusion of lammelipodia as a result of actin polymerisation, (3) formation of attachment sites, (4) retraction of the cells rear (Lambrechts *et al*, 2004)

In metastatic and invasive tumor cells, alterations in the regulation of the actin cytoskeleton contributes to dysregulated cellular migration The observed alterations within the actin system can arise via three non-mutually exclusive pathways (i) via mutations in actin, (ii) via changes in upstream regulatory signalling proteins, (iii) via changed expression levels of actin binding proteins In all cases, this disturbs the balance between the synergistic and antagonistic activities of the actin binding proteins that regulate actin dynamics Therefore, multiple pathways are likely to exist whereby increasing or decreasing one activity can render a cell more motile (Lambrechts *et al*, 2004)

1.5 Keratins

Keratins (40-70 kDa) are known as the major structural proteins of epithelial cells where they occur as intermediate sized filaments in the cytoplasm They are encoded by a large family comprising >40 genes (Yamada *et al*, 2002) Keratin intermediate filaments are among the most differentiation specific proteins synthesized in epithelial cells More than 20 different cellular keratins (known as cytokeratins to distinguish them from keratins present in hair and nails) have been identified, each of which appears to have a distinctive pattern of protein synthesis in normal epithelia (McBride *et al*, 1999, Moll *et al*, 1982) The keratin family of proteins is generally subdivided into basic type II keratins (K1-K8) and acidic type I keratins (K9-K20), which form heteropolymers consisting of at least one type I and one type II chain, e g K8 and K18 are partners which dimerise to form keratin filaments

There are two types of epitheha simple and stratified Simple epithelia are composed of a single layer of cells and they line organs such as the stomach, liver or kidney Simple epithelia are characterised by the expression of K8 and K18 Some simple epithelia also express K7, K17 and K19 while others express the intermediate filament protein vimentin (Fuchs *et al*, 1998)

Stratified epithelia (such as the epidermis) are composed of several layers with each successive layer representing a more differentiated state In this case cells progress from the basal to the upper layers increasing in differentiation as they proceed through the different layers Depending on the epithelial cell type each successive layer in stratified epithelia (which are at different levels of differentiation) are characterised by the expression of unique keratin pairs (Fuchs *et al*, 1998)

The specificity of keratin expression in different cell types depending on the differentiation state of the cells has led to the use of keratins as markers for identification of cell types and their level of differentiation

1.6 C/EBP transrıptıon factors

The C/EBPs are a famıly of transcrıptıon factors expressed ın several organs and are ınvolved ın controllıng dıfferentıatıon-dependent gene expressıon In lıver, fat and whıte blood cells of the myelomonocytıc lıneage, C/EBP factors have been demonstrated to be ımportant regulators of dıfferent aspects of dıfferentıatıon, ıncludıng prolıferatıon, cell cycle arrest and gene expressıon (Cassel and Nord, 2003, Ramjı and Foka, 2002)

C/EBP transcrıptıon factors belong to the bZIP class of basıc domaın transcrıptıon factors Sıx members (C/EBPα-ζ) constıtute the mammalıan C/EBP famıly The basıc regıon of C/EBP factors ıs a hıghly posıtıvely charged domaın that dırectly ınteracts wıth DNA All members of the C/EBP famıly have sımılar basıc regıon DNA-bındıng motıfs except C/EBPζ (whıch lacks DNA bındıng actıvıty) As a consequence of the hıgh sımılarıty ın the basıc regıon, C/EBPα, C/EBPβ and C/EBPδ have been shown to ınteract wıth vırtually ıdentıcal DNA sequences The leucıne zıpper regıon ıs also conserved between the dıfferent famıly members, whereas the amıno-termınal transactıvatıon domaın ıs more dıverse The leucıne zıpper domaın ıs ınvolved ın homo- and heterodımerısatıon and all proteıns ın the C/EBP famıly have been shown to form homo- and heterodımers (Cassel and Nord, 2003, Ramjı and Foka, 2002)

1 6 1 C/EBPs ın Lung

Of the tıssue specıfic C/EBP famıly members, three have been demonstrated to be expressed ın lung, namely C/EBPα, -β and -δ In adult lung, C/EBPα ıs expressed ın the type II cells of the alveolar epıthelıum, and lower levels are seen ın the bronchıolar epıthelıal Clara cells C/EBPβ ıs expressed ın type II cells of the alveolar regıon and expressıon ıs also seen ın the bronchıal epıthelıum C/EBPδ shows hıgh level expressıon ın the bronchıolar epıthelıum and lower levels ın alveolar type II cells (Cassel and Nord, 2003) An ımportant role for C/EBPα ın lung cell dıfferentıatıon ıs suggested by the phenotype of the C/EBPα (-/-) mouse C/EBPα (-/-) knockout mıce dısplay ımpaıred lung cellular dıfferentıatıon wıth abnormalıtıes ın the alveolar epıthelıum and hyperprolıferatıon of type II cells (Cassel and Nord 2003, Flodby *et al* , 1996) No lung phenotype has been reported ın C/EBPβ(-/-), C/EBPδ(-/-) or C/EBPβ(-/-)/C/EBPδ(-/-) double knockout mıce (Cassel and Nord, 2003)

Studies have shown that C/EBP transcription factors are involved in the regulation of lung epithelial differentiation related gene expression C/EBPs have been implicated in the transcriptional regulation of Surfactant Protein A (SP-A), Surfactant Protein D (SP-D), Clara Cell Specific Protein (CCSP) and the P450 enzyme CYP2B1 (Cassel *et al*, 2000, Cassel and Nord, 2003, He and Crouch, 2002, Rosenberg *et al*, 2002) These results indicate C/EBP transcription factors play an important role in lung epithelial development and differentiation

The observation that C/EBPα(-/-) mice display hyperproliferation of alveolar type II cells indicates it is involved in regulation of growth in airway epithelial cells Analysis of C/EBPα expression in a panel of lung cancer cell lines and primary tumor specimens showed decreased expression of C/EBPα in a large proportion of samples Induction of C/EBPα expression by stable transfection in two lung cancer cell lines led to growth reduction, differentiation and apoptosis, indicating a possible tumor suppressor role for C/EBPα (Halmos *et al*, 2002)

C/EBP expression is also induced after lung injury suggesting a role for these factors in repairing damaged epithelium after injury (Sugahara, 1999)

1 6 2 Translational regulation of C/EBP expression

Production of different C/EBP-α and -β polypeptides by alternative use of initiation codons represents a major form of translational control in the regulation of the C/EBP family C/EBP-α and -β mRNAs contain multiple translation initiation sites and also evolutionarily conserved upstream open reading frames (uORFs) which allow the production of different protein isoforms by differential initiation of translation initiation These protein isoforms display altered biological activity which makes translational control important in the regulation of C/EBP expression (Ramji and Foka, 2002) A study by Calkhoven *et al* (2000) showed that PKR and mTOR signalling pathways control the ratio of C/EBP-α and -β isoform expression through the translation initiation factors eIF2α and eIF4E

1.7 CBP/p300 Transcriptional co-activators

CREB-binding protein (CBP) and p300 are believed to participate in the activities of hundreds of different transcription factors CBP and p300 were both identified initially in protein interaction assays, the former through its association with the transcription factor CREB, and the latter through its interaction with the adenoviral protein E1A (Goodman and Smolik, 2000) The recognition that these two proteins, one involved in transcription and the other in cell transformation, had highly conserved sequences suggested that they had the potential to participate in a variety of cellular functions

CBP and p300 are transcriptional co-activator proteins that play a central role in co-ordinating and integrating multiple signal dependent events with the transcriptional apparatus, allowing the appropriate level of gene activity to occur in response to different physiological cues that influence, for example, proliferation, differentiation and apoptosis

The transcription regulating properties of p300 and CBP appear to be exerted through multiple mechanisms They act as protein bridges, thereby connecting different sequence-specific transcription factors to the transcription apparatus Providing a protein scaffold upon which to build a multicomponent transcriptional regulatory complex is likely to be an important feature of p300 and CBP control Another key property is the presence of histone acetyltransferase (HAT) activity, which endows p300/CBP with the capacity to influence chromatin activity by influencing nucleosomal histones Other proteins, including YY1 and c-Myc are also subject to regulation through acetylation by p300 and CBP (Chan and La Thangue, 2001, Yao *et al*, 2001, Vervoorts *et al*, 20003) p300 has also been reported to interact with the transcription factor C/EBPβ which results in activation of C/EBPβ and also triggers phosphorylation of p300 (Mink *et al*, 1997, Schwartz *et al*, 2003)

1 7 1 CBP/p300 in Lung

CBP has been found to be involved in the transcriptional regulation of the lung differentiation specific genes Surfactant Protein A (SP-A) and Surfactant Protein (SP-B) (Naltner *et al*, 2000a, Yi *et al*, 2002) CBP was found to stimulate the SP-B

promoter synergistically with the transcription factors TTF-1 and RAR in the H441 pulmonary adenocarcinoma cells (Naltner *et al*, 2000a) CBP, TTF-1 and SRC-1 synergistically activated SP-A promoter activity in A549 lung adenocarcinoma cells (Yi *et al*, 2002)

Immunocytochemical analysis of CBP and p300 in developing mouse lung showed nuclear staining for both CBP and p300 in almost all cell types at various stages of lung development indicating a role for CBP and p300 as general transcriptional co-activators in this organ (Naltner *et al*, 2000b)

1.8 Aims of Thesis

Previous work conducted in this laboratory has shown that treatment of the poorly differentiated lung cell line DLKP with the differentiation modulating agent BrdU causes increased expression of growth and differentiation related proteins The translation initiation factor eIF4E has been implicated in the post-transcriptional regulation of gene-expression in these cells with increased expression and phosphorylation of the translation initiation factor eIF4E detected in BrdU treated DLKP cells eIF4E is also considered to play a role as an oncogene and its expression is increased in various cancers It was therefore decided to analyse the effect of overexpression of eIF4E and phosphorylation on growth and gene expression in DLKP cells The main aims of this thesis therefore were as follows

- Conduct transient transfections, and generate stably transfected DLKP cells, with wild type (4E-HA), and non phosphorylatable S209 mutant (4E S209-HA), HA (hemagluttinin) epitope tagged human eIF4E constructs
- Analyse expression of Cytokeratin 8, 18, 19 which are subject to post-transcriptional regulation in BrdU treated DLKP cells
- Analyse expression of β1 integrin and associated α Integrin binding partners which are also differentially expressed in BrdU treated DLKP cells
- Conduct invasion assays to determine if overexpression of 4E-HA or 4E S209-HA alters the invasion characteristics of DLKP cells
- Investigate changes in transcriptional control as a result of 4E-HA and 4E S209-HA overexpression using oligonucleotide microarray analysis
- Conduct large scale analysis of protein expression using two dimensional gel electrophoresis to determine the effects of 4E-HA and 4E S209-HA overexpression on the protein expression profiles of these cells

2.0 Materials and methods

2.1 Cell Culture Methods

2 1 1 Water

Ultrapure water was used in the preparation of all media and solutions Initially the water was pre-treated which involved activated carbon, pre-filtration and anti-scaling This water was then purified by a reverse osmosis system (Millipore Milli-RO 10 Plus, Elgastat UHP) to a standard of 12 - 18 MΩ/cm resistance

2 1 2 Treatment of Glassware

All solutions for use in cell culture and maintenance were prepared and stored in sterile glass bottles Bottles (and lids) and all other glassware used for any cell-related work were prepared as follows - all glassware and lids were soaked in a 2% (v/v) solution of RBS-25 (AGB Scientific) for at least 1-hour This is a deproteinsing agent, which removes proteineous material from the bottles Glassware was scrubbed and rinsed several times in tap water, the bottles were then washed by machine using Neodisher detergent, an organic, phosphate-based acid detergent The bottles were then rinsed twice with distilled water, once with ultrapure water and sterilised by autoclaving

2 1.3 Sterilisation

Water, glassware and all thermostable solutions were sterilised by autoclaving at 121°C for 20 minutes (min) under pressure of 1bar Thermolabile solutions were filtered through a 0 22 μm sterile filter (Millipore, millex-gv, SLGV-025BS) Low protein-binding filters were used for all protein-containing solutions

2 1 4 Media Preparation

Medium was routinely prepared and sterility checked by Mr Joe Carey (technician) as in SOP NCTCC 003-02 The basal media used during routine cell culture were prepared according to the formulations shown in Table 2 1 1 Media (10x) was added to sterile ultrapure water, buffered with HEPES and $NaHCO_3$ and adjusted to a pH of 7 45 - 7 55 using sterile 1 5M NaOH and 1 5M HCl The media were then filtered through sterile 0 22μm bell filters (Gelman, 121-58) and stored in 500ml sterile bottles at 4°C Sterility checks were carried out on each 500ml bottle of medium as described in Section 2 2 7

The basal media were stored at 4°C up to their expiry dates as specified on each individual 10x medium container (3 months) Working stocks of culture media was prepared as 100ml aliquots, supplemented with L-glutamine (Gibco, 25030-024) and fetal calf serum as required This was stored for up to 2 weeks at 4°C, after which time, fresh culture medium was prepared

	DMEM (Gibco, 12501-029)	**Hams F12** (Gibco, 21700-109)
10X Medium	500ml	Powder
Ultrapure H_20	4300ml	4700ml
1M HEPES* Sigma , H-9136	100ml	100ml
7 5% $NaHCO_3$ BDH, 30151	45ml	45ml

* HEPES = N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

Table 2.1.1 Preparation of basal media

2 2 Methods Used in Maintaining Cell Lines

2 2 1 Safety Precautions

All cell culture work was carried out in a class II down-flow re-circulating laminar flow cabinet (Nuaire Biological Cabinet) and any work, which involved toxic compounds, was carried out in a class II cytoguard (Gelman) Strict aseptic techniques were adhered to at all times The laminar flow cabinet was swabbed with 70% industrial methylated spirits (IMS) before and after use, as were all items used in the cabinet Each cell line was assigned specific media and waste bottles and only one cell line was used at a time in the cabinet, which, was allowed to clear for 15min between different cell lines The cabinet itself was cleaned each week with industrial detergents (Virkon, Antec International, TEGO, TH Goldschmidt Ltd), as were the incubators

2 2 2 Culture of Adherent Cell Lines

DLKP cell lines were cultured in ATCC medium (Ham's F12/ DMEM (1 1)) supplemented with 5% FCS and 2mM L-glutamine (Gibco, 25030-024)

The cell lines employed during the course of this research were generally maintained in non-vented 75 cm^2 flasks (Costar, 3075)

2 2 3 Subculture of Adherent Cell Lines

During routine sub-culturing or harvesting of adherent lines, cells were removed from their flasks by enzymatic detachment The following protocol outlines the methods used in subculturing DLKP cell lines

Waste medium was removed from the flasks and rinsed with a pre-warmed (37°C) trypsin/EDTA (TV) solution (0 25% trypsin (Gibco, 25090-028), 0 01% EDTA (Sigma, E5134) solution in PBS (Oxoid, BR14a)) The purpose of this was to eliminate any naturally occurring trypsin inhibitor, which would be present in residual serum Fresh TV was then placed on the cells (1ml/25cm^2 flask or 2ml/75cm^2 flask) and the flasks were incubated at 37°C until the cells were seen to have detached (5 min) The trypsin was deactivated by addition of an equal volume of growth medium (*i e* containing serum) The entire solution was transferred to a 30ml sterile universal tube (Greiner, 201151) and

centrifuged at 201xg for 5 minutes The resulting cell pellet was re-suspended in pre-warmed (37°C) fresh growth medium, counted (Section 2 2 5) and used to re-seed a flask at the required cell density or to set up an assay

2 2 4 Cell Counting

Cell counting and viability determinations were carried out using a trypan-blue (Gibco, 15250-012) dye exclusion technique

An aliquot of trypan-blue was added to a sample from a single cell suspension in a ratio of 1 5 After 3 min incubation at room temperature, a sample of this mixture was applied to the chamber of a haemocytometer over which a glass coverslip had been placed Cells in the 16 squares of the four outer comer grids of the chamber were counted microscopically An average per comer grid was calculated with the dilution factor being taken into account and final cell numbers were multiplied by 10^4 to determine the number of cells per ml (volume occupied by sample in chamber is 0 1cm x 0 1cm x 0 01cm *i e* 0 0001cm^3 therefore cell number x 10^4 is equivalent to cells per ml) Non-viable cells were those, which stained blue while viable cells excluded the trypan-blue dye and remained unstained

2 2 5 Cell Freezing

To allow long term storage of cell stocks, cells were frozen and cryo-preserved in liquid nitrogen at temperatures of –180°C Once frozen properly, such stocks should last indefinitely

DLKP were frozen using the standard freezing method as follows Cells to be frozen were harvested in the log phase of growth (*i e* actively growing and approximately 60-70% confluent) and counted as described in Sections 2 2 4 Pelleted cells were re-suspended in serum and an equal volume of a DMSO/serum (1 9, v/v) freezing solution The freezing solution was slowly added (drop-wise) to the cell suspension (as DMSO is toxic to cells) A final concentration of at least $5x10^6$ cells/ml was generated The suspension was then aliquoted into cryovials (Greiner, 122 278) which were then quickly placed in the vapour phase of liquid nitrogen containers (approximately -80°C) After 2 5

to 3 5 hours, the cryovials were lowered down into the liquid nitrogen where they were stored until required

2 2 6 Cell Thawing

The following procedure was used for thawing DLKP cells Immediately prior to the removal of a cryovial from the liquid nitrogen stores for thawing, a sterile universal tube containing 5 ml growth medium was prepared This allowed for the rapid transfer and dilution of thawed cells to reduce their exposure time to the DMSO freezing solution (it is toxic at room temperature) The cryovial was partially thawed and its contents were transferred to the universal The suspension was centrifuged at 201xg for 5 min, the DMSO-containing supernatant was removed and the pellet was then re-suspended in fresh growth medium Viability counts were carried out (Section 2 2 4) to determine the efficacy of the freezing/ thawing procedures A sample was also taken for sterility analysis (Section 2 2 7) Thawed cells were placed into tissue culture flasks with the appropriate volume of medium (5ml/25cm^2 flask and 10ml/75cm^2 flask) and allowed to attach overnight

2 2 7 Sterility Checks

Sterility checks were routinely carried out on all media, supplements and trypsin used for cell culture Samples of basal media were inoculated into Columbia (Oxoid, CM331) blood agar plates, Sabauraud (Oxoid, CM217) dextrose and Thioglycollate (Oxoid, CM173) broth's which should between them detect most contaminants including bacteria, fungus and yeast Growth media (*i e* supplemented with serum and L-glutamine) were sterility checked at least 3 days prior to use by incubating samples at 37°C These were subsequently examined for turbidity and other indications of contamination Freshly thawed cells were also subjected to sterility checks

2 2 8 *Mycoplasma* Analysis

Mycoplasma examinations were carried out routinely (at least every 3 months) on all cell lines used in this study

2 2 8 1 Indirect Staining Procedure

In this procedure, *Mycoplasma*-negative NRK cells (a normal rat kidney fibroblast line) were used as indicator cells i e these cells were incubated with supernatant from test cell lines and then examined for *Mycoplasma* contamination NRK cells were used for this procedure because cell integrity is well maintained during fixation and stocks are mycoplasma negative A fluorescent Hoechst stain was utilised which binds specifically to DNA and so will stain the nucleus of the cell in addition to any *Mycoplasma* DNA present A *Mycoplasma* infection would thus be seen as small fluorescent bodies in the cytoplasm of the NRK cells and sometimes outside the cells

NRK cells were seeded onto sterile coverslips in sterile Petri dishes (Greiner, 633 185) at a cell density of $2x10^3$ cells per ml and were allowed to attach overnight at 37°C in a 5% CO_2, humidified incubator A 1ml aliquot of cell-free (cleared by centrifugation at 201xg for 5 min) supernatant from each test cell line was then inoculated onto a NRK petri dish and incubated as before until the cells reached 20 - 50% confluency (4-5 days) After this time, the waste medium was removed from the petri dishes, the coverslips (Chance Propper, 22 x 22 mm) were washed twice with sterile PBS, once with a cold PBS/Carnoys (50/50) solution and fixed with 2ml of Carnoys solution (acetic acid methanol-1 3) for 10 minutes The fixative was then removed and after air drying, the coverslips were washed twice in deionised water and stained with 2ml of Hoechst 33258 stain (BDH) (50ng/ml) for 10 minutes

From this point on, work proceeded without direct light to limit quenching of the fluorescent stain The coverslips were rinsed three times in PBS They were then mounted in 50% (v/v) glycerol in 0 05M citric acid and 0 1M disodium phosphate and examined using a fluorescent microscope with a UV filter

2 2 8 2 Direct Staining

The direct stain for *Mycoplasma* involved a culture method where test samples were inoculated onto an enriched *Mycoplasma* culture broth (Oxoid, CM403) - supplemented with 20% serum, 10% yeast extract (Oxoid L21, 15% w/v) and 10% stock solution (12 5g D-glucose, 2 5g L-arganine and 250 mls sterile-filtered UHP) This medium was designed to optimise the growth of any contaminants and was incubated at 37°C for 48

hours Samples of this broth were then streaked onto plates of *Mycoplasma* agar base (Oxoid, CM401) which had also been supplemented as above and the plates were incubated for 3 weeks at 37°C in a CO_2 environment The plates were viewed microscopically at least every 7 days and the appearance of small, "fried egg" -shaped colonies would be indicative of a mycoplasma infection

2.3 Preparation of Cell Samples for Analysis

2.3 1 Preparation of Bromodeoxyuridine (BrdU) Stock Solution

DLKP Bromodeoxyuridine (BrdU) treatment studies were carried out using 5-bromodeoxyuridine (BrdU) (Sigma, B5002) BrdU powder was reconstituted in UHP water to a stock concentration of 10mM and the resultant solution was filter sterilised through a sterile 0 22μm filter, ahquoted into sterile Eppendorfs and stored at -20^{0}C for up to 1 year

2 3 2 Preparation of cell samples for Immunocytochemical/Immunoflourescence Analysis

For immunocytochemical and immunoflourescence analysis (Section 2 8), cells were plated onto 6-well plates (Costar, 3516) at densities of 1x10^4 cells per well A 2 ml volume of medium was sufficient for each well The cells were allowed to attach and form colonies by incubating at 37^{0}C, 5% CO_2 for 24 hours The plates were covered with parafilm to prevent contamination For BrdU treatments after 24 hr, the medium was removed and 2 ml fresh medium containing either 10μM BrdU was then added to each well and the plates were then incubated for 7 days Medium was replaced every 3-4 days over the course of the assay All waste medium was retained for disposal by incineration

2.3 2 1 Fixing Cells for Immunofluorescence/Immunocytochemistry

After incubation, cells were rinsed 3X in PBS A 2 ml volume of ice cold methanol was added to each well and the plates were incubated at –20°C for 7 minutes and removed The plates were allowed to air-dry, wrapped in foil and stored at –20°C

2.3 3 Preparation of cell samples for Western Blot analysis

For Western blotting, cells were inoculated into 75cm^2 flasks at a density of 1x10^5 cells per flask and allowed to attach and form colonies For BrdU treatments, BrdU at a concentration of 10μM was then added to the cells after 24 hours and cells were grown for 7 days Otherwise, cells were incubated until approx 80% confluent Medium was replaced every 3-4 days The cells were then harvested by trypsinisation, washed in sterile PBS A, counted, pelleted and stored at -80°C until required

2 3 4 Preparation of cell samples for RT-PCR/ Microarray RNA analysis

For RNA analysis, cells were inoculated into 75cm^2 flasks at a density of 1x10^5 cells per flask and allowed to attach and form colonies Cells were incubated until approx 80% confluent Medium was replaced every 3-4 days The cells were then harvested by trypsinisation, washed in sterile PBS A and counted Approximately 10^8 cells were pelleted and lysed using 1ml of TRI REAGENT™ (SIGMA, T-9424) The samples were allowed to stand for 5 mins at RT to allow complete dissociation of nucleoprotein complexes and then snap frozen in liquid nitrogen and stored at -80°C until required

2 3 5 Preparation of cell samples for 2D electrophoresis

For preparation of samples for 2D electrophoreisis cells were inoculated into 175cm^2 flasks at a density of 5x10^5 cells per flask and allowed to attach and form colonies Medium was replaced every 3-4 days until cells were approx 80% confluent The cells were then harvested by trypsinisation, washed in ice-cold sterile PBS A, counted and pelleted The further preparation of cell samples for 2D electrophoresis is described in section 2 9 2

2 4 Growth Assay Experimental Protocol

Cells were seeded at a density of 0 5x10^5 cells/flask in 25cm^2 flasks The cells were allowed to attach overnight at 37°C incubator On each day following initial seeding, the cells were trypsinised (Section 2 2 3) and counted (Section 2 2 4) Each flask was counted on both counting chambers of a Hemocytometer and two flasks were counted each day The proliferation assay was carried out for a total of 6 days

2.5 Invasion Assay Experimental Protocol

Invasion assays were performed using BD Biocoat™Matrigel™ Invasion Chambers (Cat No 354480) according to manufacturers instructions Invasion assays were performed using 5x10^4 cells per invasion chamber and 5% foetal calf serum was used as chemottractant Cells were incubated in invasion chambers at 37^0C, 5% CO_2 for 48 hours After this time, the inner side of the invasion chamber was wiped with a wet cotton swab while the outer side was stained with 0 25% crystal violet for 10 minutes and then rinsed with PBS and allowed to dry Five fields at 10X magnification were counted for crystal violet stained cells per chamber

2.6 Overexpression Studies

eIF-4E cDNA in the BK episomal vector was a kind gift from Prof Arrigo DeBenedetti (Louisiana, USA) The 4E-HA and 4E S209-HA plasmids were gifts from Dr Rob Schneider (New York, USA) Both plasmids were obtained as a culture of pre-transformed cells resistant to geneticin (GEN) and ampicillin (AMP)

2 6 1 Plasmid Preparation

Cultures were streaked on LB agar containing 50μg/ml Ampicillin (Sigma, G9516) and incubated at 37^{0}C overnight From these, a single colony was inoculated into 10ml of LB Broth Ampicillin (50μg/ml) and grown overnight A 2ml sample of this suspension was then added to 200ml of TB Ampicillin 50μg/ml and left to grow overnight at 37°C for large-scale isolation of plasmid from transformed cells The following day the cells were pelleted for 15 mins at 2655xg The plasmid DNA was then isolated from the cells usin the Maxi-Mini Qiagen Plasmid DNA extraction Kit (Qiagen, 12143) The DNA concentration was determined by measuring the absorbance OD 260nm

2 6 2 Transfection of Cell Lines

2 6 2 1 Transient Transfections

On the day prior to transfections, cells to be transfected were plated from a single cell suspension and seeded into 25cm^2 flasks at 4x10^5 cells per flask

On the day of the assay the liposome transfection agent Fugene6 (Boehringer Mannheim, 1 814 443) was mixed with the DNA to be transfected in a 3 1 ratio and allowing 2 μg DNA/flask The components were mixed in 100 μl serum free medium (SFM) per flask to be transfected The Fugene6 was added to the SFM and care was taken not to allow it to touch the sides of the eppendorf The DNA was then added The components were mixed by gentle tapping and allowed to sit for 25 minutes at room temperature During this period, the medium was changed on the wells to be transfected and on the control wells After 25 minutes had elapsed, 100μl of the transfection mix was added to the flasks in a drop-wise fashion with constant swirling The transfections were carried out

over 72 hours and then the flasks were sampled for immunoimmunocytochemistry (72 hrs) or Western blot (at 24, 48 and 72hrs)

2 6 2 2 Generation of Stably Transfetcted Cell Lines

To generate stably transfected DLKP cells, four flasks were set up per transfection, i e three for transfection and one as a control The cells were seeded at 4 x 10^5 cells/flask and allowed to attach overnight The transfection was carried out using the liposome transfection agent Fugene6 (Boehringer Mannheim, 1 814 443) as described for transient transfections (Section 2 6 2 1) Antibiotic selection commenced 48 hours after transfection and the relevant cells and their controls were selected with initial concentrations of 200 µg/ml G418 (Sigma, G9516) The concentration of the Geneticin was incrementally increased over time until the contents of the control flasks were all dead The final selection concentrations reached was 800 µg/ml (G418)

2 6 2 3 Generation of Clonal Populations From Stably Transfected Mixed Populations

To generate clonal populations from stably transfected DLKP cells, the cells were diluted and plated out in 96-well plates, such that, the probability was that one cell would be found in every third well (i e a plating suspension of 3 3 cells/ml) These plates were incubated overnight at 37°C and at 5% CO_2 Twelve hours after plating, the plates were examined and wells containing single cells were highlighted and monitored Up to 20 clones were usually isolated and gradually expanded to 12-well plates, 6-well plates and eventually flasks

2 6 3 Quantification of DNA using a UV Spectrophotometer

The plasmid DNA was quantified using a UV spectrophotometer (Molecular Devices) at 260nm By simultaneous measurement of the OD at 280nm the relative purity of the sample could be ascertained The amount of DNA was calculated from the following formula

$$OD_{260} \times 50 \times \text{Dilution factor}/1000 = \text{DNA amount } (\mu g/\mu l)$$

The relative purity could be calculated using

$$\text{Purity} = OD_{260}/OD_{280}$$

Purity values of 1 8 – 2 would generally be expected

2.7 Immunocytochemical and Immunofluorescence Analysis

2 7 1 Immunocytochemical Analysis

The avidin-biotin complex (ABC) immunoperoxidase technique combined with the diaminobenzidine (DAB) visualisation procedure was used in all immunocytochemistry experiments The ABC method involves application of a biotin-labelled secondary antibody to cells probed with a primary antibody, followed by the addition of avidin-biotin-peroxidase complex which results in a high staining intensity due to the formation of an avidin-biotin lattice which contains peroxidase molecules The peroxidase enzyme then reacts with a DAB solution to give an insoluble, brown-coloured precipitate The formation of this brown precipitate-coloured precipitate is indicative of primary antibody reactivity

The procedure used is as follows

Cell preparations on 6-well tissue culture plates (which had been previously fixed in methanol and frozen at -20^{0}C) were allowed to thaw and equilibrate at room temperature A grease pen (DAKO, S2002) was used to encircle cells in the tissue culture plates to contain the various solutions involved during the procedure The cells were incubated for 5 minutes with a 3% H_2O_2 solution to quench any endogenous peroxidase activity that may be present in the cells and which could lead to false positive results The cells were then rinsed with UHP and placed in TBS for 5 minutes The plates were incubated for 20 minutes at room temperature (RT) with an appropriate serum diluted 1 5 in TBS to block non-specific binding This was removed and 30-50µl of optimally diluted primary antibody was administered The tissue-culture plates were placed on a tray containing moistened tissue paper and incubated at 37^{0}C for 2 hours The primary antibodies used in these studies are listed in Table 2 7 1 The wells were then rinsed in TBS/ 0 1% Tween x3 for 5 min each and then incubated for 30 min with a biotinylated secondary antibody diluted in TBS (Table 2 7 1) The wells were rinsed as before and incubated with strepABComplex/ Horse Radish Peroxidase (HRP) (DAKO, K377) for 30 min at RT, after which they were rinsed x3 in TBS/ 0 1% Tween The cells were then incubated with a DAB solution (DAKO, S3000) for 7-10 minutes Excess DAB solution was then

rinsed off with UHP water The wells were then mounted using a commercial mounting solution (DAKO, S3023)

Antibody	Host	Supplier	Dilution	Serum for Blocking
CK8	Mouse	Sigma (C5301)	1/300	Rabbit (Dako, X092)
CK18	Mouse	Sigma (C8451)	1/100	Rabbit
CK19	Mouse	Sigma (C6930)	1/50	Rabbit
FAK	Mouse	BD Transduction Laboratories (610088)	1/50	Rabbit
eIF-4E	Mouse	BD Transduction Laboratories (610270)	1/250	Rabbit
CBP	Rabbit	Santa Cruz (sc-369)	1/100	Goat
P300	Rabbit	Santa Cruz (sc-584)	1/100	Goat
C/EBPβ	Rabbit	Santa Cruz (sc-150)	1/100	Goat
ODC	Mouse	SIGMA (O1136)	1/100	Rabbit
β1 Integrin	Mouse	Serotech (MCA1188)	1/100	Rabbit
α1 Integrin	Mouse	Chemicon (MAB1973Z)	1/100	Goat
α2 Integrin	Mouse	Chemicon (MAB1950Z)	1/100	Goat
α3 Integrin	Mouse	Chemicon (MAB1952Z)	1/100	Goat
α5 Integrin	Mouse	Chemicon (MAB1956Z)	1/100	Goat
Alexa Fluor 488 Phalloidin	N/A*	Molecular Probes	1/20	N/A*
Anti Mouse (FITC)	Rabbit	Dako (F0261)	1/30	N/A
Anti Mouse (Biotin)	Rabbit	Dako (E0354)	1/300	N/A
Anti Rabbit (Biotin)	Goat	Dako (E0432)	1/300	N/A
Anti Goat (Biotin)	Rabbit	Dako (E0466)	1/300	N/A

Table 2 7 1 Antibodies used in Immunofluorescent/Immunocytochemical studies including the labelled secondary antibodies (*Alexa Fluor 488 Phalloidin is not an antibody it is a fluorescent derivative of the phallotoxin phalloidin)

2 7 2 Basic Immunofluorescence Analysis

Basic immunofluorescence was used for FAK staining, frozen 6-well sample plates were removed from the –20°C freezer and allowed to equilibrate to room temperature (~10 minutes) Grease circles (DAKO pen, DAKO Cat S2002) were then drawn within the wells (to contain the solutions used in the subsequent analysis) The cells were re-hydrated using 1x TBS for 5 minutes

This was tapped off and the relevant serum (listed in Table 2 7 1) (diluted 1/5) was added as a 'blocker' for 20 minutes at room temperature The serum was removed at this point and the primary antibody was applied (antibodies and dilutions are listed in Table 2 7 1) This was incubated overnight at 4°C in a moist environment The following day, the primary antibody was removed and the wells were washed three times in TBS-0 1% tween at 5 minutes per wash The fluorescent secondary antibodies were prepared in the dark room under dim conditions and were covered in foil upon dilution with TBS (They are light sensitive) The secondary antibodies (Table 2 7 1) were incubated for 60 minutes and the plates were wrapped in foil to maintain dim conditions All work from this point onwards was carried out in the dark to prevent 'quenching' fluorescent signal After 60 minutes incubation the antibodies were removed and the plates were washed three times in TBS-0 1% tween The wells were then mounted using fluorescent mounting medium (Dako, S3023) and covered with coverslips (Chance Propper, 22 x 22 mm)

2 7 3 Cell Fixation and Labelling for F-actin Fluorescence Analysis

Cells were plated on glass chamber slides (NUNC) and cultured at 37°C, 5%CO_2 for 5 days before use Cells were washed in PHEM buffer (PIPES 60mMol, HEPES 25mMol, EGTA 10mMol, $MgCL_2$ 2mMol) and fixed in 4% paraformaldehyde, 0 5% gluteraldehyde in PHEM for 20 mm at room temperature Cells were permeabilized with 0 1% Triton X-100 in PHEM Free aldehyde groups were reduced with 0 1% (w/v) sodium borohydride in PBS Cells were preincubated in PHEM containing 1% PBS for 30 minutes Alexa Fluor 488 Phalloidin methanolic stock was diluted 1/20 in PHEM containing 1% BSA for 20 mins at room temperature and this staining solution was then placed on cells for 30 mins at room temperature Cells were then washed twice in PHEM buffer The wells were then mounted using fluorescent mounting medium (Dako, S3023) and covered with coverslips (Chance Propper, 22 x 22 mm)

2 7 4 Confocal Microscopy

Cells fluorescently labelled with Phalloidin (F-actin) and FAK were analysed by confocal microscopy The fluorochromes (FITC and Alexa 488) were excited with a 488nm laser line generated from an ArKr laser Band pass filters and PMT detectors were optimised to detect the emitted fluorescence An xyz scan was performed for each sample and results were displayed as an average projection of the stack obtained

2 8 Western Blotting analysis

2 8.1 Sample Preparation

Cell pellets were taken from flasks of cells in culture following differentiation or transfection experiments These pellets were usually washed 2X in PBS, dried and stored at -80°C until needed

Cell pellets (Section 2 7 1) were lysed in TG lysis buffer [20mM Tris-HCl/1mM EGTA pH 8, 10% glycerol, 1% TritonX-100, 1 5mM $MgCl_2$, 0 137mM NaCl, 1mM Na_3VO_4, 1mM Pefabloc (Boehringer, 84500920-22)] and 1X Protease inhibitor cocktail (Boehringer, 1697498) on ice for 30 minutes followed by sonication in a Labsonic U (Braun) unit The cells were pulsed 3-5 times on ice using a repeating duty cycle of 0 3seconds Once 60-80% of the cells were determined to be lysed (by checking a sample of lysate under a microscope), the sonication was stopped The sonicated samples were then centrifuged at 664xg for 5 minutes on a benchtop microfuge to remove cellular debris The supernatant was carefully removed and aliquoted to eppendorf tubes Protein samples were stored at –20°C until required for quantification or analysis

2 8 2 Protein Quantification by DC Protein Assay

Protein quantification could be carried out using the DC protein assay (Biorad, 500-0116) which is based on the Lowry protein assay To quantify the protein extracted, serial dilutions of the protein were prepared in duplicate (all sample and standard dilutions were made in lysis buffer) and a standard curve was generated using serial dilutions of a 1mg/ml BSA (Sigma, A9543) stock A 5 µl volume of standards or samples were loaded into a clean 96-well plate To each well 25 µl of solution A (provided) was added followed by 200 µl solution B The plate was mixed gently and the colour was allowed to develop for 15 minutes The plates were quantified using a Spectra max plus plate reader (Molecular Devices) at 750 nm and the data was processed using Soft max Pro software

2 8 3 Acrylamide Gel Electrophoresis

Proteins were separated by SDS polyacrylamide gel electrophoresis (SDS PAGE) The resolving and stacking gels were prepared as outlined in Table 2 8 1 The gels were poured in to clean 10 cm x 8 cm gel cassettes consisting of glass plates separated by 0 75cm plastic spacers The resolving gel was poured initially, allowed to solidify and overlayed with the stacking gel Before the stacking gel was set, a comb was inserted to generate sample wells Gels were generally used immediately but could be stored at 4°C overnight if wrapped well in foil For most applications, 50 µg of protein was loaded to each well Samples were mixed with 5X loading buffer (6 25 ml 1 25 M Tris-HCl pH 6 8, 2 5 g SDS, 14 5 ml glycerol, 0 025% bromophenol blue, this was made up to 50 ml with H_2O) The samples were boiled for three minutes prior to loading and were run in parallel with protein size markers (Isis, P77085) The electrophoresis conditions were 250 V and 45 mA The gels were run for 1-1 5 hours (time depends on the size of the protein to be studied, i e larger proteins were run for longer)

Component	Resolving Gel (7 5%)	Resolving Gel (10%)	Stacking Gel (5%)
30 %Acrylamide Stock (Sigma, E344-500ML-C)	3 8 ml	5 ml	0 8 ml
UHP	8 ml	6 8 ml	3 6 ml
1 875 M Tris-HCl (pH 8 8)	3 ml	3 ml	-
1 25 M Tris-HCl (pH 6 8)	-	-	0 5 ml
10 % SDS	150 µl	150 µl	50 µl
10 % Ammonium Persulphate (Sigma, A1433)	60 µl	60 µl	17 µl
TEMED (Sigma, T8133)	9 µl	9 µl	6 µl

Table 2 8 1 Preparation of Acrylamide Gels

2 8 4 Western blotting

Following electrophoresis, gels were equilibrated in transfer buffer (25 mM Tris, 192 mM Glycine (Sigma, G7126) pH 8 3-8 5 without adjustment) for 15 minutes with agitation Protein gels were transferred to Hybond ECL nitrocellulose membrane (Amarsham, RPN 2020D) using semi-dry electroblotting (semi-dry transfer cell (Biorad)) For this, 8 sheets of Whatman 3 mm filter paper (Whatman, 1001824) were soaked in transfer buffer and placed on the cathode plate of a semi-dry blotting apparatus Excess air was removed from between the filters by rolling a glass pipette over the filter paper Nitrocellulose, cut to the same size of the gel, was soaked in transfer buffer and placed over the filter paper, making sure there were no air bubbles The acrylamide gel was placed over the nitrocellulose and 8 more sheets of pre-soaked filter paper were placed on top of the gel Excess air was again removed by rolling the pipette over the filter paper The proteins were transferred from the gel to the nitrocellulose at a current of 34mA at 15V for 20-25 mm

All incubation steps from now on, including the blocking step, were carried out on a revolving apparatus (Stovall, Bellydancer) to ensure even exposure of the nitrocellulose blot to all reagents

The nitrocellulose membranes were blocked for 1-2 hours at room temperature with fresh, filtered, 5% non-fat dried milk (Cadburys, Marvel skimmed milk) in TBS (8 76 g NaCl, 6 09 g Tris-HCL in 1L UHP)/ 0 1% Tween (Sigma P1379) pH 7 5

After blocking, the membranes were rinsed with TBS-tween and incubated with primary antibody (Table 2 5 3) overnight at 4^0C The primary antibody was removed and the membranes rinsed 3 times with TBS/ 0 1% Tween The membranes were then washed for 15 min and then twice for 5 minutes in TBS/ Tween Bound antibody was detected using enhanced chemiluminescence (ECL)

Following chemiluminescent detection, blots were again washed and blocked as described and re-probed for an internal standard eg GAPDH This was carried out as before

2 8 5 Enhanced Chemiluminescence Detection

Protein bands were developed using the Enhanced Chemiluminescence Kit (ECL) (Amersham, RPN2109) according to the manufacturer's instructions

Hoseradish Peroxidase Conjugated Secondary antibody (diluted appropriately, Table 2 8 6) was added to the blots with shaking for 1 hour at room temperature The secondary antibody was removed and the membranes were washed as before A sheet of cellophane was flattened over a smooth surface, eg a glass plate, making sure all air bubbles were removed The membrane was then placed on the cellophane, and excess fluid removed An equal volume of ECL (1 5ml) detection reagent 1 and reagent 2 were mixed and covered over the membrane Charges on the cellophane ensured the fluid stayed on the membrane The reagent was removed after one minute and the membrane wrapped in cellophane The membrane was exposed to autoradiographic film (Kodak, X-OMAT S, 500 9907) in an autoradiographic cassette for various times (depending on the level of signal) The autoradiographic film was then developed
The exposed film was developed for 5min in developer (Kodak, LX24) diluted 1 6 5 in water The film was briefly immersed in water and transferred to a Fixer solution (Kodak, FX-40) diluted 1 5 in water, for 5minutes The film was transferred to water for 5 min and then air-dried

2 8 6 Antibodies Used for Western Blotting

Antibody	Host	Supplier	Dilution *
FAK	Mouse	BD Transduction Laboratories (610088)	1/50
eIF-4E	Mouse	BD Transduction Laboratories (610270)	1/250
CBP	Rabbit	Santa Cruz (sc-369)	1/100
Anti-HA	Mouse	Roche (1666606)	1/1000
GAPDH	Mouse	Abcam (ab8245)	1/10,000
β-actin	Mouse	Sigma (A5441)	1/10,000
Mouse IgG	Sheep	Sigma (A6782)	1/1,000
Rabbit IgG	Goat	Sigma (A4914)	1/5,000
Goat IgG	Rabbit	Sigma (A5420)	1/1,000-1/5,000

Table 2 8 6 Antibodies used for Western blotting
*All antibodies were diluted in TBS

2 9 Proteomics-2D Gel Electrophoresis

2 9 1 Chemicals

Cy2, Cy3, Cy5, immobilized pH gradient strips, Ampholytes, were purchased from GE Healthcare Urea and iodoacetamide were bought from Fluka Chemical Corp (Milwaukee, WI) CHAPS, Tris and DTT were obtained from Sigma

2 9 2 Sample Preparation and Protein Labeling

Cells at ~80% confluence were washed twice in 0 5x phosphate-buffered saline, lysed in lysis buffer (4% (w/v) CHAPS, 7 M urea, 2M thiourea, 10 mM Tris-HCl, pH 8 5) and then homogenized by passing through a 25-gauge needle six times Insoluble material was removed by centrifugation at 20,817xg for 20 min at 10°C Protein concentration was determined using the BSA protein assay kit (Pierce) Cell lysates were labelled with *N*-hydroxy succinimidyl ester-derivatives of the cyanine dyes Cy2, Cy3 and Cy5 following the protocol described previously Typically, 50 µg of lysate was minimally labelled with 400 pmol of either Cy3 or Cy5 for comparison on the same 2D gel Labelling reactions were performed on ice in the dark for 30 min and then quenched with a 50-fold molar excess of free lysine to dye for 10 min on ice A pool of all samples was also prepared and labeled with Cy2 to be used as a standard on all gels to aid image matching and cross-gel statistical analysis The Cy3 and Cy5 labelling reactions (50 µg of each) from each lysate were mixed and run on the same gels with an equal amount (50 µg) of Cy2-labeled standard

2 9 3 Protein Separation by 2D Gel Electrophoresis and Gel Imaging

Immobilized linear pH gradient (IPG) strips, pH 4-7, were rehydrated in rehydration buffer (7 M urea, 2M thiourea, 2% CHAPS, 0 5% IPG Buffer, 50 mM DTT) overnight, according to the manufacturers guidelines Isoelectric focusing was performed using a IPGphor apparatus (GE Healthcare) for a total of 40 kV-h at 20°C, 50 mA Strips were equilibrated for 15 min in 50 mM Tris-HCl, pH 8 8, 6 M urea, 30% (v/v) glycerol, 1% (w/v) SDS containing 65 mM DTT and then for 15 min m the same buffer containing 240

mM iodoacetamide. Equilibrated IPG strips were transferred onto 18x 20-cm 12% uniform polyacrylamide gels poured between low fluorescence glass plates. Strips were overlaid with 0.5% (w/v) low melting point agarose in running buffer containing bromphenol blue. Gels were run using the Ettan Dalt 12 appartus (GE Healthcare) at 1.8 W/gel at 10°C until the dye front had run off the bottom of the gels (approximately 18h). All the images were collected on a Typhoon 9400 Variable Mode Imager (GE Healthcare). Statistics and quantitation of protein expression were carried out in Decyder software (GE Healthcare).

2.9.4 Spot digestion and Mass Spectrometric Analyses

Excision of protein spots, trypsin digestion and protein identification by mass spectrometric analysis using an Ettan MALDI-ToF Pro instrument from GE Healthcare was performed according to an established methodology. Preparative gels containing 300µg of protein were fixed in 30% (v/v) methanol, 7.5% (v/v) acetic acid overnight and washed in water and total protein was detected by post-staining with SyproRuby dye (Molecular Probes) for 3 h at room temperature or Colloidal Coomassie (Sigma) for 2 hours. Excess dye was removed by the appropriate destaining and washing methods. Sypro Ruby gels were imaged using a Typhoon 9400 Variable Mode Imager (Amersham Biosciences, Inc.) at the appropriate excitation and emission wavelengths for the stain. The subsequent gel image was imported into the BVA module of DeCyder software and was matched to images generated from DIGE analysis. Spots of interest were selected and confirmed using this software for subsequent picking using an Ettan Spot Picker. Colloidal Coomassie stained gels were scanned using an Image Scanner (GE Healthcare) flatbed scanner. The gel files were imported into ImageMaster 2D Platinum Version 5.0 software for subsequent spot detection. Spots of interest generated by DIGE analysis were matched to their counterparts in the ImageMaster software and these spot locations were exported for subsequent picking using an Ettan Spot Picker. Gel plugs were placed into a presilconized 1.5 mL plastic tube for destaining, desalting and washing steps. The remaining liquid above the gel plugs was removed and sufficient acetonitrile was added in order to cover the gel plugs. Following shrinkage of the gel plugs, acetonitrile was

removed and the protein-containing gel pieces were rehydrated for 5 min with a minimal volume of 100 mM ammonium bicarbonate An equal volume of acetonitrile was added and after 15 min of incubation, the solution was removed from the gel plugs and the samples then dried down for 30 min using a vacuum centrifuge Individual gel pieces were then rehydrated in digestion buffer (12 5ng trypsin per µl of 10% Acetromtrile 40mM Ammonium Bicarbonate) to cover the gel pieces More digestion buffer was added if all the initial volume had been absorbed by the gel pieces Exhaustive digestion was carried out overnight at 37 °C After digestion, the samples were centrifuged at 12,000 g for 10 min using a bench top centrifuge The supernatant was carefully removed from each sample and placed into clean and silconized plastic tubes Samples were stored at -70 °C until analysed by MS For MALDI-Tof analysis, mixtures of tryptic peptides from individual samples were desalted using Millipore C-18 Zip-Tips (Milhpore) and eluted onto the sample plate with the matrix solution [5 mg/mL a-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0 1% trifluoroacetic acid (v/v)] Mass spectra were recorded using the MALDI ToF instrument operating in the positive reflector mode at the following parameters accelerating voltage 20 kV, and pulsed extraction on (focus mass 2500) Internal and external calibration was performed using trypsin autolysis peaks at m/z 842 50, m/z 2211 104 and Pep4 mix respectively The mass spectra were analysed using MALDI evaluation software (Amersham Biosciences) and protein identification was achieved with the PMF Pro-Found search engine for peptide mass fingerprints Peptide samples of insufficient abundance for MALDI-Tof identification were subjected to LC-MS/MS using an Ettan MDLC system (GE Healthcare) attached to an LTQ mass spectrometer (Thermo Electron) with an ESI ion source Peptide sequences were identified using SEQUEST algorithm incorporated into BioWorks software (version 3 1) (Thermo Electron) and the Swiss-Prot human protein database

2 9.5 Silver staining of 2D electrophoresis gels

All cell lysates to be used for DIGE analysis were screened initially by silver staining to ensure that these samples were of good quality Cell samples for silver staining were prepared as for DIGE analysis but are not labelled with Cy dyes Samples are separated by 2D Gel Electrophoresis as for Cy Dye labelled proteins and processed as follows

- Fixing gels are placed in fixing solution for a minimum of 30 minutes
- Washing Using the outlet on the gel box, the fixing solution was carefully drained out A volume of water (150 ml) was added to rinse and the gel was placed on a belly dancer for five minutes The gel was rinse a total of three times
- Sensitizing After the third wash, the gel box was drained and approximately 200 ml of sensitizing solution was added before returning gel to the belly dancer for 30 minutes
- Washing Then using the method outlined above, three 10-minute washes were carried out with distilled water
- Silver Reaction Following the washes, silver reaction solution (200 ml) was added and the gel returned to the belly dancer for 20 minutes
- Washing Following the silver step, a further two washes were carried out Each wash was a minimum of 5 minutes
- Developing Developer (200 ml) was added to the gel and allowed to develop on the belly dancer until protein spots appear, something in the region of 20 minutes
- Stopping When the desired amount of spots had appeared on the gel, the developing was stopped with the addition of stopping solution (200ml) and the gel returned to the belly dancer for 10 minutes
- Storage For medium to long-term storage the gels were kept in distilled water All equipment used in the procedure was cleaned using warm water and detergent, before rinsing with distilled water

2 9 5 1 Preparation of 1L of Fixing solution (Required for 6 gels)

A volume of 400ml of ethanol and 100 ml of acetic acid were added to a 1L of distilled water and mixed well

2 9 5 2 Preparation of 1L of Sensitizer solution (Required for 6 gels)

To 300ml of ethanol, 2g sodium thiosulphate, and 68g sodium acetate were added together in 1L of distilled water and mixed with a stirring bar until in solution

2 9 5 3 Preparation of 1L of Silver solution (Required for 6 gels)

To 1L of distilled water, 2 5g of silver nitrate was added and mixed well Immediately before use 400µl of formaldehyde was added and the solution mixed well

2 9.5.4 Preparation of 1L of Developer solution (Required for 6 gels)

To 1L of distilled water, 25g of sodium carbonate was added and mixed by swirling

2 9 5 5 Preparation of 1L of Stopper solution (Required for 6 gels)

To 1L of distilled water, 14 6g of Na_2EDTA was added and mixed well by swirling

2.10 RNA Analysis

2.10.1 Preparation of RNA for Analysis

RNA is easily degraded by RNase enzymes which are ubiquitous, thus the following precautions were taken prior to RNA work.

All solutions for RNA related work, i.e. that would come in to contact with the RNA, were prepared from sterile UHP that had been treated with 0.1% diethyl pyrocarbonate (DEPC) (Sigma, D5758) before autoclaving. Solutions for RNA work were made in bottles that had been baked at 180°C for 8 hours or more. All eppendorfs PCR tubes used *etc.* were RNase free and pre-autoclaved prior to use as were Gilson pipette tips. Disposable nitrile gloves were worn at all times during RNA work (to protect the operator and to prevent RNase degradation). The gloves were changed frequently during RNA manipulation.

2.10.2 RNA Isolation Using TriReagent

RNA was extracted from cultured cells in 75cm^2 flasks. Cells were initially trypsinised, washed in PBS A, pelleted and then lysed in TriReagent and stored at –80°C until required.

The following protocol outlines the method whereby pure RNA was isolated from TriReagent. The frozen TriReagent samples were allowed to thaw at room temperature and upon thawing, were allowed to sit for at least 5 minutes to ensure complete dissociation of nucleoprotein complexes. A volume of 0.2 ml of chloroform was added per ml of TriReagent in the sample. This was shaken vigorously for 15 s and the samples were allowed to stand for 15 minutes at room temperature. The sample was then centrifuged at 17,949xg for 15 minutes at 4 °C. Following centrifugation, the sample separated in to three layers, the upper aqueous layer containing the RNA, the interphase (DNA) and the red layer containing the protein. The upper layer was thus carefully

removed to a fresh eppendorf and 0 5 ml of isopropanol (Fluka, 59304) was added to the aqueous RNA solution per ml of TriReagent used initially The tubes were mixed well and allowed to stand at room temperature (15-20 minutes) This effected the precipitation of the RNA, which was then recovered by centrifugation at 17,949xg for 10 minutes The RNA was washed (2X) in 75% EtOH and air dried for 5-10 minutes The recovered RNA pellet was then dissolved in 12-20 µl DEPC treated H_2O with repeated pipetting To aid re-suspension of the RNA, the sample was also heated to 55°C for 10 minutes followed by cooling on ice RNA was aliquoted and stored at –80°C until required

2 10 2 1 RNA Purification for Oligonucleotide Microarray Analysis

For reproducible results using Affymetrix Oligonucleotide Microarrays, the initial RNA had to be of extremely high quality, thus the RNeasy (Qiagen 74104) system was used to further purify RNA samples isolated using TriReagent The RNeasy purification kit is based on the guanidine thiocyanate method of RNA extraction The procedure was performed according to manufacturers instructions

2 10 3 RNA Quantification

RNA (like DNA) was quantified using a UV spectrophotometer The OD_{260} was used to quantify the RNA in the sample using the following equation

$$OD_{260} \times 40 \times \text{Dilution Factor}/1000 = \text{RNA content } (\mu g/\mu l)$$

By simultaneously measuring the OD_{280} the purity of the sample could be estimated

$$\text{Purity} = OD_{260}/OD_{280}$$

This was typically in the range of 1 8-2 0 A ratio of <1 6 indicated that the RNA may not be fully in solution The RNA was diluted to 1 µg/µl stocks for reverse transcription (RT-reaction)

2 10 4 Gel Electrophoresis of RNA to Determine Quality

All solutions to be used for the gel electrophoresis of RNA were prepared in DEPC treated water including the electrophoresis buffer and the gels The samples (containing approximately 5 µg RNA/well) were run on 0 8% agarose gels To sink the RNA in to the wells, pre-autoclaved glycerol was mixed with the RNA to a final concentration of 10% (v/v)

2 10 5 Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Analysis

2 10 5 1 Reverse Transcription of RNA (cDNA Synthesis)

To form cDNA, the following components were mixed in a 0 5 ml eppendorf tube (Eppendorf, 0030 121 023) and heated to 70 °C for 10 minutes followed by cooling on ice

1 µl Oligo dT 12-18 primers (0 5 µg/µl)
1 µl RNA (1 µg/µl)
3 µl DEPC H_2O

This step gets rid or RNA secondary structure and allows the oligo dT to bind the poly $(A)^+$ tail of the RNA
As this mixture was heating the following reaction mix was generated (all volumes listed in master mix assume 1 µg total RNA)

4 µl 5X buffer (Sigma, P2317)
2 µl 100 mM DTT (Sigma, D6059)
1 µl RNasin (40 U/µl) (Sigma, R2520)
1 µl dNTPs (10 mM each) (Sigma, DNTP-100)
6 µl DEPC H_2O
1 µl MMLV-RT (200U/µl) (Sigma, M1302)

Once the RNA mixture had cooled (~2 minutes) 15 µl of the master mix was added and mixed by flicking The resultant mixture was given a rapid centrifuge to collect the material in the bottom of the tube and then incubated at 37°C for 1 hour The resultant cDNA was stable at 4°C but for prolonged storage was maintained at –20°C

2 10 5.2 Polymerase Chain Reaction (PCR)

The cDNA was then analysed for the expression of genes of interest by PCR

The standardised PCR mix is listed below and did not change significantly with any of the PCRs carried out in this thesis

12 25 µl H_2O

2 5 µl 10X PCR buffer (Sigma, P2317)

1 5 µl 25 mM $MgCl_2$ (Sigma, M8787)

4 µl 1 25 mM dNTP

0 5 µl each of the forward and reverse primers (250 ng/µl) for the target gene (Oswel DNA Service, Southhampton)

0 5 µl each of the forward and reverse primers to the housekeeping gene of interest i e β Actin (25 ng/µl), (also synthesised by Oswel DNA Service)

0 25 µl Taq Polymerase (5U/µl) (Sigma, D4545)

The samples were mixed and centrifuged before being placed on the thermocycler (Biometra)

The PCR protocol used is outlined below

95 °C for 3 minutes (Denaturation step)

25-30 cycles of

95 °C for 30 s (Denaturation)

52-60 °C for 30 s (Annealing)

72 °C for 30 s (Extension)

and

72 °C for 7 minutes (Extension)

PCR products were stored at 4 °C until they were analysed by gel electrophoresis

2 10 5 3 Gel Electrophoresis of PCR Products

Typically 2% agarose (Sigma, A9539) gels were used for PCR gel electrophoresis, these gels were prepared and run in 1X TBE (10 8g Tris base, 5 5 g Boric Acid, 4 ml 0 5M EDTA and made up to 1L with UHP) and were melted in a laboratory microwave Upon cooling, the gel was supplemented with 5 µl ethidium bromide (10 mg/ml) (to allow visualisation of the DNA The gel was then poured in to the electrophoresis unit (Biorad) and allowed to set By placing a comb in to the top of the gel prior to hardening, sample wells were formed

To run the samples, 2 µl of 6X loading buffer (50% Glycerol, 1 mg/ml bromophenol blue, 1 mM EDTA) was added to 10 µl PCR product and loaded to the gel with an appropriate size marker (Sigma, D0672) The gels were electrophoresed at 120-150 mV for 1-2 hours (depending on size of the target gene, i e to get adequate separation) Once the internal control and target bands were seen to have migrated to the required extent, the gel was taken to the gel analyzer (an EpiChemi II Darkroom, UVP Laboratory Products) and photographed

2 10 6 Affymetrix GeneChip® Oligonucleotide Microarray Analysis

The microarray gene expression experiments which were performed in this body of work were performed using Affymetrix® Human Genome U133A GeneChips® Affymetrix GeneChip probe microarrays are manufactured using technology that combines photolithography and combinatorial chemistry Tens to hundreds to thousands of different oligonucleotide probes are synthesised and each of these oligonucleotides is located in a specific area on the microarray slide, called a probe cell Each probe cell contains millions of copies of a given oligonucleotide and each feature size on the Affymetrix U133A GeneChip is 18 microns Due to advances in microarray design Affymetrix have since launched a new GeneChip, U133 Plus 2, which has decreased the feature size of the probes from 18 microns to 11 microns The new U133 Plus 2 GeneChips are now comprised of the old Affymetrix U133A and U133B GeneChips on a single slide The reduction in feature size to 11 microns has resulted in an increase in feature definition, with improved sharpness and signal uniformity

The most important aspect in efficient probe design is the quality of the sequence information used Probe selection and array design are two major factors in reliability, sensitivity, specificity and versatility of expression probe arrays Probes selected for gene expression arrays by Affymetrix are generated from sequence and annotation data obtained from multiple databases such as GenBank, RefSeq and dbEST Sequences from these databases are collected and clustered into groups of similar sequences Using clusters provided by UniGene database as a starting point, sequences are further subdivided into subclusters representing distinct transcripts

This categorisation process involves alignment to the human genome, which reveals splicing and polyadenylation variants The alignment also extends the annotation information supplied by the databases pinpointing low quality sequences These areas are usually trimmed for subsequent generation of high quality consensus sequences or

alternatively Affymetrix employ quality ranking to select representative sequences, called exemplars, for probe design

In general, Affymetrix use 11 to 16 probes which are 25 bases in length for each transcript The probe selection method used by Affymetrix for their U133 GeneChips takes into account probe uniqueness and the hybridisation characteristics of the probes which allow probes to be selected based on probe behaviour Affymetrix use a multiple linear regression (MLR) model in the probe design that was derived from thermodynamic model of nucleic acid duplex formation This model predicts probe binding affinity and linearity of signal changes in response to varying target concentrations An advantage of this type of model-based probe selection system is that it provides a physical and mathematical foundation for systematic and large-scale probe selection Also, an essential criterion of probe selection by Affymetrix for quantitative expression analysis is that hybridisation intensities of the selected probes must be linearly related to target concentrations

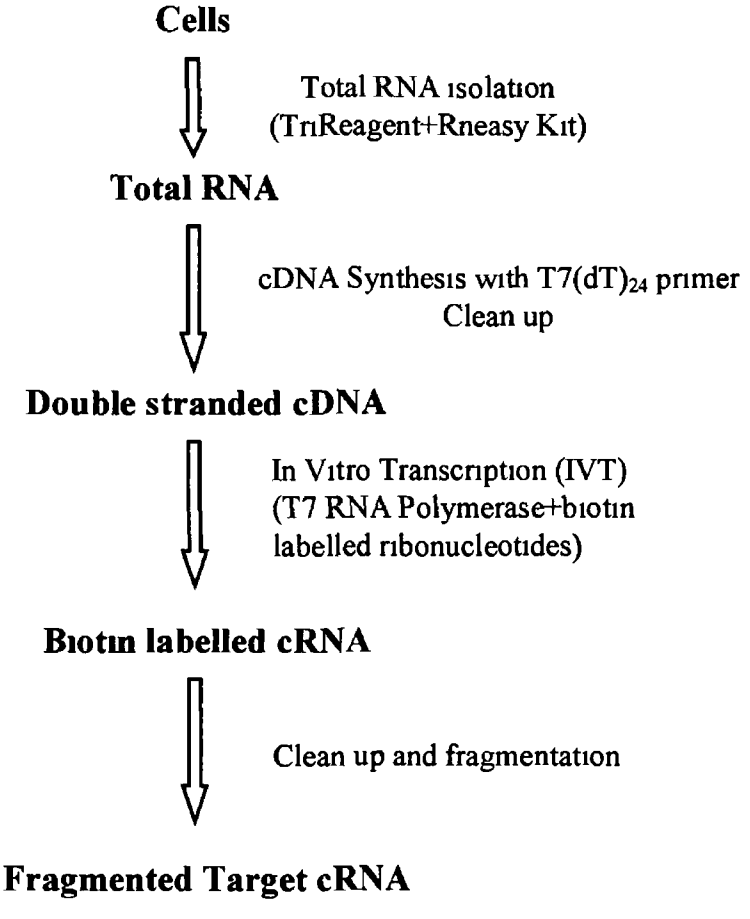
A core element of Affymetrix microarray design is the Perfect/Mismatch probe strategy For each probe that is designed to be perfectly complimentary to a given target sequence, a partner probe is also generated that is identical except for a single base mismatch in its center These probe pairs, called the Perfect Match probe (PM) and the Mismatch probes (MM), allow the quantitation and subtraction of signals caused by non-specific cross-hybridisation The differences in hybridisation signals between the partners, as well as their intensity ratios, serve as indicators of specific target abundance

2 10 6 1 Preparation of samples for microarray analysis

RNA was extracted from 4E-HA 5, 4E S209-HA 6 and pcDNA 2 cells using TriReagent (section 2 10 2) and was then further purified using RNeasy spin columns (Qiagen) (Section 2 10 2 1) RNA quality was verified by OD 260/280 readings and by analysis on the Agilent 2100 Bioanalyser (Fig 3 1 2 12 2)

Fragmented biotin labelled target cRNA was generated from 10 μg of total RNA (Section 2 10 6 2) and was then used to hybridise to Affymetrix HGU133A GeneChip arrays Fragmented and unfragmented cRNA quality was confirmed by agarose gel electrophoresis (Section 2 10 4)

Preparation of Target cRNA for Affymetrix GeneChips



2 10 6 2 Sample and Array Processing

After RNA isolation, quantification and purification using the Qiagen Rneasy isolation method (Section 2 10 1-4), cDNA was synthesised using the GeneChip T7-Oligo (dT) Promoter Primer Kit (Affymetrix, 900375) from 10μg total RNA First strand cDNA synthesis was then performed using the SuperScript Choice Kit (BioSciences, 11917-010) First strand cDNA synthesis involved 'primer hybridisation' where the T7-Oligo (dT) primer was incubated with the RNA and DEPC-treated H_2O at 70^0C for 10 mins, followed by a short incubation in ice, 'temperature adjustment' where 5X first strand buffer, DTT and dNTP mix were added to the RNA mix and incubated at 42^0C for 2 mins and 'First Strand synthesis' where SuperScript II RT was added to the mix and incubated at 42^0C for 1 hour Second strand cDNA synthesis was performed and purified using GeneChip Sample Cleanup module (Affymetrix, 900371) as recommended by the manufacturers instructions

cRNA was then synthesised and biotin-labelled using the Enzo BioArray HighYield RNA Transcript Labelling Kit (Affymetrix, 900182) Biotin-labelled cRNA was purified using the GeneChip Cleanup Module Kit (Affymetrix, 900371) and quantified The value obtained was adjusted to reflect carryover of unlabelled total RNA A sample of biotin-labelled cRNA was taken for gel electrophoresis analysis The labelled cRNA was then fragmented before hybridisation onto the Affymetrix GeneChip probe microarrays The aliquot of fragmented sample RNA was stored at -20^0C until ready to perform the hybridisation step

Hybridisation of cRNA onto the Affymetrix GeneChip probe human microarrays (Affymetrix, HU133A and HU133 Plus 2) was performed in the Conway Institute, University College Dublin, where the Affymetrix Hybridisation Oven and Fluidics Station is set up along with the Affymetrix GeneChip Scanner, which exported the data directly into the Affymetrix analysis software, MicroArray Suite 5 1 (MAS 5 1)

2 10 6 3 Processing and analysis of microarray expression data

I would like to thank Eoin Ryan who performed the processing, statistical analysis and clustering of Microarray Data in these experiments

The processing and analysis of the expression data was conducted as follows
The expression data from the 9 GeneChips were scaled to 100 using Affymetrix MAS 5 0 software This process adjusts the trimmed mean of each data set to 100 so that different GeneChips are directly comparable This is known as a linear normalisation
After this step, various QC parameters were extracted from the expression data and plotted as in Figure 3 1 2 12 4

One of the most important of the QC parameters is the Scaling Factor Affymetrix technical support advises that chips should not be compared if the Scaling Factors show more than a three-fold difference All QC parameters were found to be within acceptable limits
Scaled gene expression data were subsequently exported into Genespring expression data analysis software Further normalisation of array data was conducted prior to expression analysis The median value of each probe set from the control samples (pcDNA 2) was given a value of 1 This meant that a probe set with an average two fold increase in either of the other experimental samples would have a 'normalised value of 2' after this normalisation A probe set with a two-fold downregulation would have a normalised value of 0 5
At this stage, there were 22,283 genes (probe sets) in the analysis An initial filter was applied to remove genes that are not flagged as present in at least three of the nine samples analysed These genes were further filtered to remove genes that do not cross a two-fold threshold (up or down) across the experiment
This left 927 genes that appeared to be more than 2-fold up or down-regulated in 4E-HA 5 or 4E 209-HA 6 cells in comparison to pcDNA 2 control cells A Welch ANOVA statistical test with a p-value cut off of 0 05 was used to find statistically significant genes

Genes that passed the statistical test were clustered using the Pearson Correlation (settings Separation 1, Minimum Distance 0 001, merge similar branches) This clustering was used to generate a heat map graphical representation (Fig 3 1 2 12 5) of differentially expressed genes grouped (clustered) according to their expression pattern

3.0 Results

3 1 Overexpression of eIF4E in DLKP

Previous studies in this laboratory have shown that treatment of DLKP cells with the differentiation modulating agent 5'-bromo-2'-deoxyuridine (BrdU) induces post-transcriptional upregulation of the expression of growth and differentiation related proteins Treatment of DLKP cells with BrdU induces post-transcriptional upregulation of expression of the cytokeratins 8, 18, 19, and also β1 integrin (Meleady and Clynes 2000, 2001, McBride *et al*, 1999) Treatment of DLKP cells with BrdU was also found to induce increased expression and phosphorylation of the translation initiation factor eIF4E As eIF4E is known to be involved in translational regulation of gene expression it was decided to analyse the effects of increased eIF4E levels on growth and gene expression in these cells

3.1.1 Overexpression of eIF4E in BK shuttle vector (BK-4E)

For initial DLKP eIF4E overexpression experiments, DLKP cells were stably transfected with eIF4E cDNA in a BK episomal shuttle vector (BK-4E) DLKP cells were also stably transfected with an empty BK shuttle vector as a control (DLKP-BK) These were kind gifts from Prof Arrigo DeBenedetti, Louisianna, U S A (DeBenedetti and Rhoads, 1990) Stably transfected cells were selected with geneticin (800 μg/ml)

Immunocytochemical characterisation of eIF4E, Keratin 8, and Ornithine Decarboxylase (ODC) expression was carried out on uncloned bulk populations of BK-4E and DLKP-BK cells (Figs 3 1 1 1-3 1 1 3) Immunocytochemical analysis showed increased expression of eIF4E, Keratin 8 and ODC in BK-4E transfected DLKP cells ODC expression has previously been shown to be translationally regulated by eIF4E (Shantz *et al*, 1996)

Several unsuccesful attempts were made at generating clonal populations of BK-4E and and DLKP-BK cells These cells did not survive after limiting dilution and studies were discontinued with these cells

(A) DLKP-BK

(B) BK-4E

Figure 3.1.1.1 Immunocytochemical analysis of eIF4E expression in BK-4E, eIF4E overexpressing DLKP cells. eIF4E expression is increased in BK-4E cells (B), compared to DLKP-BK control cells (A).

(A) DLKP-BK

(B) BK-4E

Figure 3.1.1.2 Immunocytochemical analysis of Ornithine Decarboxylase (ODC) expression in BK-4E, eIF4E overexpressing cells. ODC expression is increased in BK-4E cells (B), compared to DLKP-BK control cells (A).

(A) DLKP-BK

(B) BK-4E

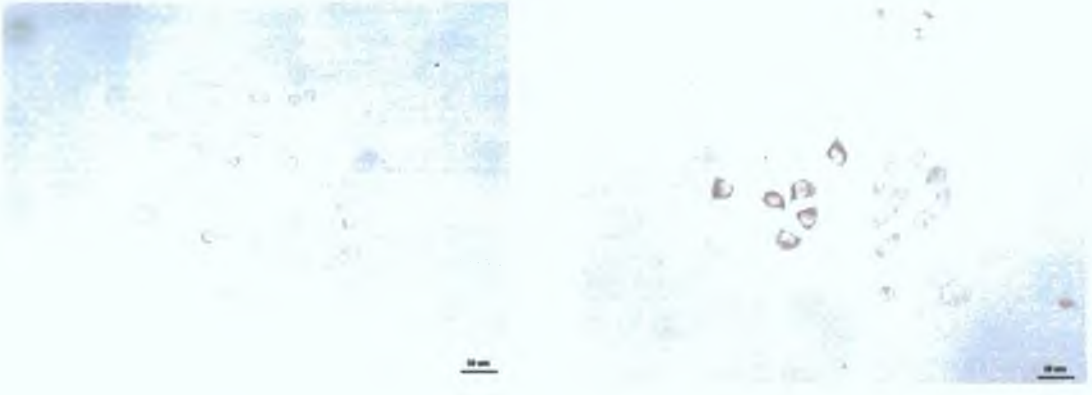


Figure 3.1.1.3 Immunocytochemical analysis of Keratin 8 expression in BK-4E, eIF4E overexpressing cells. K8 expression is increased in BK-4E cells (B), compared to DLKP-BK control cells (A).

3 1 2 Overexpression of HA tagged eIF4E (4E-HA) in DLKP cells

DLKP cells were subsequently stably transfected with plasmids expressing influenza hemagglutinin epitope (HA)-tagged wild type eIF4E (4E-HA) and also a mutant of eIF4E that cannot be phosphorylated at Ser 209, Ser 209→Ala eIF4E (4E S209-HA) The 4E-HA and 4E S209-HA plasmids were gifts from Dr Rob Schneider, New York, USA (Cuesta et al, 2000) These HA-tagged eIF4E constructs have been shown to be effective in regulating protein translation in previous experiments (Cuesta et al, 2000) Ser 209 is the main regulatory phosphorylation site of eIF4E but its exact role and importance in translational control is unknown DLKP cells were also transfected with an empty pcDNA plasmid vector as a control

3 1 2 1 Transient Transfection of 4E-HA in DLKP cells

DLKP cells were transiently transfected with pcDNA contol, 4E-HA and 4E S209-HA cDNAs Cells transiently transfected for 24, 48 and 72 hrs were analysed by Western blot using anti-eIF4E and anti-HA antibody (Figs 3 1 2 1-3 1 2 6) Western blot analysis shows 4E-HA and 4E S209-HA proteins were effectively expressed in transiently transfected cells, though 4E S209-HA protein appears to be expressed at a lower level than 4E-HA protein

eIF4E, Keratin 8, β1 integrin and Ornithine decarboxylase expression in transiently transfected cells 72hrs post-transfection was analysed by immunocytochemistry (Figs 3 1 2 7-3 1 2 10)

Immunocytochemical analysis of eIF4E levels was performed using anti-eIF4E antibody, which detects both endogenous wild-type eIF4E and transfected HA-tagged eIF4E (Fig 3 1 2 7) Staining for eIF4E was increased in 4E-HA transiently transfected cells compared to pcDNA control transfected cells eIF4E expression appeared to be heterogenous in these cells with some goups of cells staining more intensely than others Staining for eIF4E in 4E S209-HA transfected cells did not appear to be stronger than in pcDNA controls This is likely to be due to the lower levels of transfected protein in these cells (Fig 3 1 2 3) which may not be detected by immunocytochemistry

Immunocytochemical analysis of Keratin 8 levels in 4E-HA transiently transfected cells shows increased expression (Fig 3 1 2 8) Keratin 8 staining is heterogenous in these cells, which correlates with staining for eIF4E Keratin 8 shows intense staining in certain 4E-HA transfected cells Keratin 8 staining in 4E S209-HA transiently transfected cells shows no increase compared to pcDNA control tranfected cells (Fig 3 1 2 8)

β1 integrin expression in 4E-HA and 4E S209-HA transiently transfected cells shows no increase in staining for β1 integrin compared to pcDNA transfected cells (Fig 3 1 2 9)

ODC expression was increased in 4E-HA transiently transfected cells but not in 4E S209-HA transfected cells (Fig 3 1 2 10)

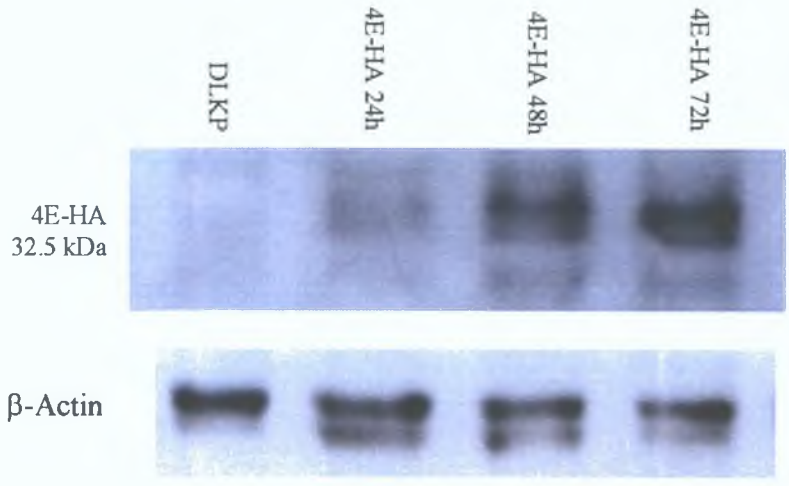


Figure 3.1.2.1 Western Blot analysis of 4E-HA levels in 4E-HA transiently transfected DLKP cells using anti-HA antibody. 4E-HA protein is highly expressed in transiently transfected DLKP cells after 48 and 72 hrs

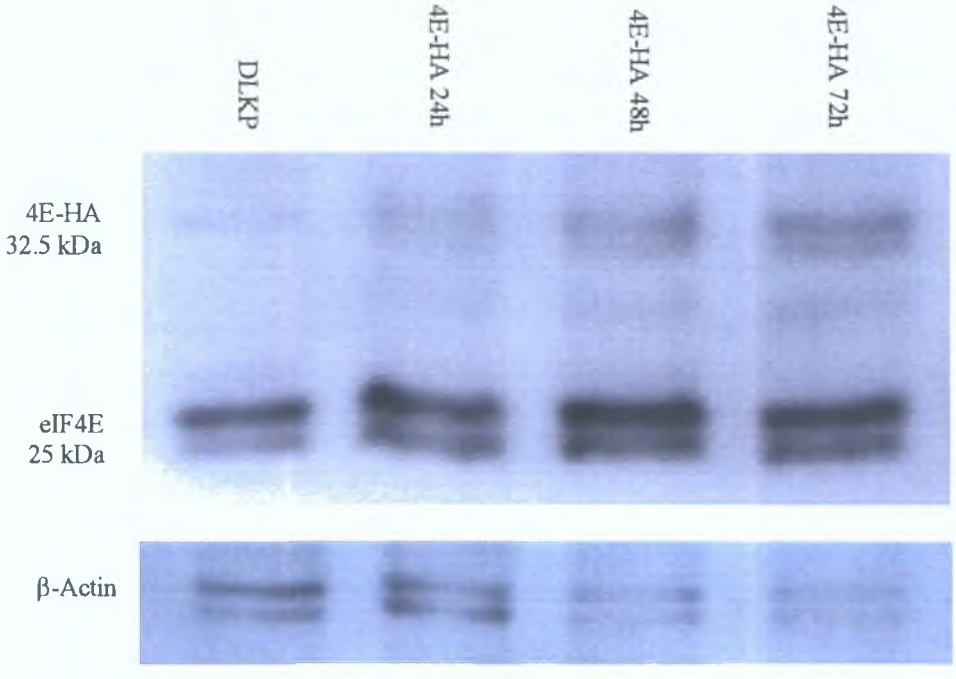


Figure 3.1.2.2 Western Blot analysis of 4E-HA transiently transfected DLKP cells using anti-eIF4E antibody. Anti eIF4E antibody detects wild type eIF4E protein (25 kDa) and HA-tagged transfected eIF4E (32.5 kDa). 4E-HA protein is expressed in transiently transfected DLKP cells.

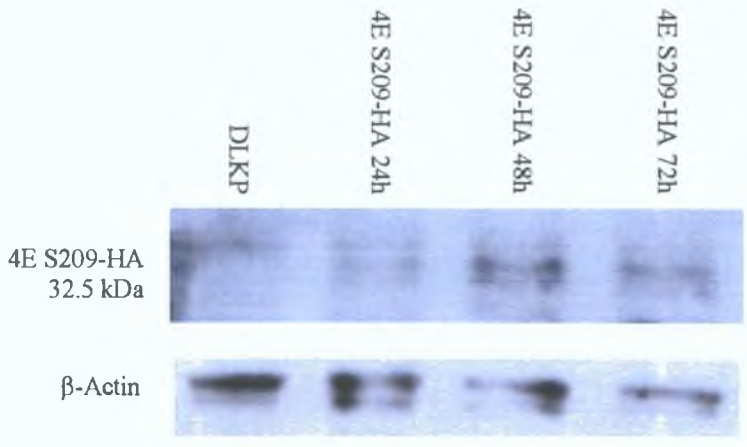


Figure 3.1.2.3 Western Blot analysis of 4E S209-HA levels in 4E-HA transiently transfected DLKP cells using anti-HA antibody. 4E S209-HA protein is expressed in transiently transfected DLKP cells.

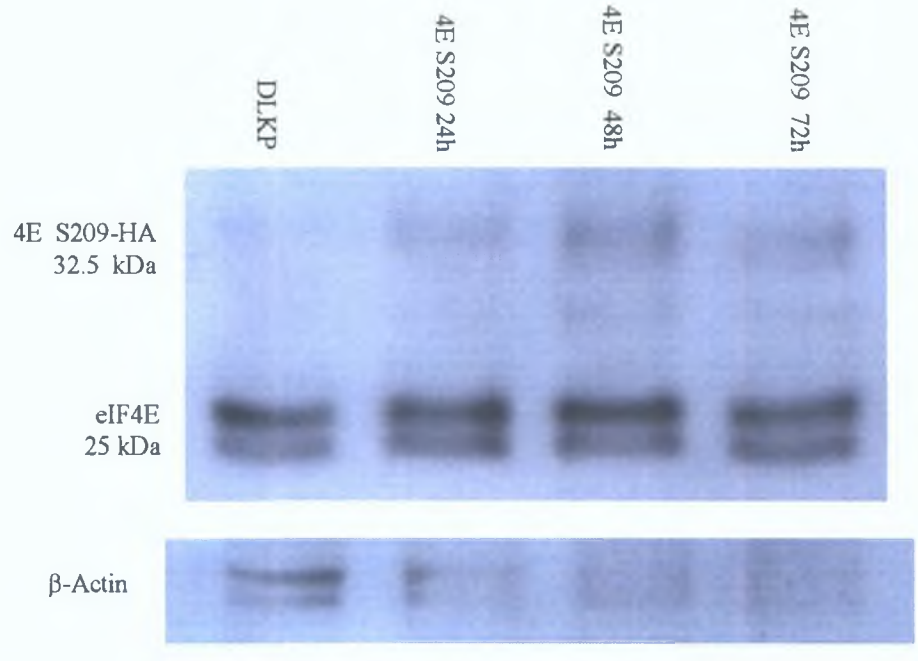


Figure 3.1.2.4 Western Blot analysis of 4E S209-HA transiently transfected DLKP cells using anti-eIF4E antibody. Anti eIF4E antibody detects wild type eIF4E protein (25 kDa) and HA tagged transfected eIF4E (32.5 kDa). 4E S209-HA protein is expressed in transiently transfected DLKP cells.

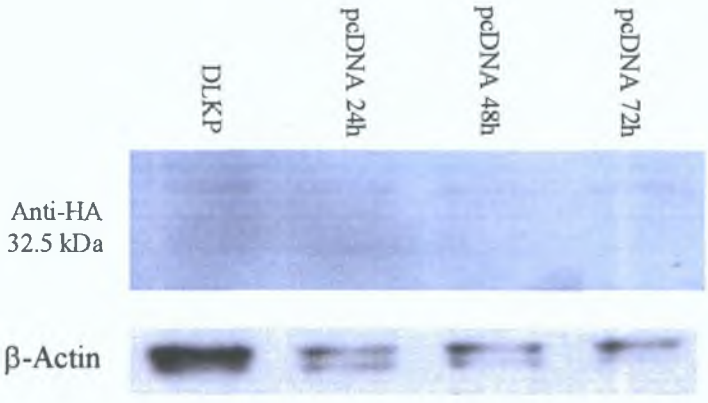


Figure 3.1.2.5 Western Blot analysis of pcDNA transiently transfected DLKP cells using anti-HA antibody. No 4E-HA protein is present in pcDNA transiently transfected cells.

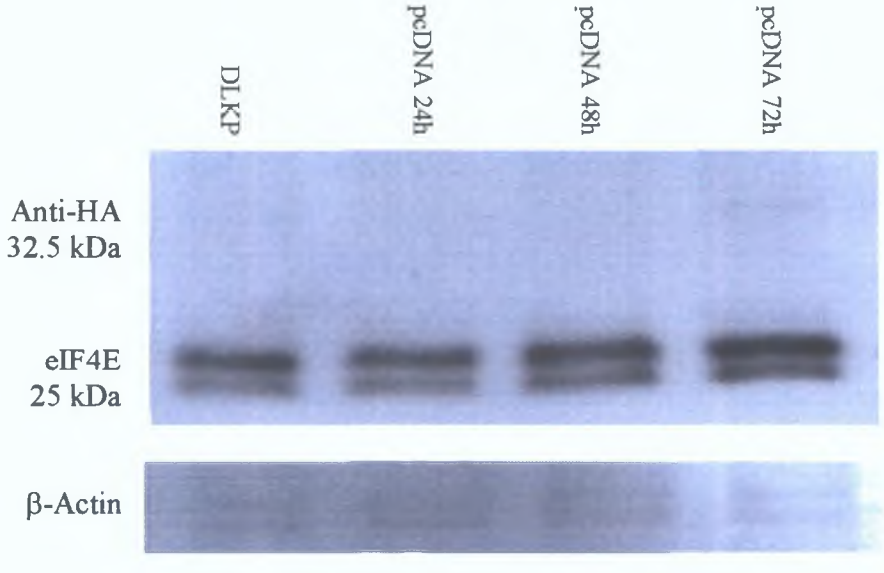
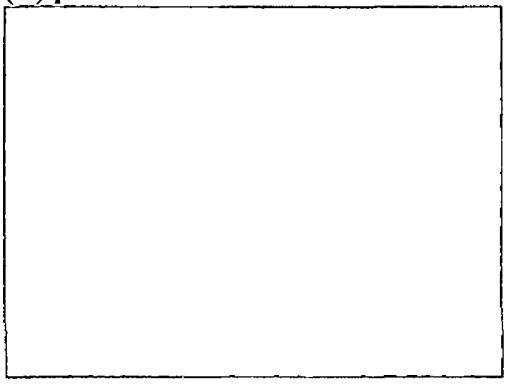
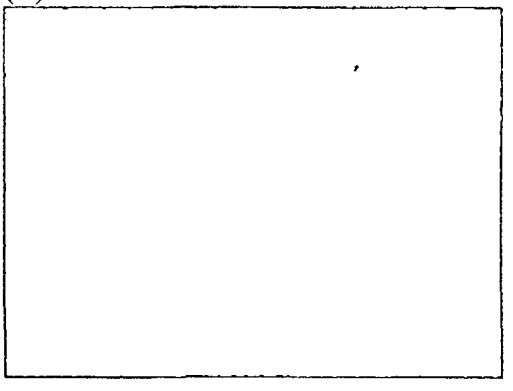


Figure 3.1.2.6 Western Blot analysis of pcDNA transiently transfected DLKP cells using anti-eIF4E antibody. Anti eIF4E antibody detects wild type eIF4E protein (25 kDa) and HA tagged transfected eIF4E (32.5 kDa). No 4E-HA protein is present in pcDNA transiently transfected cells.

(A) pcDNA 72 hr



(B) 4E S209-HA 72 hr



(C) 4E-HA 72hr

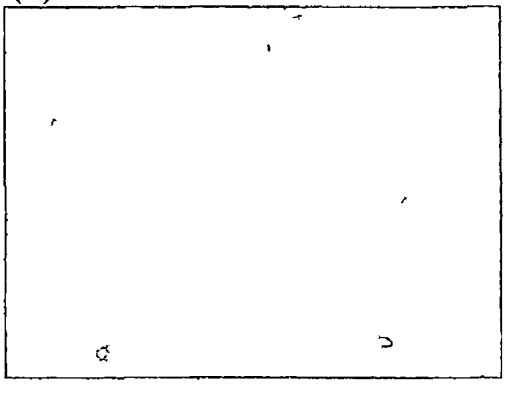


Figure 3 1 2 7 Immunocytochemical Analysis of eIF4E expression in 4E-HA transiently transfected DLKP cells Anti eIF4E antibody was used to stain transiently transfected cells Anti eIF4E antibody detects both endogenous wild type eIF4E and HA tagged transfected eIF4E eIF4E expression is increased in 4E-HA transiently transfected cells compared to pcDNA controls (A) and 4E S209-HA transfected cells (B) 4E-HA transfected cells display heterogenous staining for eIF4E (20X magnification)

(A) pcDNA 72hr

(B) 4E S209-HA 72hr

(C) 4E-HA 72hr

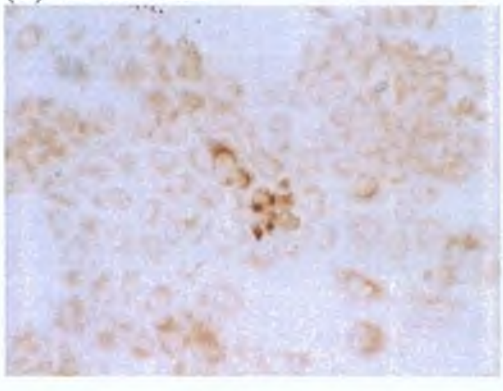
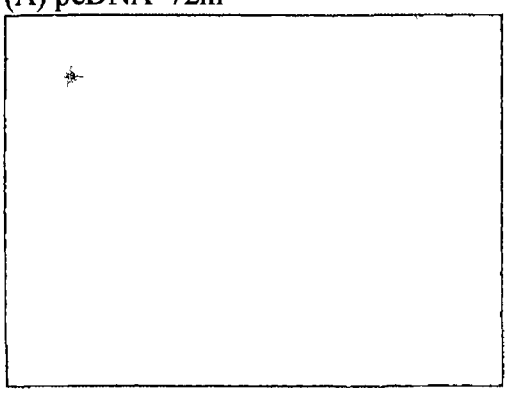
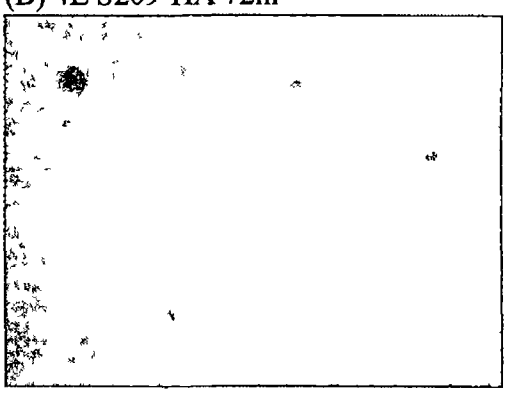


Figure 3.1.2.8 Immunocytochemical Analysis of K8 expression in 4E-HA transiently transfected DLKP cells. K8 expression is increased in 4E-HA transfected cells (C) compared to pcDNA controls (A) and 4E S209-HA transfected cells (B). Keratin 8 expression is heterogenous in these cells, this correlates with heterogenous eIF4E expression seen in these cells (Fig 3.1.2.7). (20X magnification).

(A) pcDNA 72hr



(B) 4E S209-HA 72hr



(C) 4E-HA 72hr

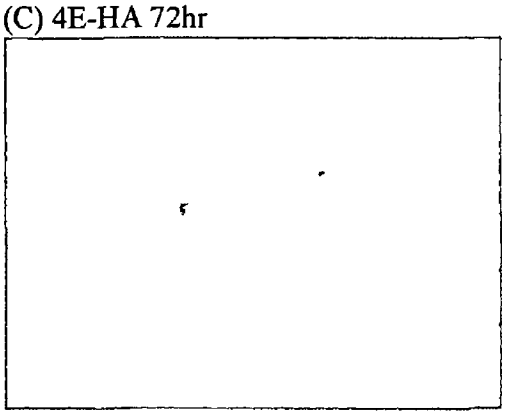


Figure 3 1 2 9 Immunocytochemical Analysis of β1 Integrin expression in 4E-HA transiently transfected DLKP cells β1 Integrin expression was low in transiently transfected pcDNA, 4E S209-HA and 4E-HA cells (20X magnification)

(A) pcDNA 72hr

(B) 4E S209-HA 72hr

(C) 4E-HA 72hr

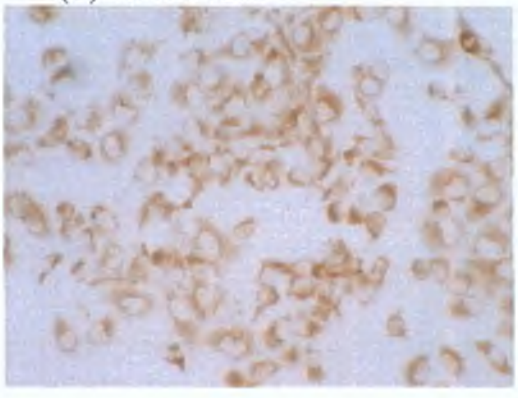


Figure 3.1.2.10 Immunocytochemical Analysis of ODC expression in 4E-HA transiently transfected DLKP cells. ODC expression was low in transiently transfected pcDNA control (A) and 4E S209-HA cells (B). 4E-HA transiently transfected cells (C) show increased ODC expression. (20X magnification).

3 1 2 2 Preliminary Screen of Uncloned Stable 4E-HA Transfected DLKP cells

DLKP cells were transfected with pcDNA control plasmid, 4E-HA or 4E S209-HA cDNAs Stably transfected cells were then selected with geneticin supplemented media (800 μg/ml)

Western blot analysis of uncloned mixed populations of 4E-HA and pcDNA stably transfected cells for 4E-HA levels shows that 4E-HA is being expressed effectively in the uncloned parental 4E-HA transfected cells (Fig 3 1 2 2 1)

Uncloned mixed populations of DLKP cells transfected with 4E-HA (Parental 4E-HA) and pcDNA control vector (Parental pcDNA) were analysed by immunocytochemistry for a number of proteins of interest prior to selection of clones for further analysis Immunocytochemical analysis shows increased expression of keratin 8, β1-integrin and ODC in 4E-HA transfected cells (Figs 3 1 2 2 2-3 1 2 2 4) Keratin 8 and β1-integrin have previously been shown to be upregulated in DLKP cells after treatment with the differentiation-modulating agent BrdU Ornithine Decarboxylase is known to be subject to translational regulation and has been shown to be upregulated in eIF4E overexpressing cell lines (Shantz *et al*, 1996)

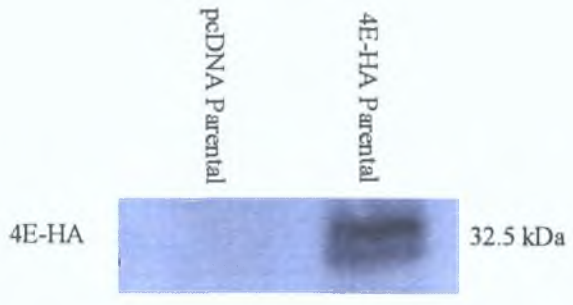


Figure 3.1.2.2.1 Western Blot Analysis of 4E-HA expression in uncloned parental 4E-HA and pcDNA transfected DLKP cells. 4E-HA protein is expressed in uncloned 4E-HA transfected cells.

(A) pcDNA Parental cells

(B) 4E-HA Parental cells

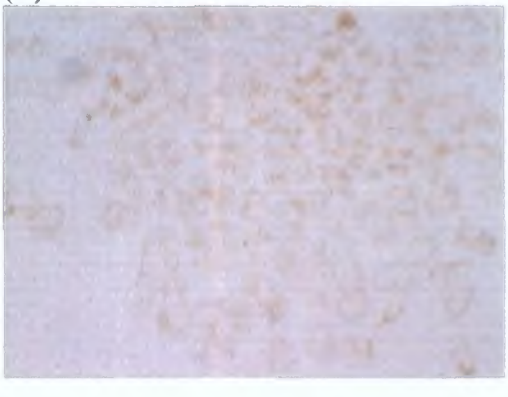


Figure 3.1.2.2.2 Immunocytochemical Analysis of Keratin 8 expression in Parental 4E-HA and pcDNA. Keratin 8 expression is increased in 4E-HA Parental cells (B) compared to pcDNA Parental control cells (A). (20X magnification).

(A) pcDNA Parental cells

(B) 4E-HA Parental cells

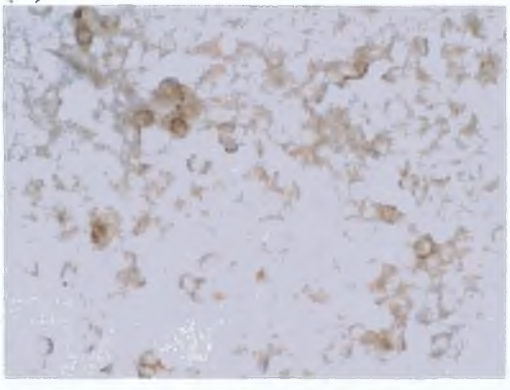
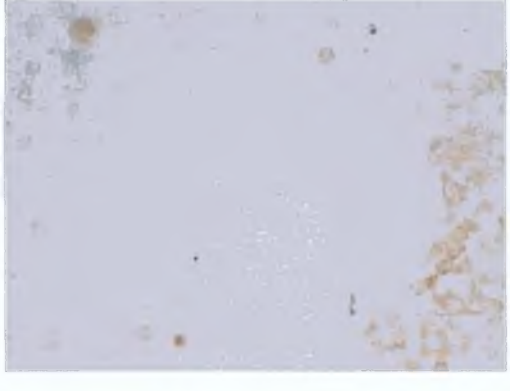


Figure 3.1.2.2.3 Immunocytochemical Analysis of β1 Integrin expression in Parental 4E-HA and pcDNA. pcDNA Parental cells (A) are negative for β1 Integrin expression. 4E Parental cells (B) show expression of β1 Integrin. (20X magnification).

(A) pcDNA Parental cells



(B) 4E-HA Parental cells

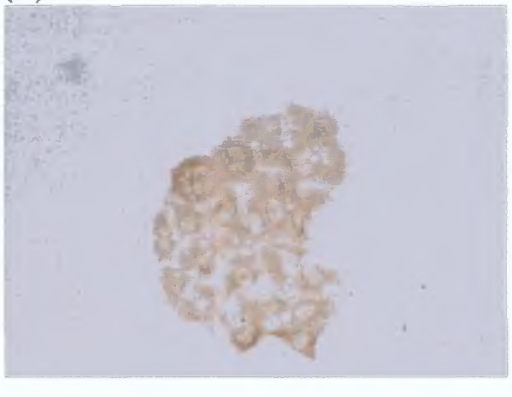


Figure 3.1.2.2.4 Immunocytochemical Analysis of ODC expression in Parental 4E-HA and pcDNA. ODC expression is increased in 4E-HA Parental cells (B), compared to pcDNA Parental control cells (A). (20X magnification).

3 1 2 3 Selection of Stably Transfected 4E-HA Overexpressing Clones

Clonal populations of DLKP cells transfected with pcDNA empty control vector and 4E-HA or 4E S209-HA cDNAs, were generated by limiting dilution Clones were then analysed by Western blotting to determine expression levels of transfected protein Western blot analysis of 4E-HA transfected clones using anti-eIF4E antibody, showed expression of transfected 4E-HA protein in 4E-HA Clone 5 (Fig 3 1 2 3 1) In agreement with Western blotting using anti-eIF4E Ab (Fig 3 1 2 3 1), Western blot analysis of 4E-HA transfected clones using anti-HA antibody showed 4E-HA Clone 5 expressed the highest level of 4E-HA protein and was therefore selected for further analysis (Fig 3 1 2 3 3) Clone 4E-HA 10 was also selected for further analysis as a lower 4E-HA expressing clone Expression of 4E-HA protein in 4E-HA transfected clones apart from 4E-HA 5 was not detected using anti-eIF4E antibody as this antibody was less sensitive than anti-HA antibody for detecting HA tagged 4E

The 4E S209-HA transfected cells were analysed by Western blot using anti-HA antibody to detect expression levels of 4E S209-HA protein (Fig 3 1 2 3 2) Of the 4E S209-HA clones, clone 6 and clone 12 expressed the highest level of 4E S209-HA protein and were selected for further analysis

Western blot analysis of the selected pcDNA control clones, 4E-HA or 4E S209-HA transfected-clones using anti-HA antibody on the same gel shows that pcDNA transfected clones do not express HA tagged 4E, 4E S209-HA clones 6 and 12 and 4E-HA clone 10 express similar levels of HA tagged 4E and 4E-HA clone 5 expresses a much higher levels of HA tagged 4E (Fig 3 1 2 4)

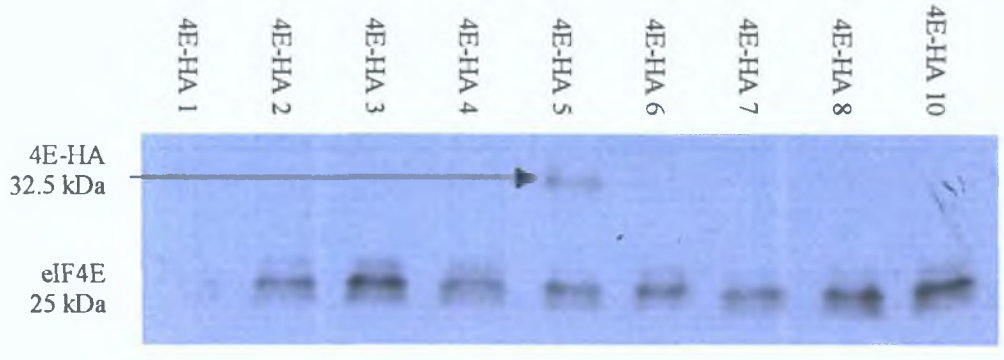


Figure 3.1.2.3.1 Western blot analysis of 4E-HA transfected clones using anti eIF4E antibody. Anti eIF4E antibody detects wild type eIF4E protein (25 kDa) and also detects HA tagged transfected eIF4E (32.5 kDa). HA tagged eIF4E protein is detected in Clone 4E-HA 5.

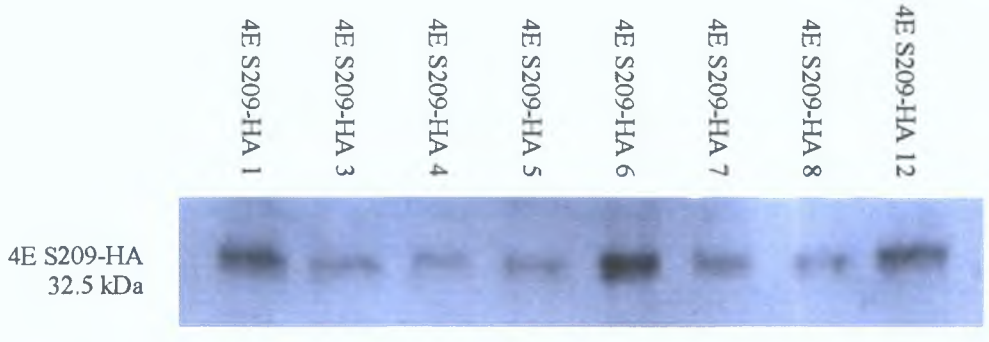


Figure 3.1.2.3.2 Western blot analysis of 4E S209-HA protein in 4E S209-HA transfected clones using anti-HA antibody. Clone 4E S209-HA 6 expresses the highest level of 4E S209-HA protein. Anti-HA antibody detects HA tagged transfected eIF4E (32.5 kDa).

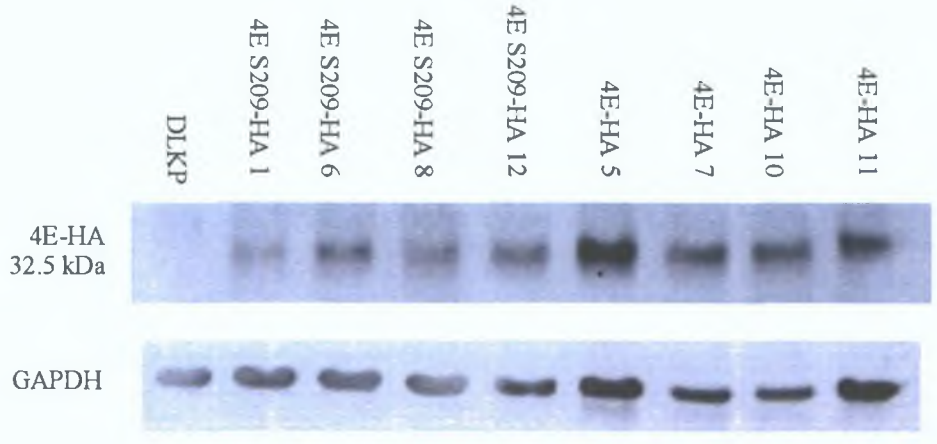


Figure 3.1.2.3.3 Western blot analysis of 4E-HA protein levels in 4E-HA transfected clones using anti-HA antibody. Anti-HA antibody detects HA tagged transfected eIF4E (32.5 kDa).

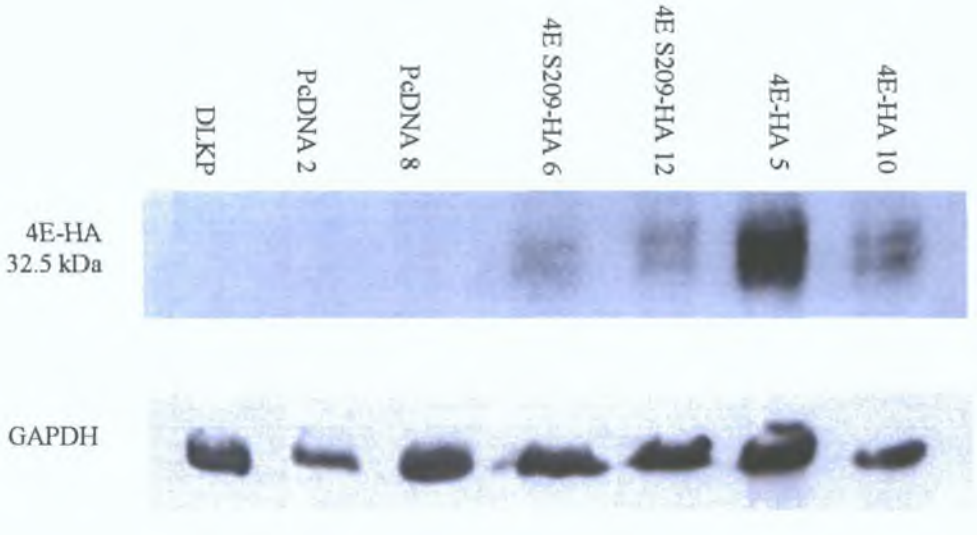


Figure 3.1.2.3.4 Western blot analysis of 4E-HA protein levels in 4E-HA and 4E S209 -HA transfected clones using anti-HA antibody. Anti-HA antibody detects HA tagged transfected eIF4E (32.5 kDa).

3.1.2.4 Analysis of 4E-HA Phosphorylation in 4E-HA Overexpressing Stably Transfected Clones

The 4E-HA overexpressing clones chosen for further analysis, 4E-HA Clone 5 and 4E S209-HA clone 6, were analysed by 2 Dimensional (2D) Electrophoresis followed by Western blotting Phosphorylated and non-phosphorylated forms of proteins are separated in the first dimension by iso-electric focusing due to changes in pI caused by additional phosphate groups Proteins are then separated according to molecular weight in the second dimension using poylacrylamide gel electrophoresis (PAGE) and then identified by Western blot anlaysis Analysis of 4E-HA 5 and 4E S209-HA 6 cell lysates with anti HA antibody after 2D electrophoresis showed the presence of phosphorylated and non-phosphorylated 4E-HA protein in 4E-HA 5 cells (Fig 3 1 2 4 1) Only non-phosphorylated 4E S209-HA protein was detected in 4E S209-HA 6 cells (Fig 3 1 2 4 2)

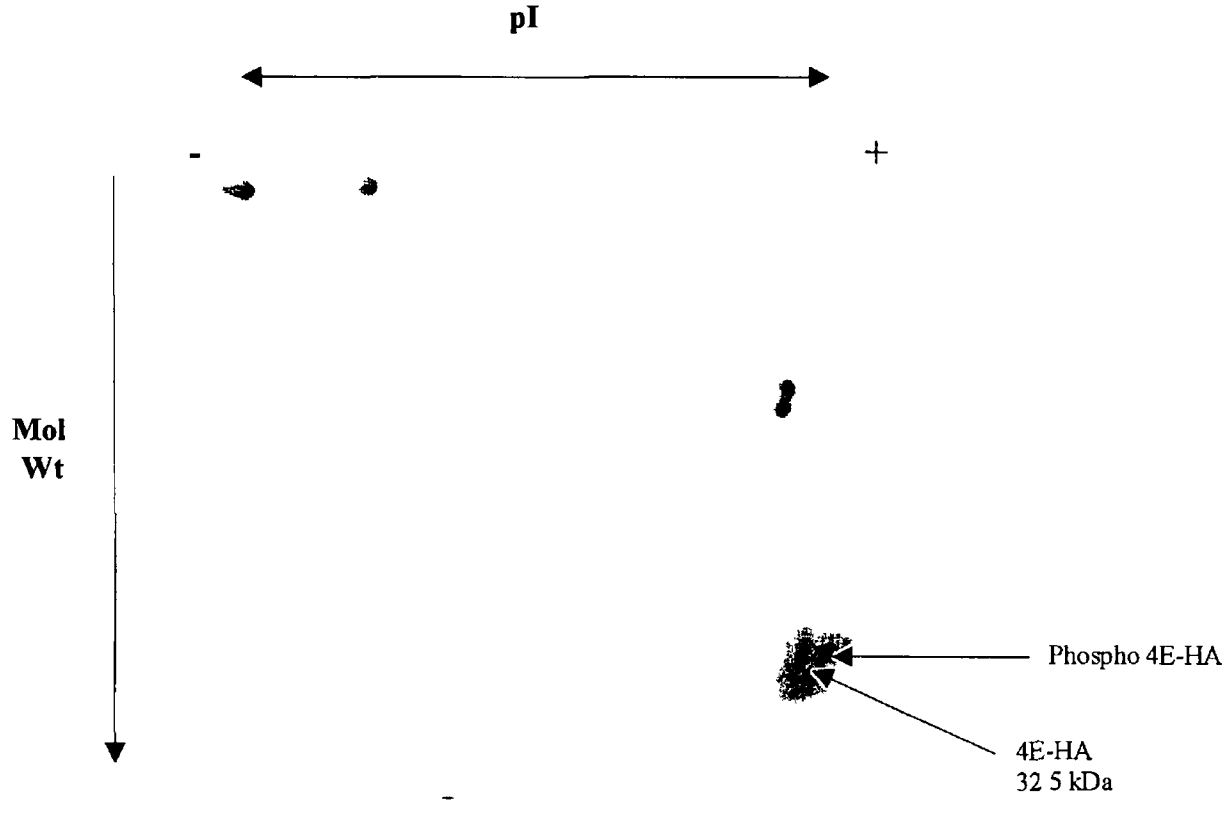


Figure 3 1 2 4 1 Phosphorylation status analysis of 4E-HA protein in 4E-HA 5 cells shows this protein is present in a phosphorylated and unphosphorylated form

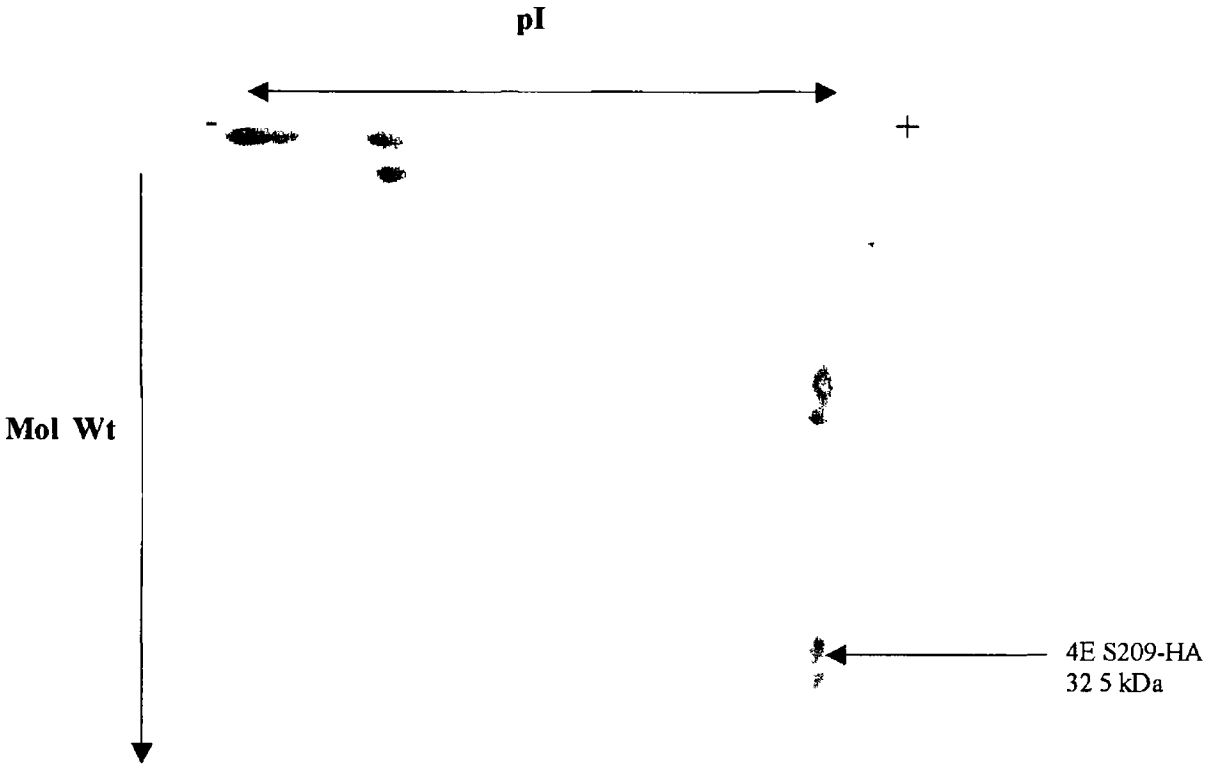
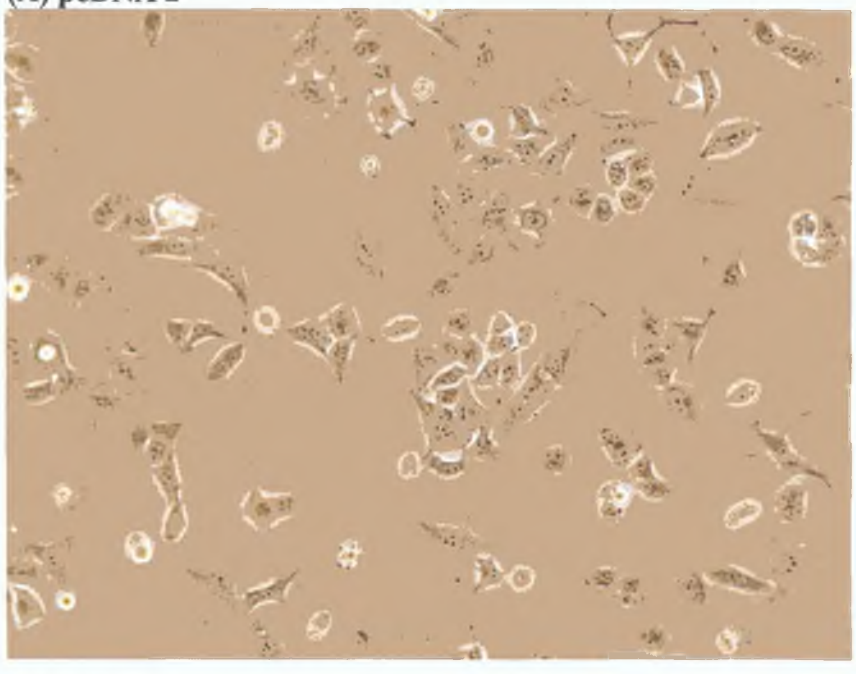


Figure 3 1 2 4 2 Phosphorylation status analysis of 4E S209-HA protein in 4E S209-HA 6 cells shows this protein is only present in an unphosphorylated form

3 1 2 5 Morphological phenotypes of 4E-HA transfected cells.

DLKP cells transfected with 4E-HA or 4E S209-HA were examined for changes in morphology The 4E S209-HA overexpressing clones possess a smooth edged rounded morphology with few cellular projections compared to pcDNA controls or 4E-HA overexpressing clones (Figs 3 1 2 5 1-3 1 2 5 3) The 4E-HA 5 cells which express high levels of 4E-HA protein showed numerous large cells containing multiple cellular protrusions

(A) pcDNA 2



(B) pcDNA 8

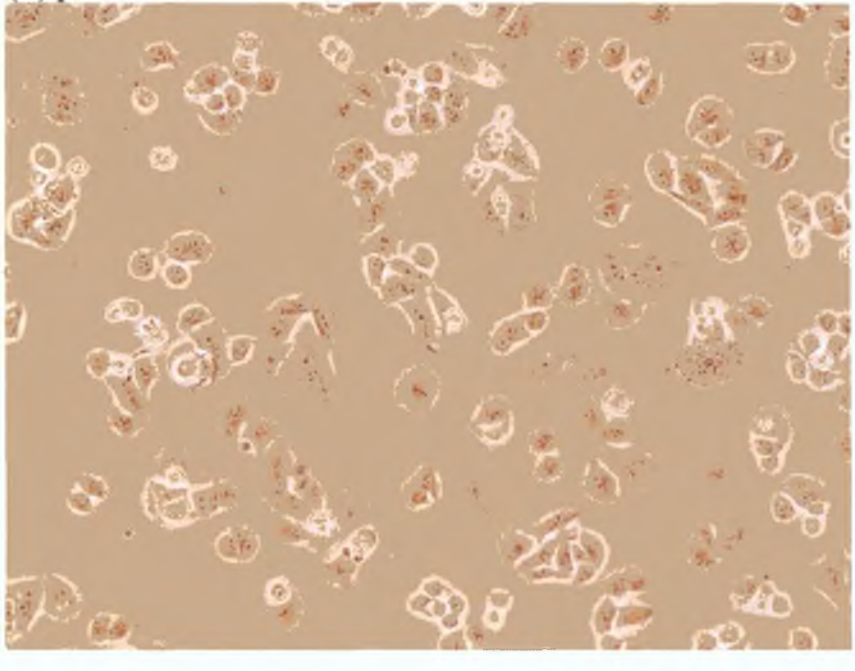
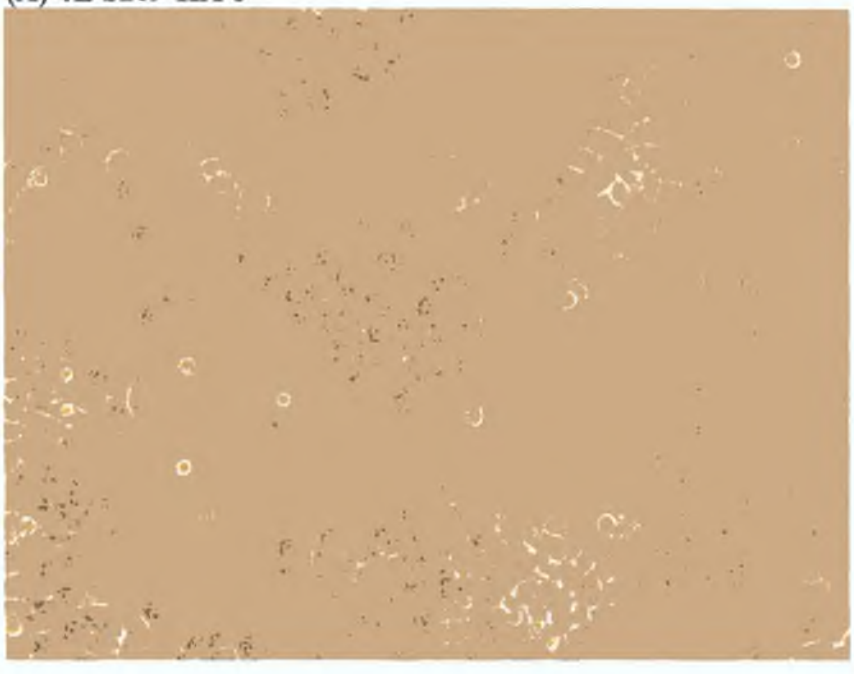


Figure 3.1.2.5.1 Morphological analysis of pcDNA control transfected DLKP cells. pcDNA control transfected cells possess an irregular morphology. (20X magnification).

(A) 4E S209-HA 6



(B) 4E S209-HA 12

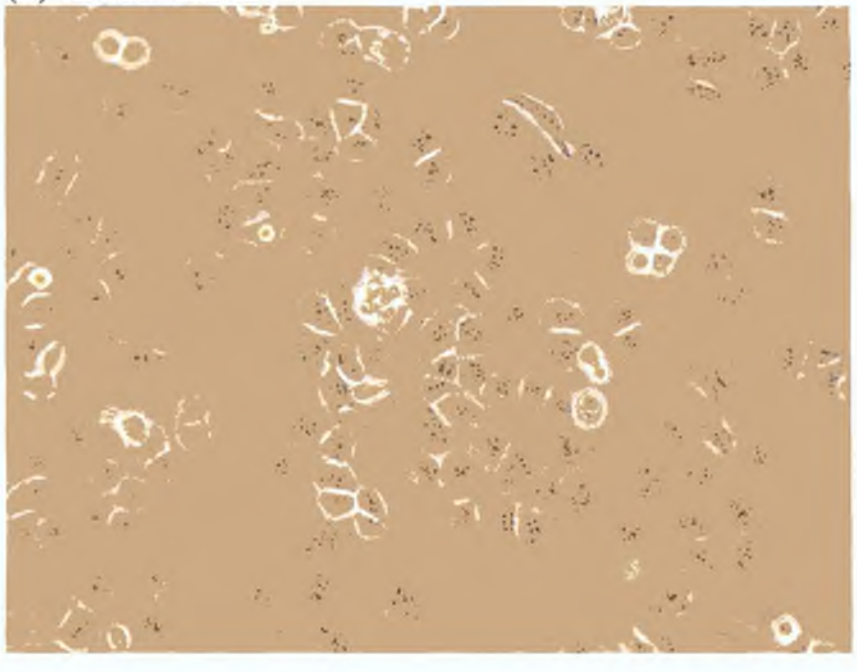
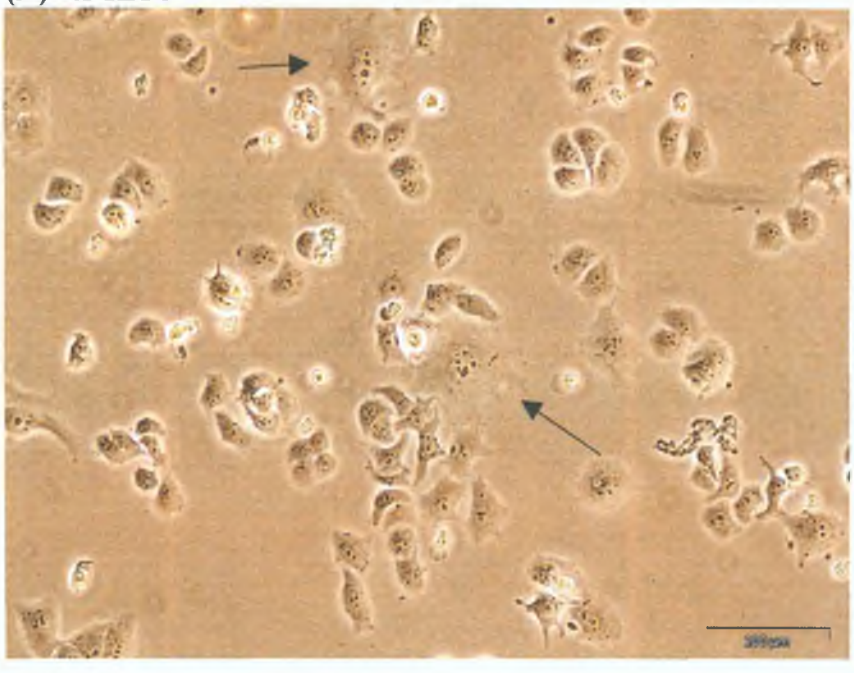


Figure 3.1.2.5.2 Morphological analysis of 4E S209-HA overexpressing DLKP cells. 4E S209-HA transfected cells have smooth rounded edges with few cellular protrusions. (20X magnification).

(A) 4E-HA 5



(B) 4E-HA 10

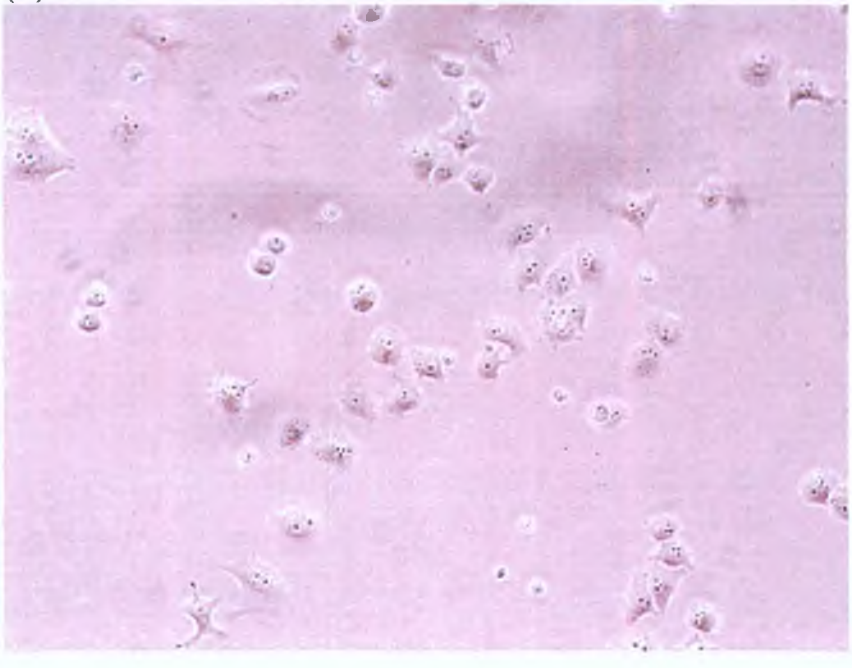


Figure 3.1.2.5.3 Morphological analysis of 4E-HA overexpressing DLKP cells. 4E-HA cells possess an irregular morphology. 4E-HA 5 cells also contain a proportion of large cells with multiple cellular protrusions. Arrows indicate large 4E-HA 5 cells (A) with multiple cellular protrusions. (20X magnification).

3 1 2 6 Keratin expression in 4E-HA overexpressing stably transfected clones

Treatment of DLKP cells with BrdU induces post-transcriptional upregulation of expression of the cytokeratins 8, 18 and 19 (Meleady and Clynes, 2001, McBride *et al*, 1999) BrdU treated DLKP cells also express elevated levels of eIF4E protein and enhanced phosphorylation of eIF4E (Walsh *et al*, 2003) Keratin 8, 18 and 19 expression was therefore analysed in 4E-HA overexpressing stably transfected DLKP clones to determine if increases in keratin expression seen in BrdU treated DLKP cells were replicated in 4E-HA overexpressing cells

Immunocytochemical analysis shows increased staining for keratin 8 in 4E-HA clone 5 (Fig 3 1 2 6 1) The 4E-HA clone 10 and both pcDNA controls and 4E S209-HA cells only show weak background staining (Fig 3 1 2 6 1) RT-PCR analysis of keratin 8 mRNA levels in 4E-HA 5 cells compared to 4E S209-HA 6, pcDNA 2 and DLKP cells shows no change in mRNA levels for keratin 8 (Fig 3 1 2 6 2)

Immunocytochemical analysis showed no change in the level of keratin 18 and 19 expression in 4E S209-HA and 4E-HA overexpressing cells compared to pcDNA controls with low levels of staining present in both (Fig 3 1 2 6 3-3 1 2 6 4)

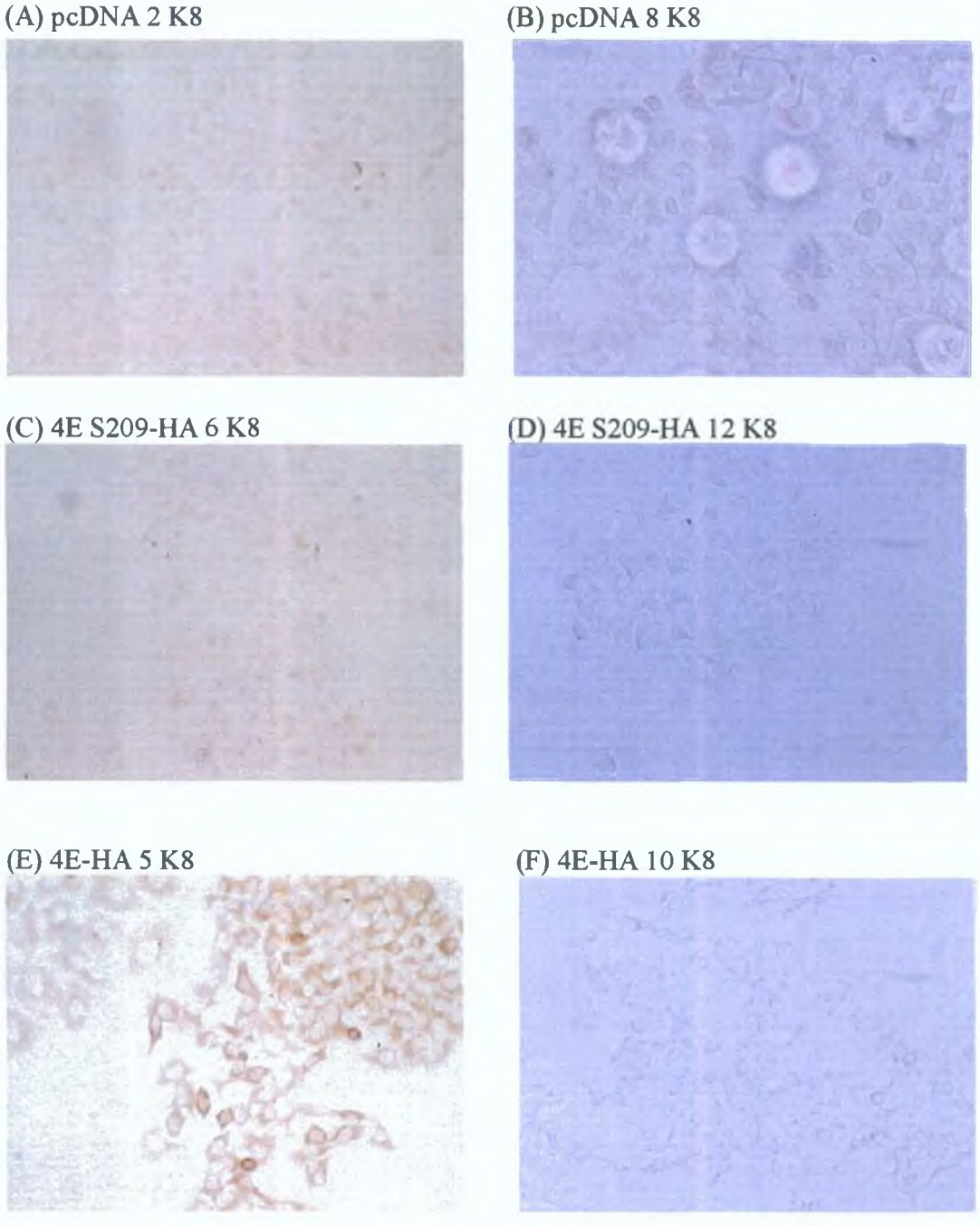


Figure 3.1.2.6.1 Immunocytochemical analysis of Keratin 8 expression in 4E-HA overexpressing clones. Increased staining for Keratin 8 was observed in 4E-HA 5 cells (E). No increased staining was observed in 4E-HA 10 (F) cells or 4E S209-HA overexpressing cells (C, D). (20X magnification).

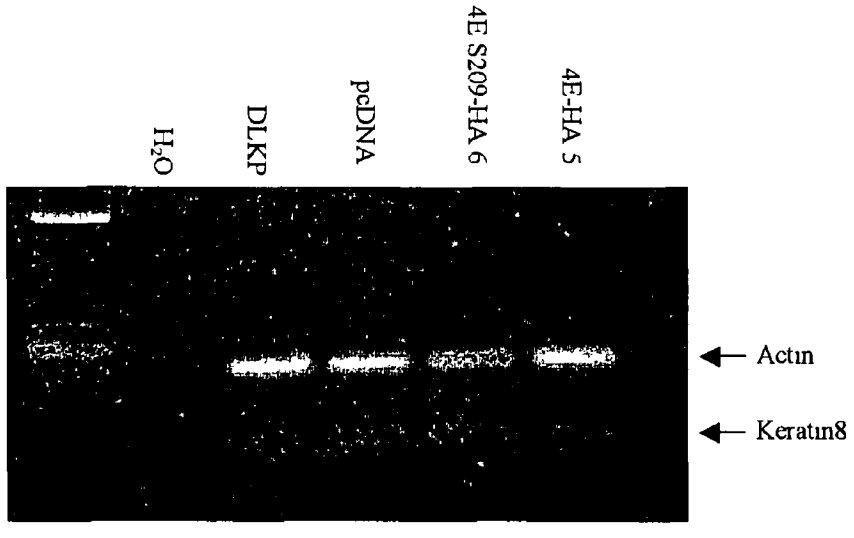


Figure 3 1 2 6 2 RT-PCR analysis of Keratin 8 Expression in 4E-HA overexpressing stably transfected clones Keratin 8 mRNA levels are unchanged in 4E-HA overexpressing clones or pcDNA transfected cells

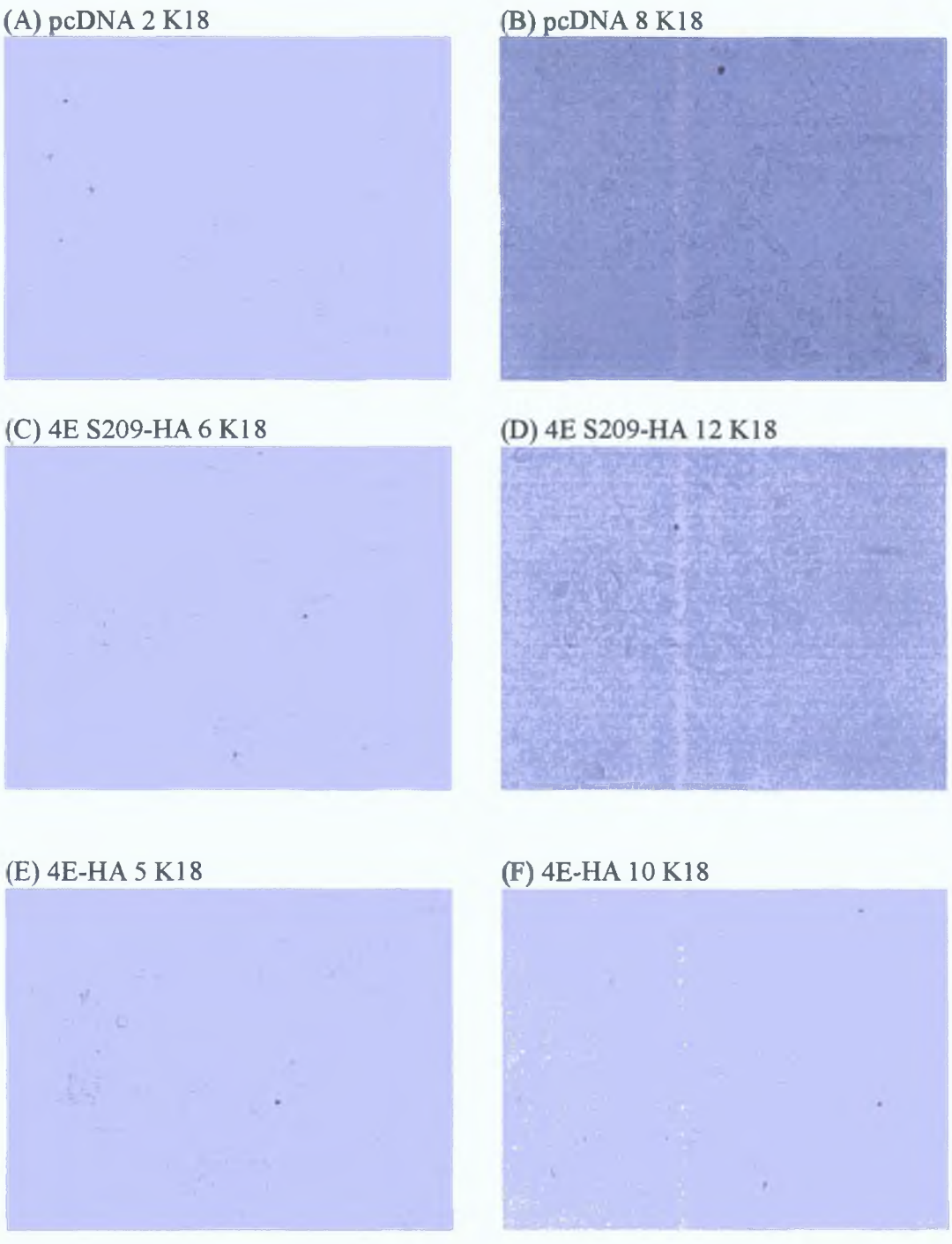


Figure 3.1.2.6.3 Immunocytochemical analysis of keratin 18 expression in 4E-HA overexpressing clones. No increase in Keratin 18 expression was observed in 4E-HA or 4E S209-HA overexpressing cells. (20X magnification).

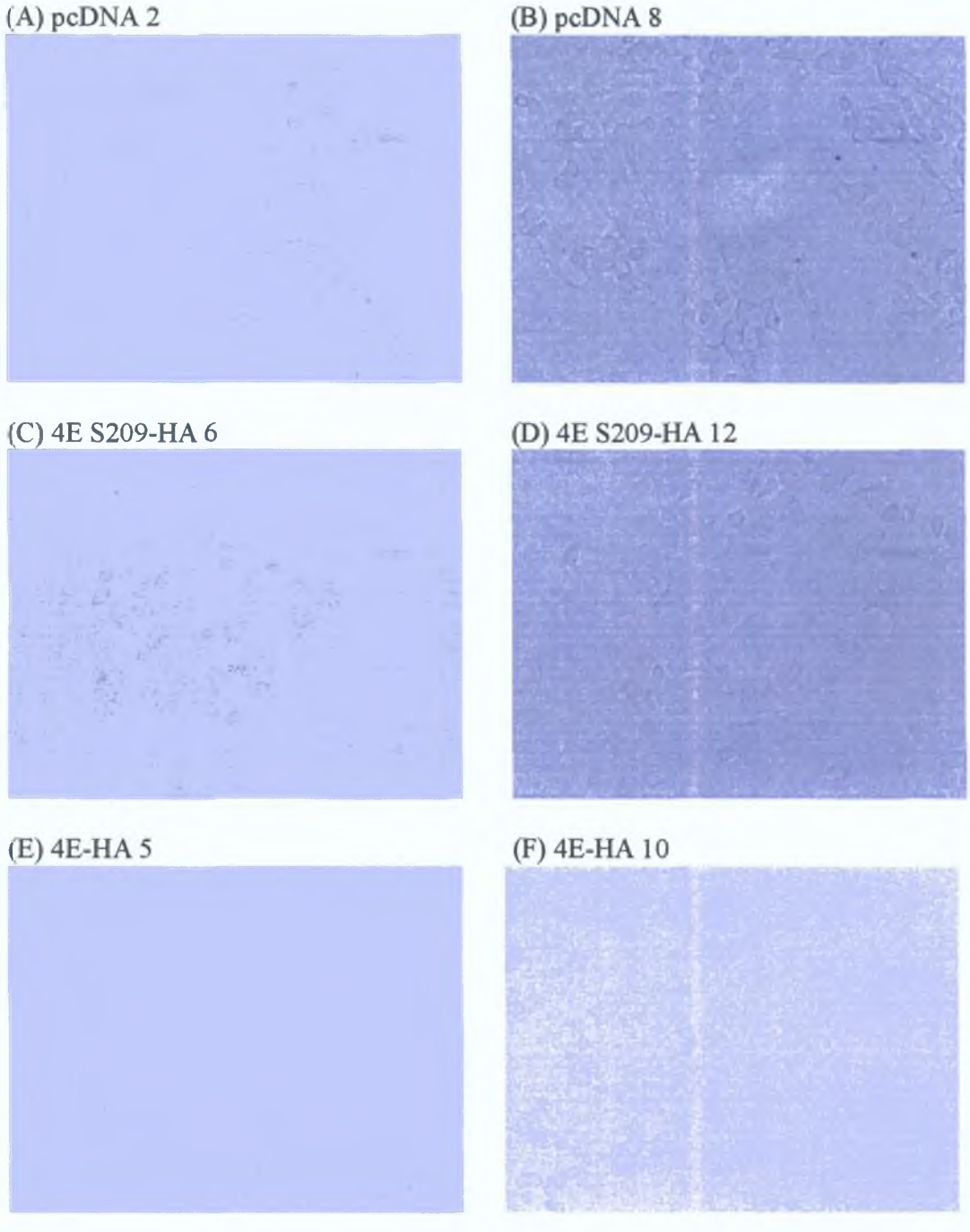


Figure 3.1.2.6.4 Immunocytochemical analysis of Keratin 19 expression in 4E-HA overexpressing clones. No increase in Keratin 19 expression was observed in 4E-HA or 4E S209-HA clones. (20X magnification).

3 1.2 7 Integrin expression in 4E-HA Overexpressing Stably Transfected Clones

β1 integrin and α2 integrin have previously been shown to be upregulated in BrdU treated DLKP cells (Meleady and Clynes, 2000) BrdU treated DLKP cells also express elevated levels of eIF4E protein and enhanced phosphorylation of eIF4E (Walsh *et al*, 2003) β1 integrin and α2 integrin expression was therefore analysed in 4E-HA overexpressing stably transfected DLKP clones to determine if increases in integrin expression seen in BrdU treated DLKP cells were replicated in 4E-HA overexpressing cells The β1 integrin binding partners α1, α3, and α5 have also been analysed here as they are known to be expressed in the pulmonary epithelium and are implicated in the the regulation of growth and development of pulmonary epithelial cells (Coraux *et al*, 1998, Sheppard, 2003)

Immunocytochemical analysis of β1 integrin expression shows increased staining for β1 integrin in 4E-HA clone 5 cells The 4E-HA clone 10, 4E S209-HA overexpressing cells and pcDNA control cells exhibit weak background staining for β1 integrin (Fig 3 1 2 7 1)

Expression of α1, α2 and α5 integrin was undetected in 4E-HA, 4E S209-HA overexpressing cells and pcDNA controls (Fig 3 1 2 7 2, 3 1 2 7 3, 3 1 2 7 5) Strong staining for α3 integrin was seen in the 4E-HA overexpressing clones 5 and 10 wheareas, it is undetected in 4E S209-HA overexpressing cells and pcDNA control cells (Fig 3 1 2 7 4)

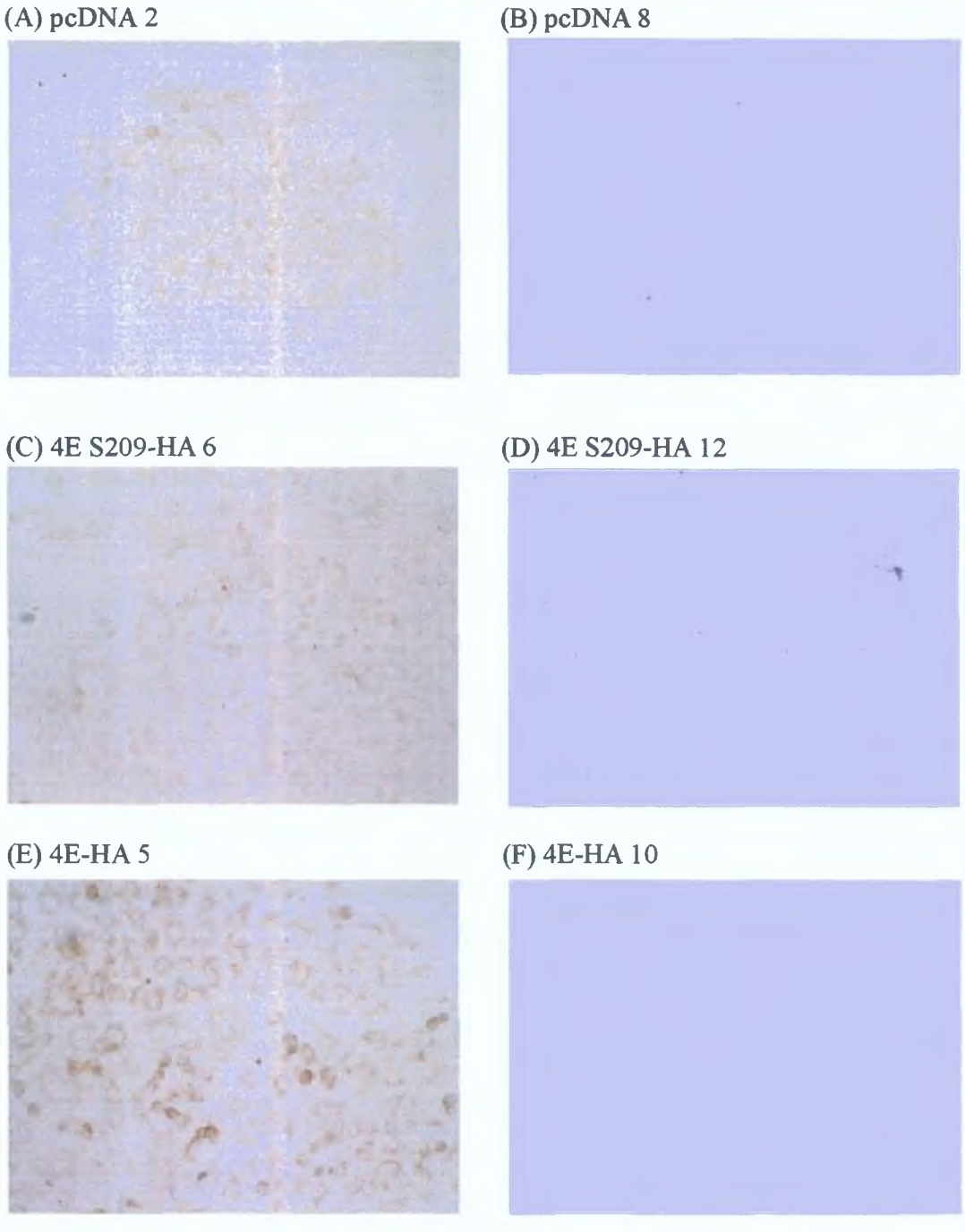


Figure 3.1.2.7.1 Immunocytochemical analysis of β1 integrin expression in 4E overexpressing cells. Increased staining for β1 integrin was observed in 4E-HA 5 cells (E). No increase in staining was observed in 4E-HA 10 (F) cells or 4E S209-HA overexpressing cells (C, D). (20X magnification).

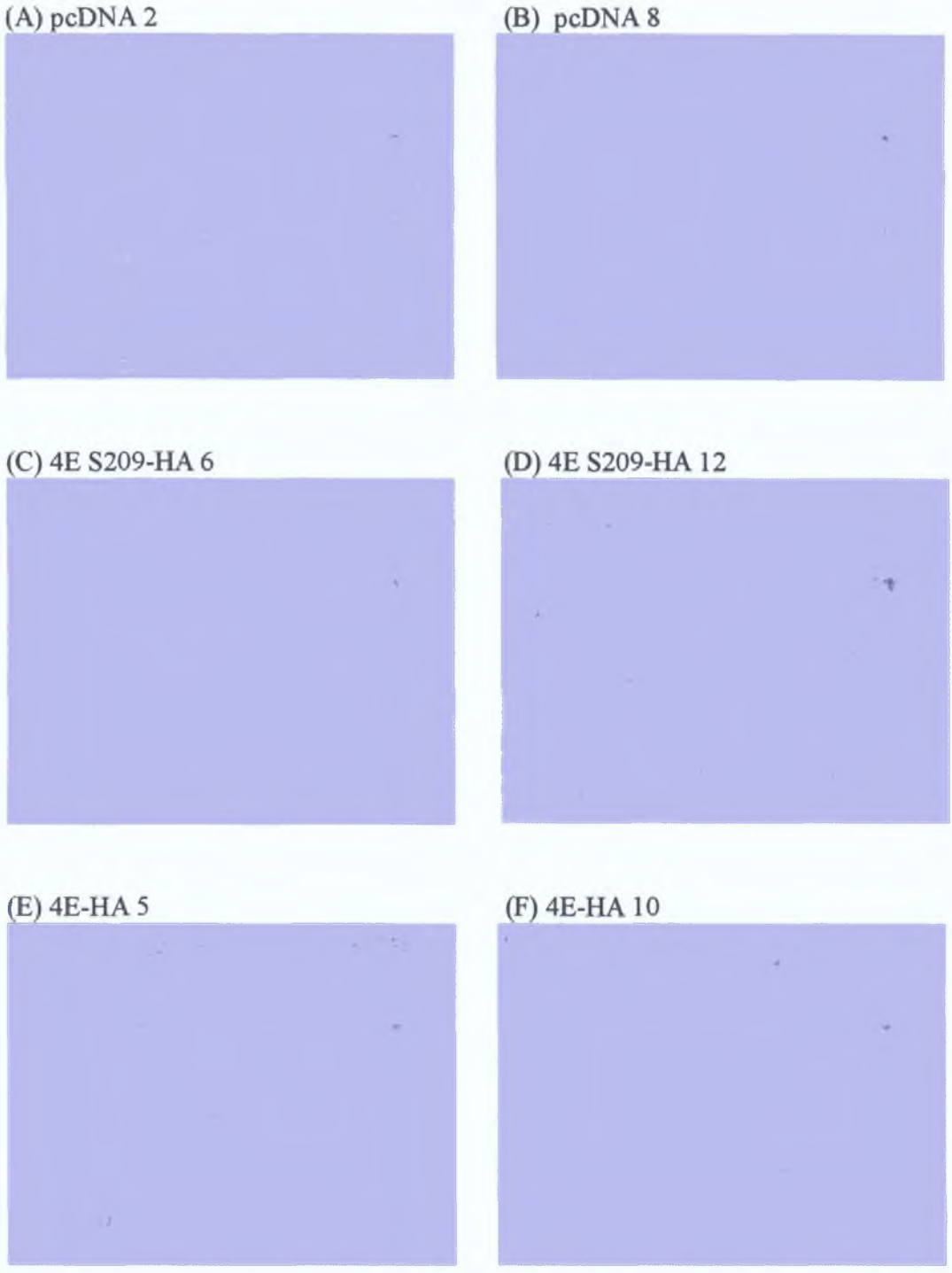


Figure 3.1.2.7.2 Immunocytochemical analysis of α1 integrin expression in 4E overexpressing cells. Staining for α1 integrin was not detected in pcDNA controls (A,B), 4E S209-HA (C,D) and 4E-HA overexpressing cells (E, F). (20X Magnification).

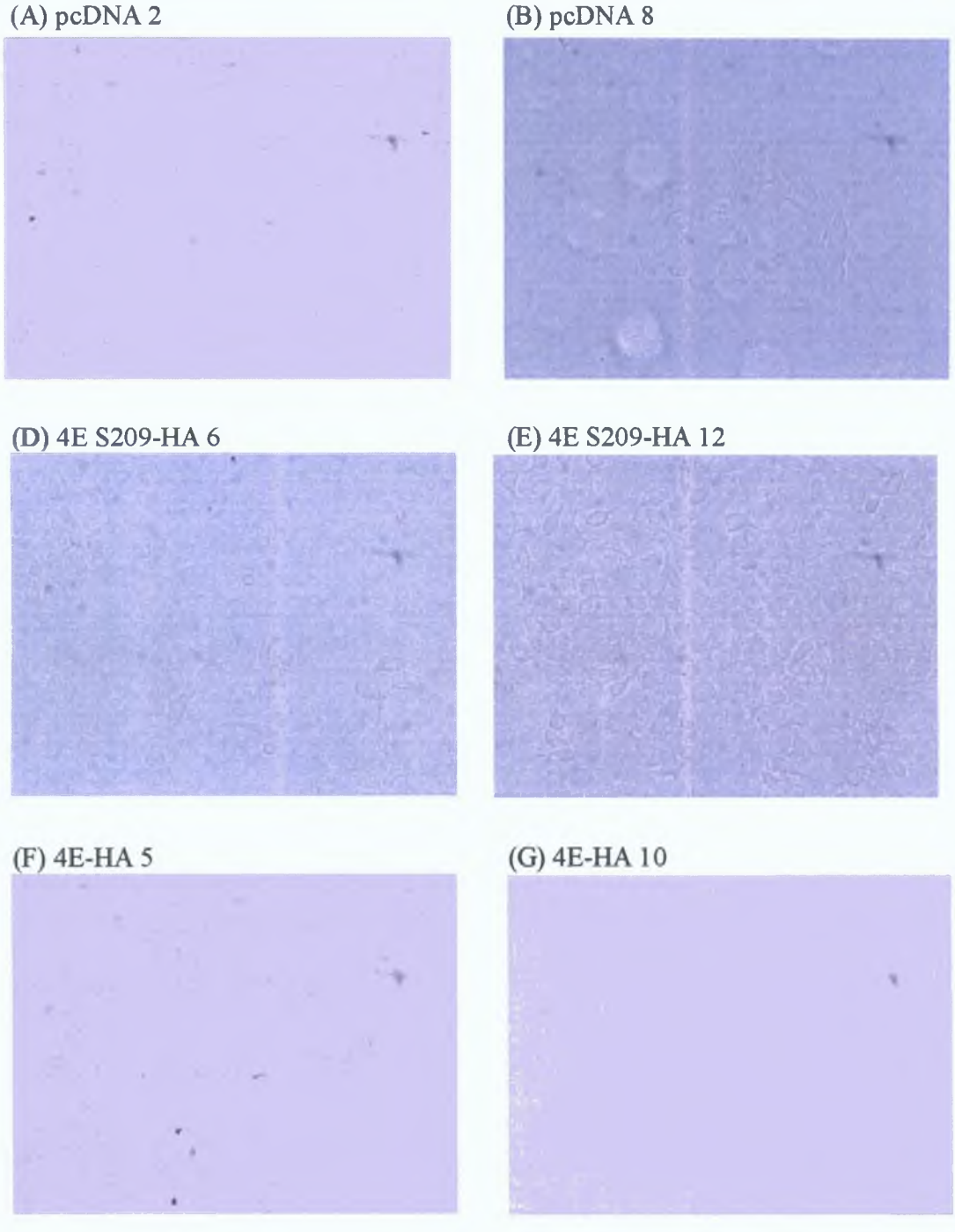


Figure 3.1.2.7.3 Immunocytochemical analysis of α2 integrin expression in 4E overexpressing cells. Staining for α2 integrin was not detected in pcDNA controls (A, B), 4E S209-HA (C, D) and 4E-HA overexpressing cells (E, F). (20X Magnification)

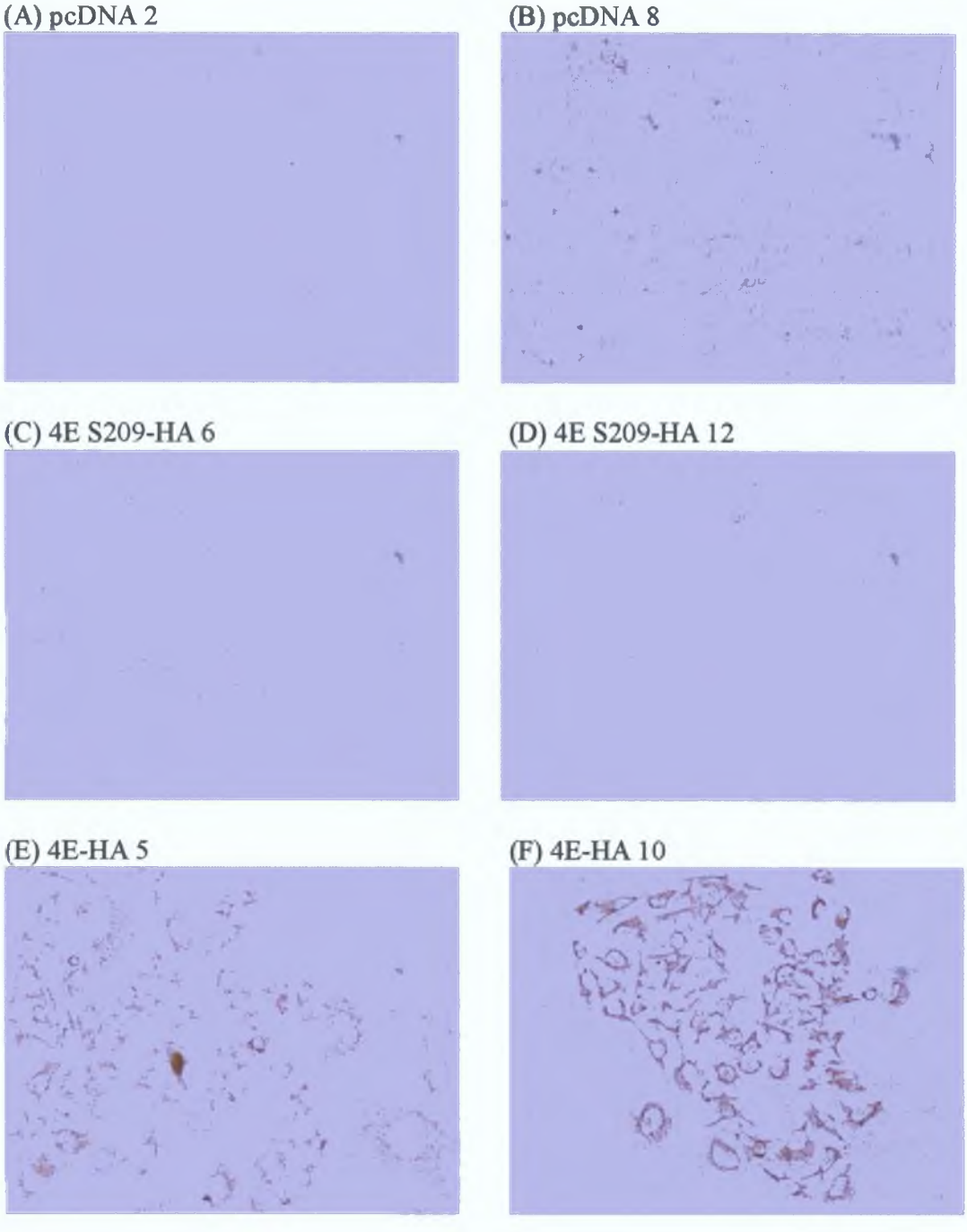


Figure 3.1.2.7.4 Immunocytochemical analysis of α3 integrin expression in 4E overexpressing cells. Strong staining for α3 integrin was detected in 4E-HA overexpressing clones 5 and 10 (E, F). No staining for α3 integrin was detected in pcDNA controls (A, B) or 4E S209-HA overexpressing cells (C, D). (20X Magnification).

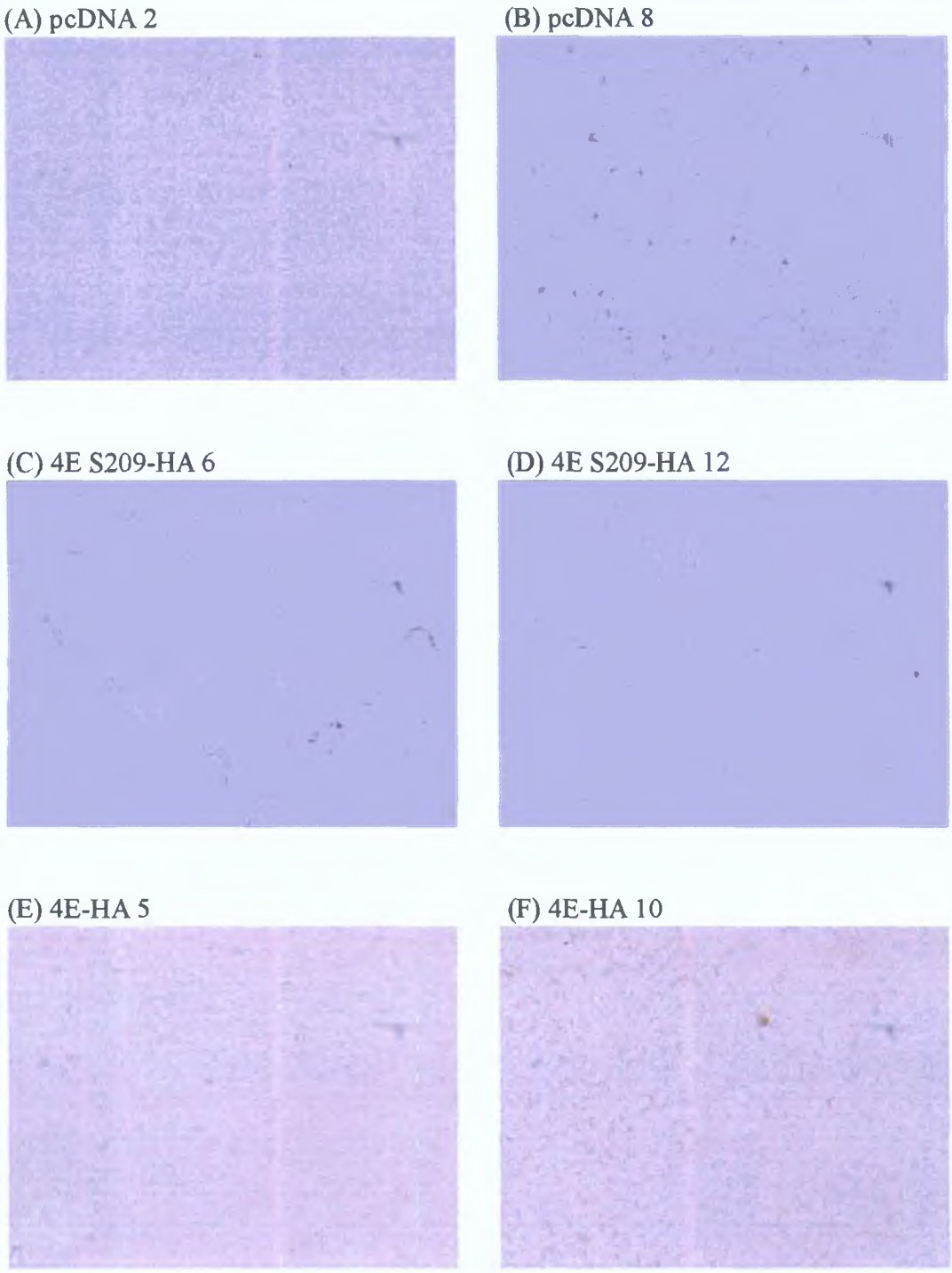


Figure 3.1.2.7.5 Immunocytochemical analysis of α5 integrin expression in 4E overexpressing cells. Staining for α5 integrin was not detected in pcDNA controls (A, B), 4E S209-HA (C, D) and 4E-HA (E, F) overexpressing cells. (20X Magnification).

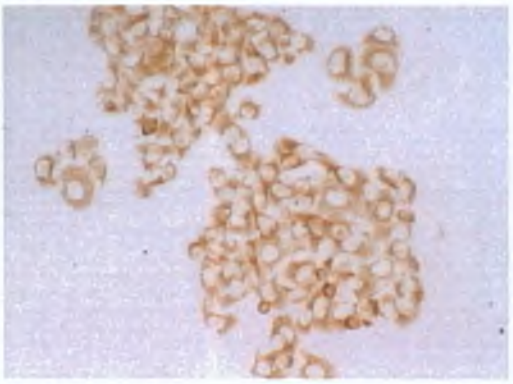
3 1 2 8 Ornithine Decarboxylase Expression (ODC) in 4E-HA overexpressing stably transfected clones

Ornithine Decarboxylase (ODC) is a key enzyme in the regulation of polyamine biosynthesis Polyamine levels are known to be involved in the regulation of growth, differentiation and transformation of cells (Shantz and Pegg, 1999) Expression of ODC is reported to be subject to translational regulation by eIF4E (Shantz and Pegg, 1999) It was therefore decided to examine expression of ODC in 4E-HA overexpressing cells to determine if any changes in expression levels were present

Analysis of ODC expression by immunocytochemistry shows upregulation of expression in 4E-HA overexpressing cells and also in 4E S209-HA cells (Fig 3 1 2 8 1)

(A) pcDNA 2 ODC

(B) 4E S209-HA 6 ODC



(C) 4E-HA 5 ODC

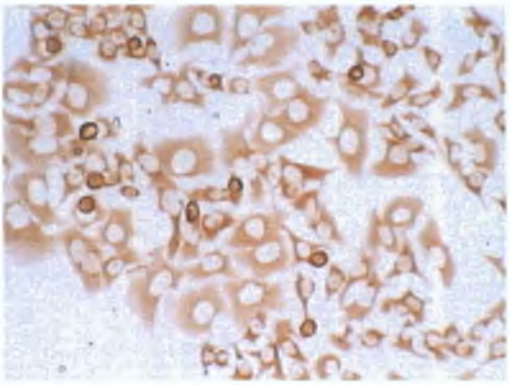


Figure 3.1.2.8.1 Immunocytochemical analysis of ODC expression in 4E overexpressing DLKP cells. Staining for ODC is increased in 4E S209-HA 6 (B) and 4E-HA 5 (C) overexpressing cells compared to pcDNA 2 controls (A). (20X magnification).

3 1 2 9 Growth rate analysis of stably transfected 4E-HA overexpressing clones

Previous studies have shown that overexpression of eIF4E increases the growth rate of cell lines The growth rates of pcDNA control, 4E-HA and 4E S209-HA transfected clones were compared to determine the effect of 4E overexpression and 4E phosphorylation on growth and proliferation in these cells Both 4E-HA and 4E S209-HA overexpressing clones had higher growth rates than pcDNA 2 control cells (Fig 3 1 2 9 1) After 6 days the growth rate of 4E-HA 5 cells levelled off as these cells reached confluency whereas the higher growth rate of 4E S209-HA 6 cells was maintained This was probably due to the fact that 4E S209-HA 6 cells were smaller and had not achieved confluency at this stage allowing them to maintain a high growth rate This experiment was performed once

TIME IN DAYS	PCDNA 2	4E S209-HA 6	4E-HA 5
1	4 875	5 75	4 938
2	8 719	12 313	9 531
3	18 813	22 625	20 75
4	25 813	42 6875	39 25
5	39 375	69 625	86 25
6	101 25	147 25	103 625

Table 3 1 2 9 1 Growth curve for 4E-HA overexpressing clones over a 6 day period Results are expressed as average cell number ($x10^4$) per flask (n=2)

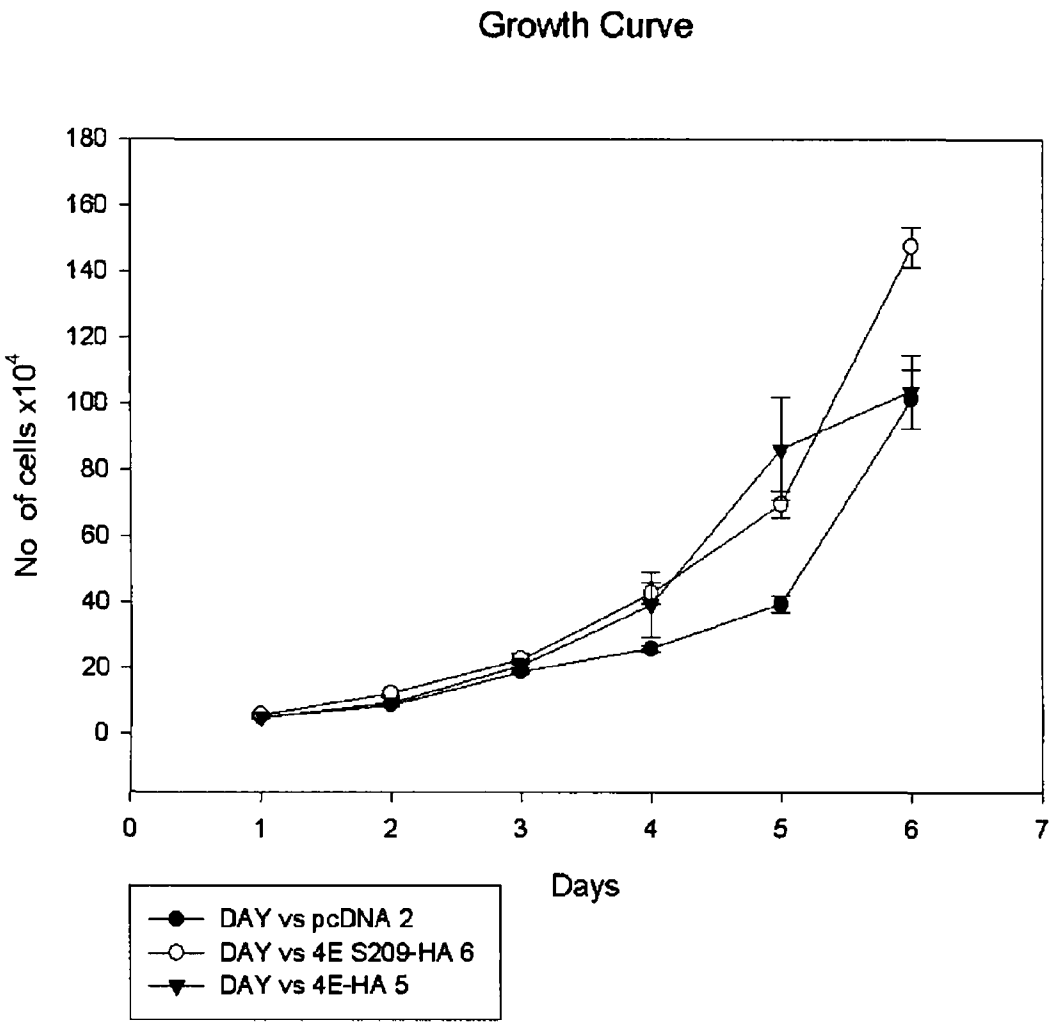


Figure 3 1 2 9 1 Growth curve of 4E-HA overexpressing clones Results are expressed as average cell number per flask x10^4 (n=2)

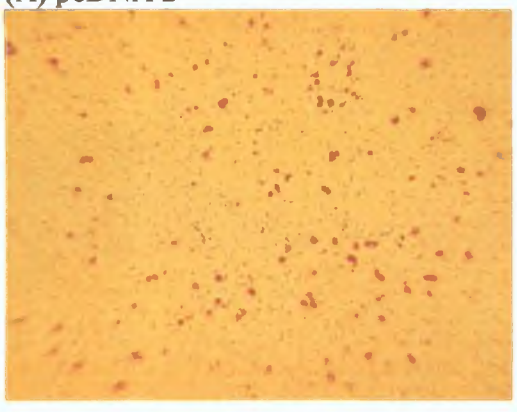
3.1.2.10 Invasion assays of 4E-HA overexpressing stably-transfected clones

eIF4E is known to be involved in the regulation of invasiveness and metastasis of cancer cells (DeBenedetti and Graff, 2004) It was therefore decided to analyse the effect of 4E-HA overexpression on the invasiveness of DLKP cells

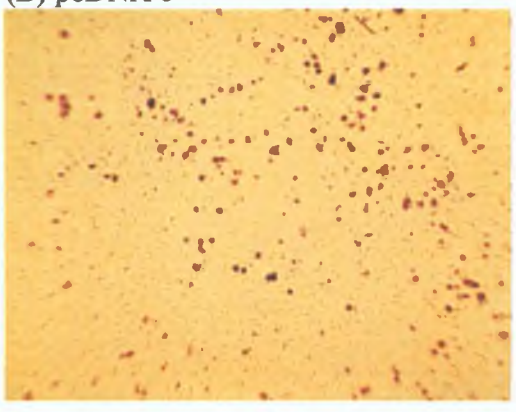
An *in vitro* system for the study of cell invasion through basement membrane was used to determine the invasiveness of 4E-HA overexpressing DLKP cells This consisted of cell culture well inserts containing an 8 μm pore-size PET membrane coated with a uniform layer of BD Matrigel^{TM} Basement Membrane Matrix Cells that had migrated through the PET membrane were stained with crystal violet and 5 fields at 10X magnification were counted per well

4E-HA Clone 5 cells showed highly increased invasiveness compared to all other cells tested (Figs 3 1 2 10 1-3 1 2 10 3)

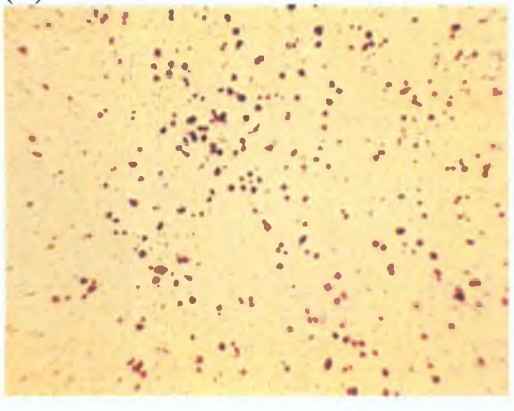
(A) pcDNA 2



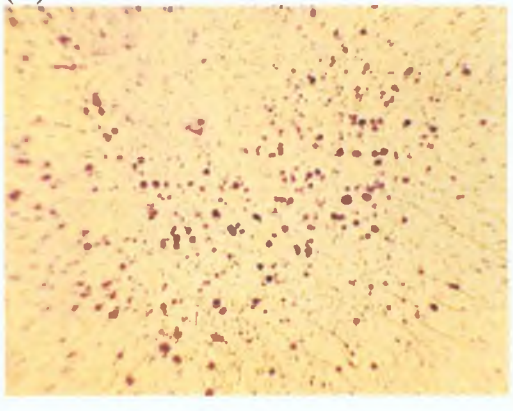
(B) pcDNA 8



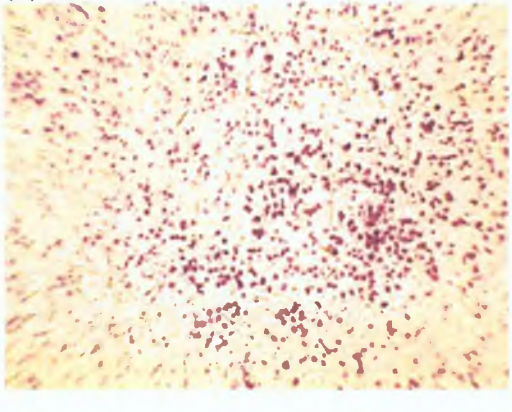
(C) 4E S209-HA 6



(D) 4E S209-HA 12



(F) 4E-HA 5



(G) 4E-HA 10

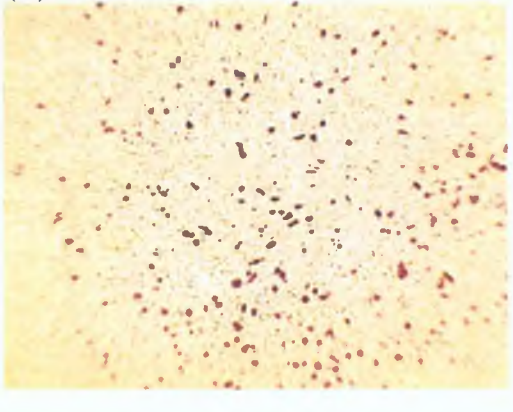


Figure 3.1.2.10.1 Invasion assays of 4E-HA overexpressing cells. Cells that have passed through Matrigel coated membrane were stained with crystal violet before counting (4X Magnification).

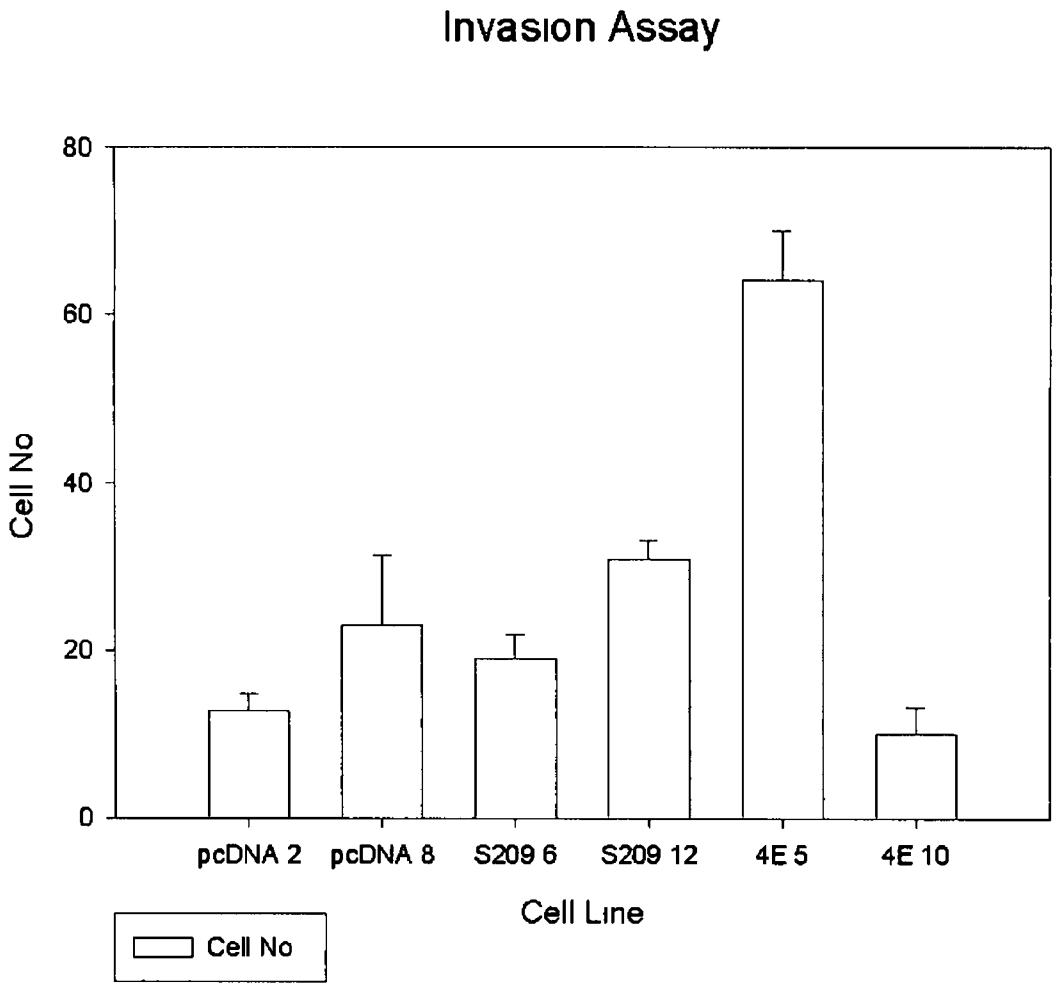


Figure 3 1 2 10 2 Invasion assay of 4E-HA overexpressing cells Invasion assays were performed in triplicate Five fields at 10X magnification were counted for each invasion chamber Results are expressed as average cell number ± standard deviation (n=3)

Cell Line	Invasiveness %
4E-HA 5	100%
4E S209-HA 12	48 28%
pcDNA 8	35 79%
4E S209-HA 6	29 55%
pcDNA 2	17 68%
4E-HA 10	15 61%

Figure 3.1 2 10.3 Invasiveness expressed as a percentage of invasiveness of 4E-HA clone 5

3.1.2.11 Proteomic analysis of 4E-HA overexpressing cells

In order to further identify differences in protein expression as a result of overexpression of 4E-HA and 4E S209-HA in DLKP cells it was decided to analyse protein expression levels in these cells using 2D-Electrophoresis In this study, we have used the recently developed ETTAN DIGE (Amersham) 2D electrophoresis system (Figure 3 1 2 11 1)

Proteomic analysis was carried out on two 4E-HA transfected DLKP clones (4E-HA 5, 4E-HA 10), two 4E S209-HA clones (4E S209-HA 6, 4E S209-HA 12) and two pcDNA control plasmid transfected clones (pcDNA 2, pcDNA 8)
The main analysis of our results was performed by pooling the results from the two clones of each group together into three groups i e 4E-HA, 4E S209-HA and pcDNA The expression values of protein spots in each group were then compared against each other e g 4E vs S209, pcDNA vs 4E, pcDNA vs S209 A t-test analysis of protein spot expression values in comparisons was applied to ensure statistical significance of results

We have also analysed differences in protein expression between the highly invasive 4E-HA overexpressing clone 4E-HA 5 and the low level invasive 4E-HA overexpressing clone 4E-HA 10 in order to identify proteins proteins which maybe involved in regulating the invasiveness of these cells

I would like to thank Dr Andrew Dowd, who performed the Dige labelling, isoelectric focusing, SDS-PAGE electropheresis and mass spectrometry protein identification for the main 4E-HA, 4E S209-HA, pcDNA cell comparison experiment described in this section and also aided in the analysis of protein expression data on Decyder Software™

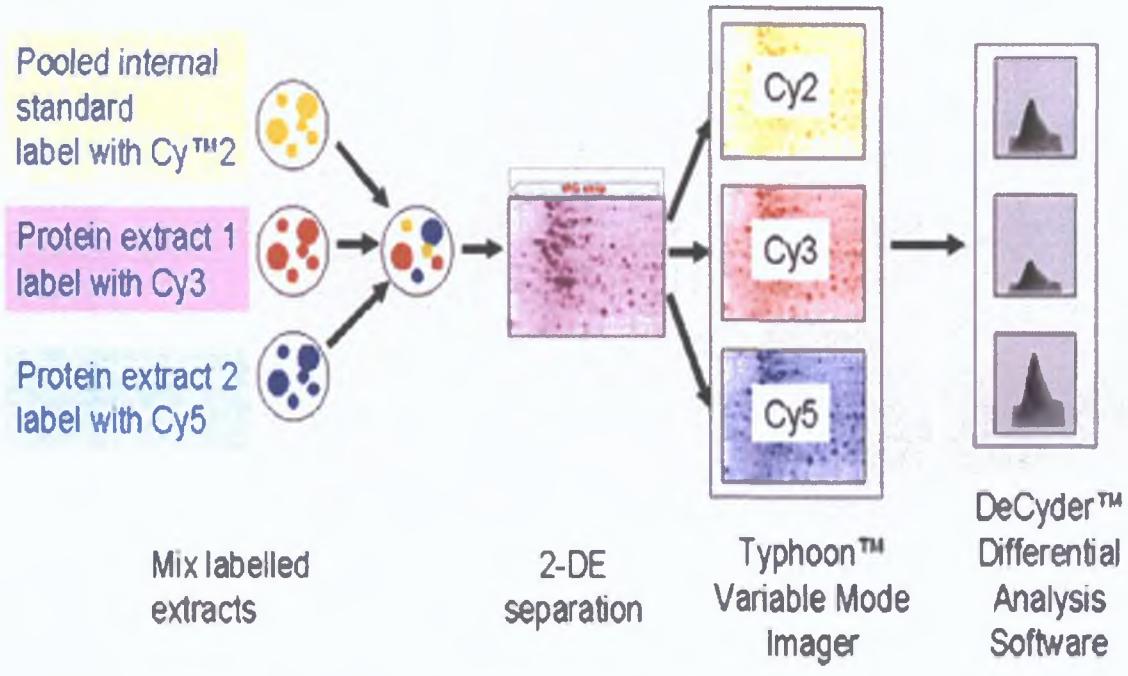


Figure 3.1.2.11.1 ETTAN DIGE (Amersham) 2D electrophoresis system.

Prior to analysis by DIGE, all protein extracts were analysed by 2D electrophoresis followed by silver staining to ensure extracts used were of good quality and had not been adversely affected by protein extraction (e g protein degradation) (Fig 3 1 2 11 2)

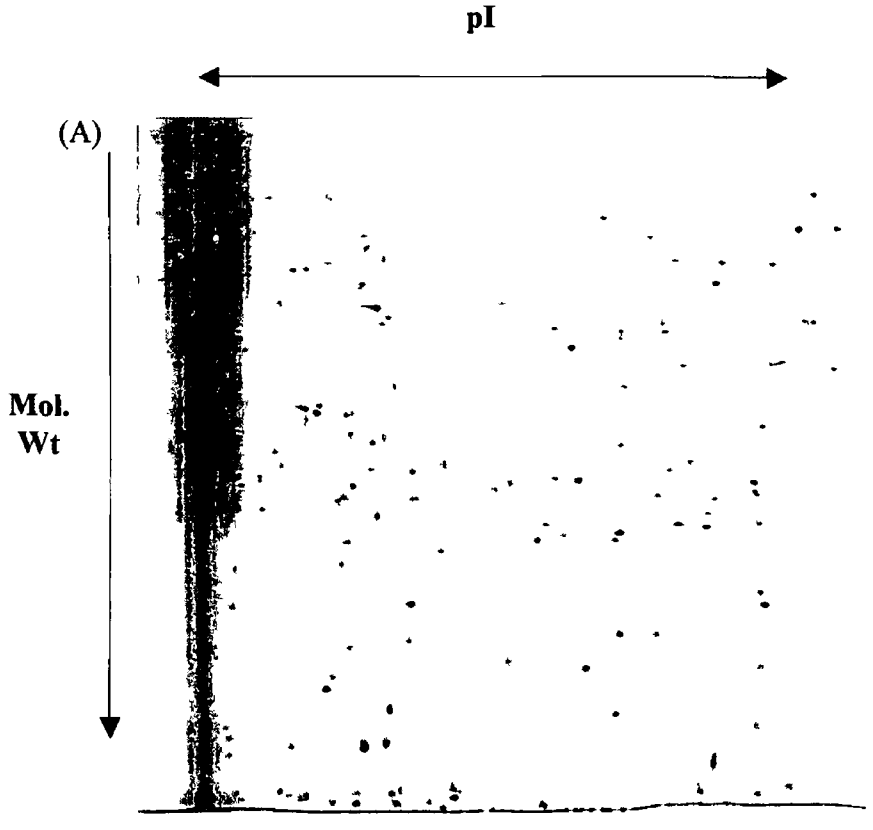


Figure 3.1 2 11 2 All protein lysates for DIGE analysis were run on 2D electrophoresis gels and visualised by silver staining to ensure the quality of proteins samples (A) Good quality sample showing clear well defined protein spots

The experimental design for DIGE analysis of pcDNA 2, pcDNA 8, 4E S209-HA 6, 4E S209-HA 12, 4E-HA 5 and 4E-HA 10 cells is outlined in Table 3 1 2 11 1 This involved two sessions running 6 2D gels simultaneously Four samples of each cell line were analysed to allow for statistical analysis of protein expression using DeCyder software (Figure 3 1 2 11 3)

Gel Number	Cy2 label	Cy3 label	Cy5 label
1	Pooled Internal Std 50μg	4E-HA 5 (Sample 1) 50μg	pcDNA 2 (Sample 3) 50μg
2	Pooled Internal Std 50μg	4E S209-HA 6 (Sample 1) 50μg	4E-HA 5 (Sample 3) 50μg
3	Pooled Internal Std 50μg	pcDNA 2 (Sample 1) 50μg	4E S209-HA 6 (Sample 3) 50μg
4	Pooled Internal Std 50μg	4E-HA 5 (Sample 2) 50μg	pcDNA 2 (Sample 4) 50μg
5	Pooled Internal Std 50μg	4E S209-HA 6 (Sample 2) 50μg	4E-HA 5 (Sample 4) 50μg
6	Pooled Internal Std 50μg	pcDNA 2 (Sample 2) 50μg	4E S209-HA 6 (Sample 4) 50μg
7	Pooled Internal Std 50μg	4E-HA 10 (Sample 1) 50μg	pcDNA 8 (Sample 3) 50μg
8	Pooled Internal Std 50μg	4E S209-HA 12 (Sample 1) 50μg	4E-HA 10 (Sample 3) 50μg
9	Pooled Internal Std 50μg	pcDNA 8 (Sample 1) 50μg	4E S209-HA 12 (Sample 3) 50μg
10	Pooled Internal Std 50μg	4E-HA 10 (Sample 2) 50μg	pcDNA 8 (Sample 4) 50μg
11	Pooled Internal Std 50μg	4E S209-HA 12 (Sample 2) 50μg	4E-HA 10 (Sample 4) 50μg
12	Pooled Internal Std 50μg	pcDNA 8 (Sample 2) 50μg	4E S209-HA 12 (Sample 4) 50μg

Table 3 1 2 11.1 Ettan DIGE experimental design for analysis of the effect of 4E-HA and 4E S209-HA overexpression Samples 1-4 represent biological replicates

DeCyder differential analysis software was used to identify protein spots which showed a 1 5 fold or more change in expression with a t-test score of 0 05 or less between

(a) pcDNA control transfected cells vs 4E-HA overexpressing cells (Section 3 1 2 11 1)
(b) pcDNA control transfected cells vs 4E S209-HA overexpressing cells (Section 3 1 2 11 2)
(c) 4E-HA overexpressing cells vs 4E S209-HA overexpressing cells (Section 3 1 2 11 3)
(d) 4E-HA clone 5 vs 4E-HA clone 10 (Section 3 1 2 11 4)

After differentially expressed protein spots were identified, these targets were then subjected to mass spectrometry analysis to determine their identities

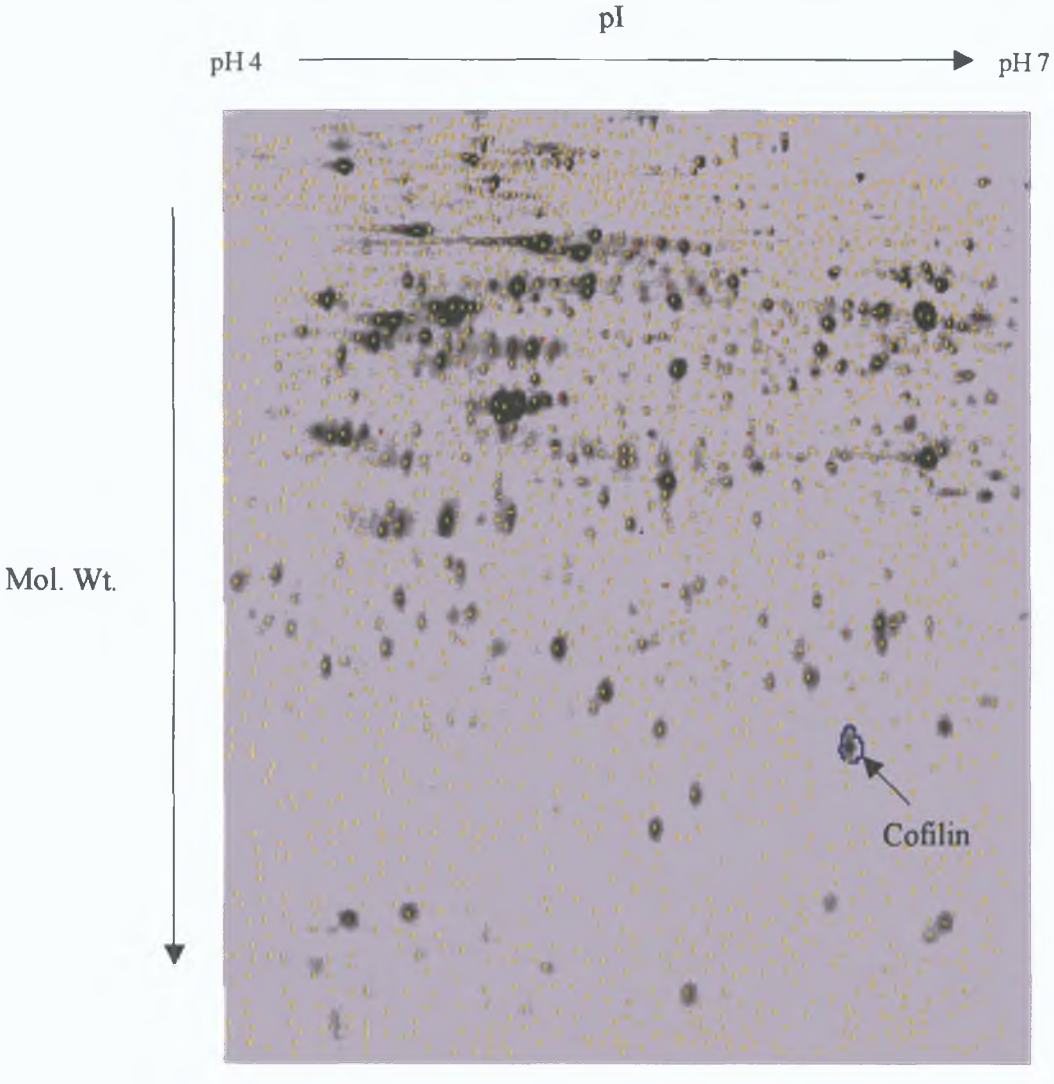


Figure 3.1.2.11.3. Representative DIGE 2D Gel Image. The protein spot identified as cofilin is circled and indicated with an arrow.

3 1 2 11 1 Differentially regulated proteins in 4E-HA overexpressing cells compared to pcDNA control transfected cells

Comparison of protein expression in 4E-HA overexpressing cells to pcDNA control transfected cells showed upregulation of three proteins, the mRNA processing protein G3BP and two protein chaperones, heat shock protein APG and chaperonin (Hsp60 protein 1) Three downregulated proteins were identified as Aldehyde Dehydrogenases and one downregulated protein was unidentified (Table 3 1 2 11 2)

Master No	Protein Name	Accession No	4E-HA vs pcDNA	Function
			Fold Change (T-test score)	
mRNA Processing				
803	G3BP	gi|5031703|	1 86 (0 029)	mRNA processing/degradation
Protein Folding				
297	Heat Shock Protein APG	gi|31541941|	1 9 (0 0094)	Chaperone Activity
1236	Chaperonin (Hsp60 protein 1)	gi|31542947|	1 51 (0 021)	Chaperone Activity
Metabolism				
1097	Aldehyde Dehydrogenase 1A1	gi|21361176|	-1 67 (0 05)	Free retinal binding
1100	Aldehyde Dehydrogenase 1	gi|2183299|	-1 76 (0 00064)	Free retinal binding
1117	Aldehyde Dehydrogenase 1A1	gi|21361176|	-2 11 (0 0016)	Free retinal binding
Unidentified				
2164	-	-	-1 7 (0 049)	-

Table 3 1 2 11 2 Differentially Expressed Proteins in 4E-HA overexpressing cells compared to pcDNA control cells

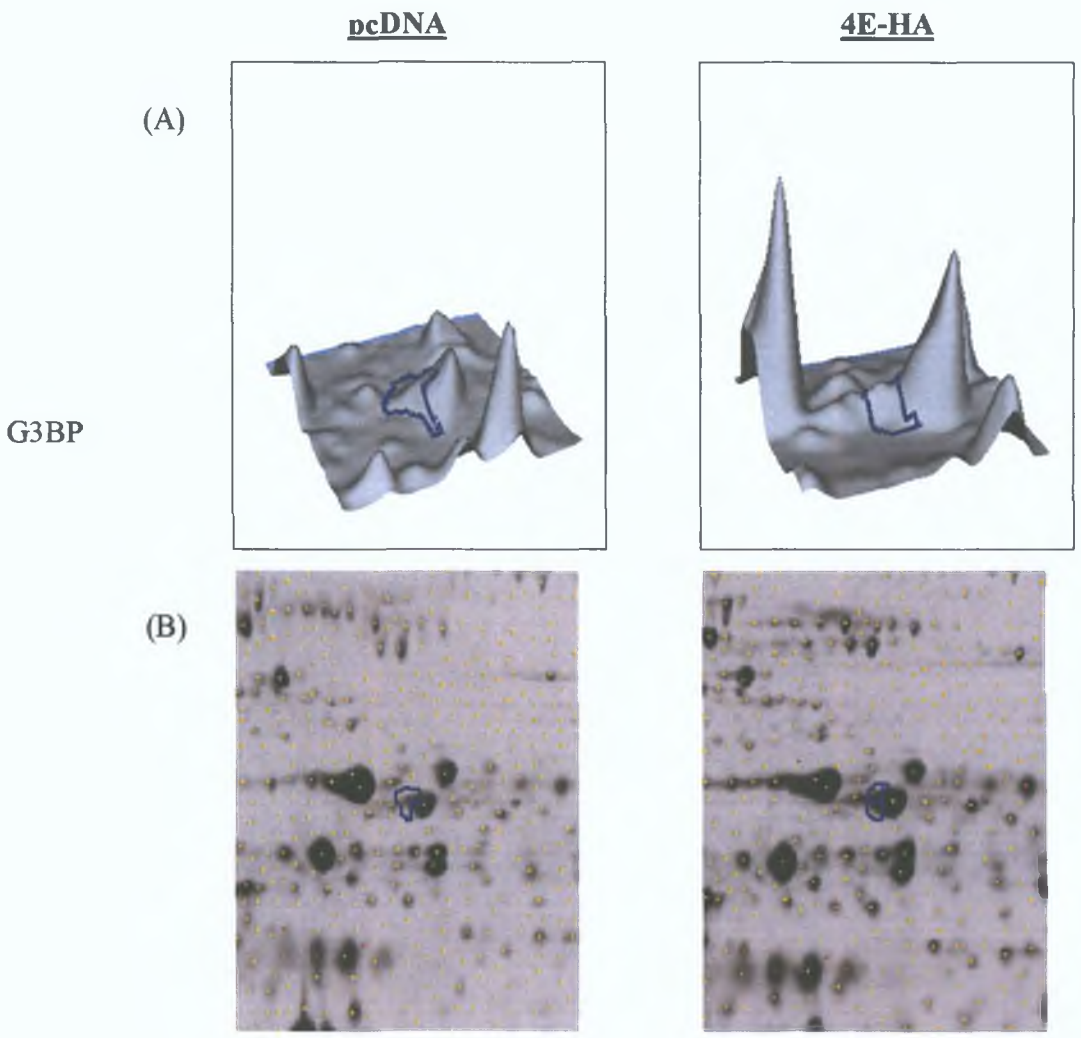


Figure 3.1.2.11.4 Decyder 3D view (A) and graph view (B) of G3BP expression in pcDNA and 4E-HA. The protein identified as G3BP is circled in purple.

3 1 2 11.2 Differentially regulated proteins in 4E S209-HA overexpressing cells compared to pcDNA control transfected cells

Comparison of protein expression in 4E-S209 HA overexpressing cells to pcDNA control transfected cells showed twelve protein spots were downregulated and seven upregulated in the 4E S209-HA overexpressing cells (Table 3 1 2 11 3) Of the the twelve downregulated protein spots, seven have been identified and are involved in cellular functions such as cytoskeletal regulation, mRNA processing and regulation of metabolic pathways

Seven protein spots were upregulated, five of which have been identified Three upregulated proteins are involved in regulation of protein degradation via the ubiquitin-proteasomal degradation pathway (Figure 3 1 2 11 5) One is an activator of protein chaperones and another is a nucleotide metabolism enzyme The Decyder graph view of the expression levels of the protein degradation protein PA28 beta is shown in Figure 3 1 2 11 6

Master No	Protein Name	Accession No	4E S209-HA vs pcDNA	Reported Function
			Fold Change (T-test score)	
Cytoskeletal				
2861	Cofilin	gi|5031635|	-4 77 (0 00099)	Actin filament organisation
1600	Moesin	gi|4505257|	-1 57 (0 024)	Actin cytoskeleton membrane interaction
2276	Tropomyosin3	gi|55665783|	-1 51 (0 00088)	Actin filament stabilisation
3290	Tubulin Specific Chaperone A	gi|30583547|	-1 91 (0 00016)	β-Tubulin processing
mRNA Processing				
1299	HNRPF	gi|15990432|	-1 98 (0 013)	mRNA processing
1296	HNRPF	gi|16876910|	-1 69 (0 043)	mRNA processing
Protein Degradation				
2312	Proteasome Activator PA28 beta chain	gi|2136005|	2 45 (4 5E-07)	Protein Degradation
2553	Ubiquitin carboxy-terminal hydrolase L1	gi|4185720|	1 91 (0 00016)	Protein Degradation
2313	Proteasome activator subunit 1 (PA28 alpha chain)	gi|30581141|	1 96 (0 00021)	Protein Degradation
Metabolism				
2062	Purine Nucleoside Phosphorylase	gi|55925942	2 19 (0 00080)	Purine Nucleotide Salvage
1808	DDAH1	gi|21707415|	-1 73 (0 0022)	Nitric Oxide Biosynthesis
2535	Glyoxalase 1	gi|15030212|	-1 46 (0 019)	Methylglyoxal detoxification
Protein Folding				
1539	AHA1	gi|6912280|	1 98 (0 0058)	Chaperone Activator
Unidentified				
2313	-		1 96 (0 00021)	
2596	-		-1 65 (0 00062)	
3310	-		-1 52 (0 0076)	
1670	-		-2 15 (0 022)	
1440	-		1 57 (0 026)	
1688	-		-1 57 (0 035)	
993	-		-1 88 (0 036)	

Table 3 1 2 11 3 Differentially Expressed Proteins in 4E S209-HA overexpressing clones compared to pcDNA control clones

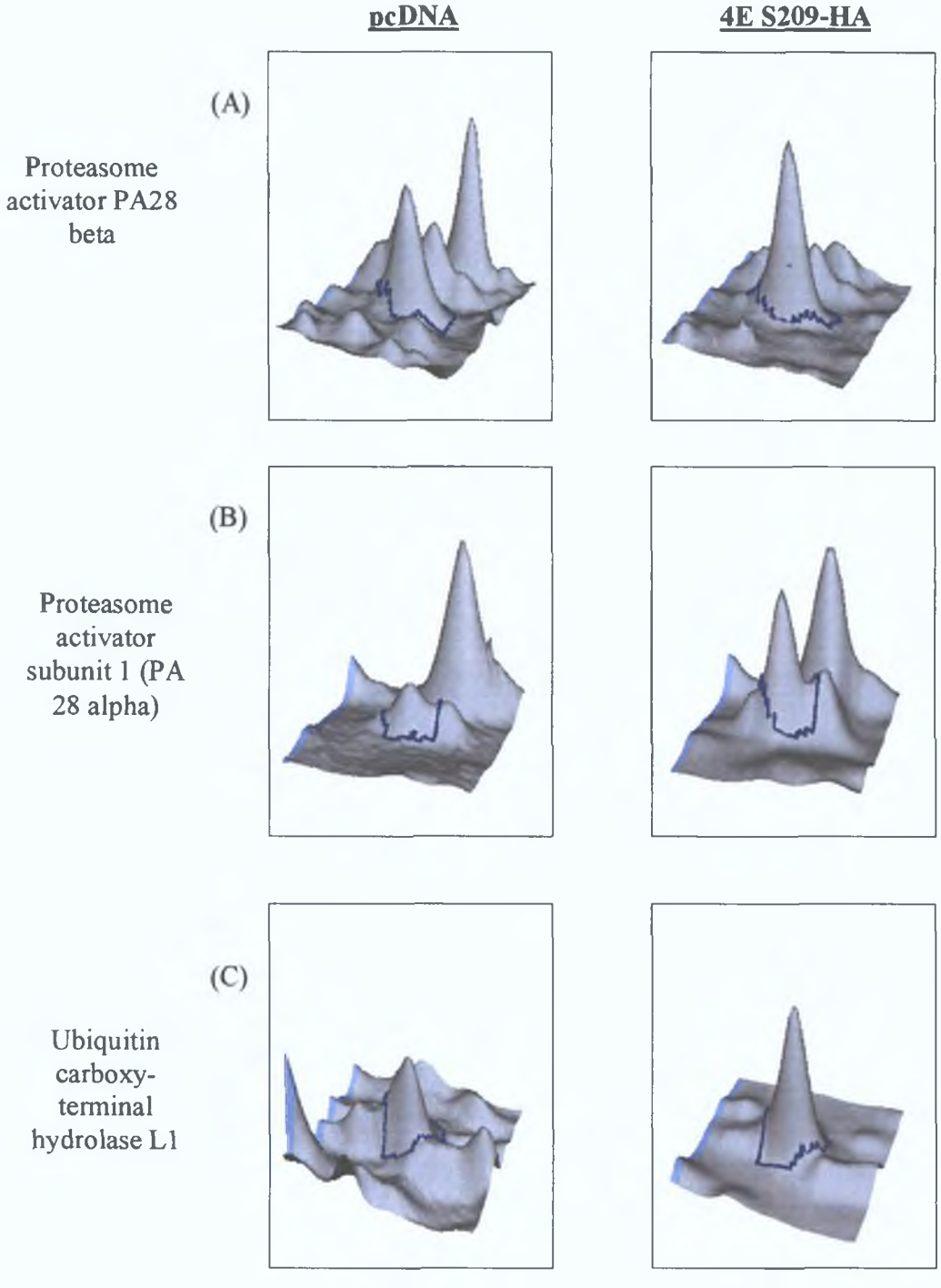


Figure 3.1.2.11.5 Decyder 3D view of protein degradation proteins upregulated in 4E S209-HA transfected cells compared to pcDNA control cells.

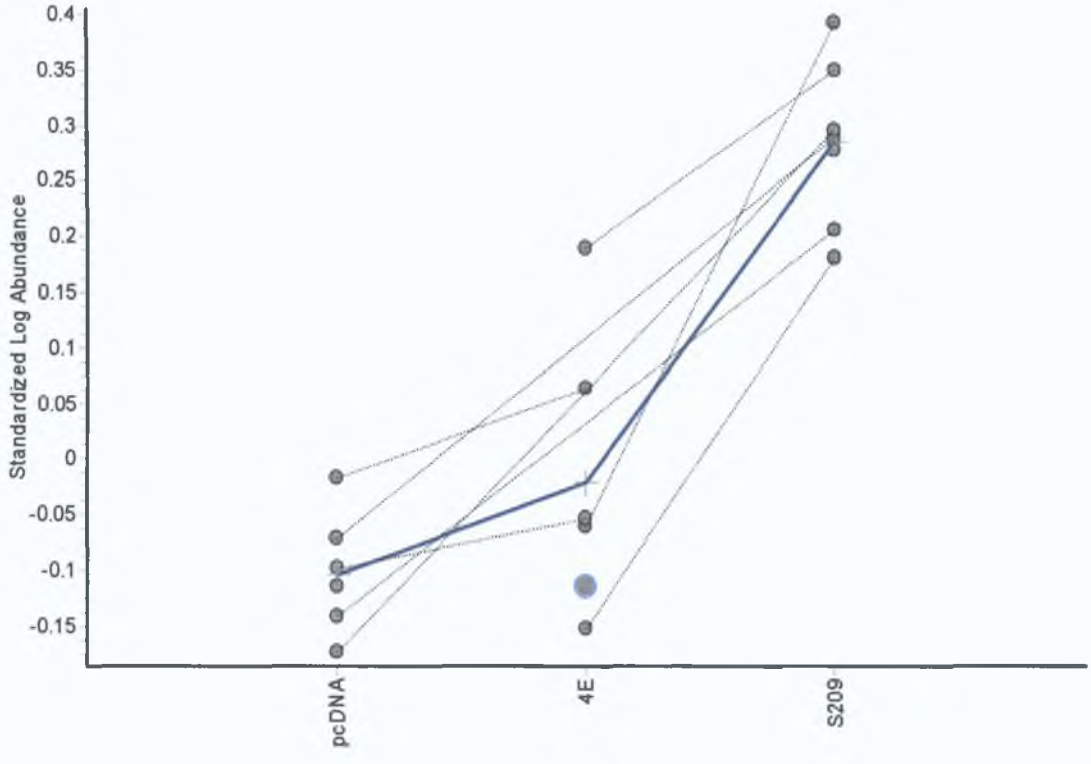


Figure 3.1.2.11.6 DeCyder graph of expression level of protein identified as Proteasome Activator PA28 beta in pcDNA, 4E-HA and 4E S209-HA transfected cells.

3 1 2 11 3 Differentially regulated proteins in 4E S209-HA overexpressing cells compared to 4E-HA overexpressing cells

Comparison of 4E-HA overexpressing cells to 4E S209-HA overexpressing cells showed down-regulation of 31 proteins and up-regulation of 15 proteins Of these 46 differentially expressed proteins 26 have been identified (Table 3 1 2 11 4)
Of the down-regulated proteins, eight have been identified as cytoskeletal proteins, four are involved in the processing of mRNAs, two are protein chaperones, two are metabolic proteins and one is a protein degradation pathway protein (possible actin)
Of the upregulated proteins, three are involved in regulation of protein degradation via the ubiquitin-proteasomal degradation pathway, five are identified as aldehyde dehyrogenase 1 or 1a1 and one is the nucleotide metabolism enzyme Purine Nucleoside Phosphorylase Figures 3 1 2 11 7 and 3 1 2 11 8 show a Decyder 3D and image view of Cofilin and Moesin expression respectively Figure 3 1 2 11 9 shows a Decyder graph view of Cofilin expression

Master No.	Protein Name	Accession Number	4E S209-HA vs 4E-HA Fold Change (T-test score)	Reported Function
Cytoskeletal				
2861	Cofilin	gi|5031635|	-5.38 (0.0069)	Actin filament organisation
1600	Moesin	gi|4505257|	-1.71 (0.015)	Actin cytoskeleton membrane interaction
1333	Solute Carrier Family 9, isoform 3 regulator 1 (EBP-50)	gi|4759140|	-2.24 (0.0084)	Actin cytoskeleton membrane interaction
2276	Tropomyosin3	gi|55665783|	-1.5 (0.033)	Actin filament stabilisation
993	EPB41L2	gi|21961573|	-1.98 (0.0066)	Actin organisation
3290	Tubulin Specific Chaperone A	gi|30583547|	-1.86 (0.0012)	β-Tubulin processing
1666	Mutant beta actin	gi|28336|	-1.61 (0.0029)	Cytoskeletal Protein
1347	Actin-like 6A isoform 1	gi|4757718|	-1.76 (0.0066)	Chromatin Modification
mRNA Processing				
803	G3BP	gi|5031703|	-2.36 (0.038)	mRNA processing/degradation
1296	HNRPF	gi|16876910|	-1.84 (0.0065)	mRNA processing
1299	HNRPF	gi|15990432|	-1.73 (0.0034)	mRNA processing
1072	HNRPK	gi|14165435|	-1.56 (0.041)	mRNA processing
Protein Degradation				
2312	Proteasome Activator PA28 beta chain	gi|2136005|	2.07 (8.8E-05)	Protein Degradation
2553	Ubiquitin carboxy-terminal hydrolase L1	gi|4185720|	1.5 (0.0016)	Protein Degradation
2313	Proteasome activator subunit 1 (PA28 alpha chain)	gi|30581141|	1.85 (0.00026)	Protein Degradation
1300	Ubiquitin transfer carrier member	gi|16924319| blast says actin	-2.08 (0.0096)	Protein Degradation
Protein Folding				
297	Heat Shock Protein APG	gi|31541941|	-2.25 (0.012)	Protein Folding
747	HSP APG1	gi|31541941|	-2.3 (0.023)	Protein Folding
Metabolism				
1097	Aldehyde Dehydrogenase 1A1	gi|21361176|	2.4 (0.0026)	Free retinal binding
1100	Aldehyde Dehydrogenase 1	gi|2183299|	1.68 (0.0031)	Free retinal binding
1117	Aldehyde Dehydrogenase 1A1	gi|21361176|	2.17 (0.0001)	Free retinal binding
1167	Aldehyde Dehydrogenase 1A1	gi|21361176|	2.2 (0.0003)	Free retinal binding
1177	Aldehyde Dehydrogenase 1A1	gi|21361176|	2.28 (0.00033)	Free retinal binding
2062	Purine Nucleoside Phosphorylase	gi|55925942|	1.73 (0.0033)	Purine Nucleotide Salvage
1808	DDAH1	gi|21707415|	-1.44 (0.0041)	Nitric Oxide Biosynthesis

2535	Glyoxalase 1	gi|15030212|	-2 01 (0 0034)	Methylglyoxal detoxification
Unidentified				
3310		-	-1 56 (0 0047)	
1670		-	-2 06 (0 00041)	
1440		-	1 78 (0 0016)	
1688		-	-1 65 (0 0052)	
1868		-	2 87 (0 00023)	
523		-	-2 78 (0 00063)	
1156		-	1 7 (0 0022)	
2280		-	1 95 (0 0039)	
3310		-	-1 56 (0 0047)	
1688		-	-1 65 (0 0052)	
1272		-	-2 17 (0 0064)	
1123		-	1 71 (0 0058)	
911		-	-2 01 (0 0088)	
917			-1 86 (0 013)	
997		-	-2 31 (0 014)	
1136		-	1 63 (0 023)	
749		-	-2 36 (0 029)	
2077		-	2 47 (0 035)	
653		-	-3 25 (0 034)	
2814		-	-1 84 (0 04)	
1213		-	-1 76 (0 044)	

Table 3 1 2 11 4 Differentially Expressed Proteins in 4E-HA overexpressing clones compared to 4E S209-HA clones

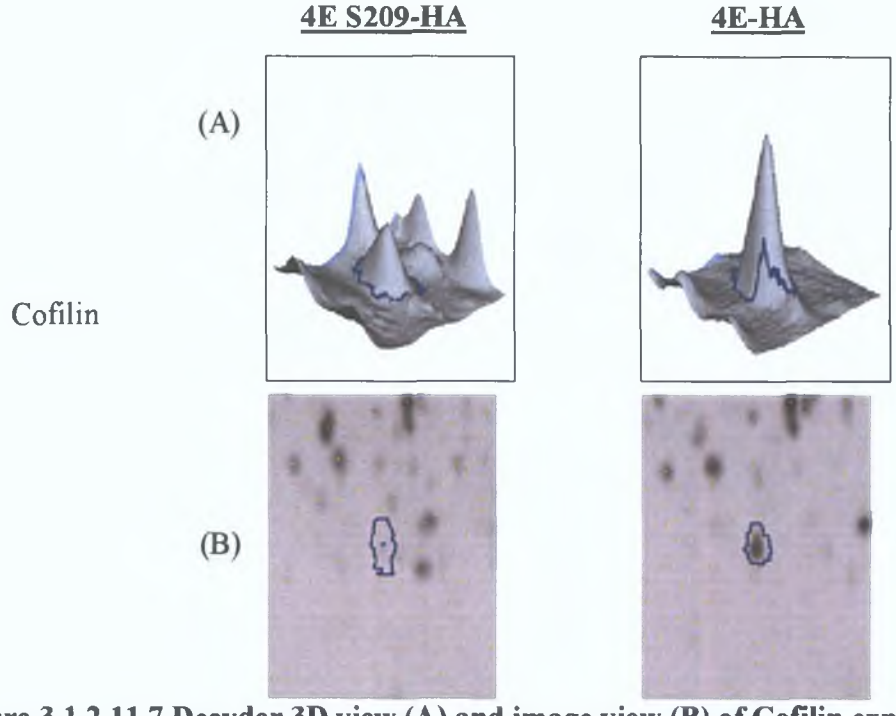


Figure 3.1.2.11.7 Decyder 3D view (A) and image view (B) of Cofilin expression in 4E-HA and 4E S209-HA samples

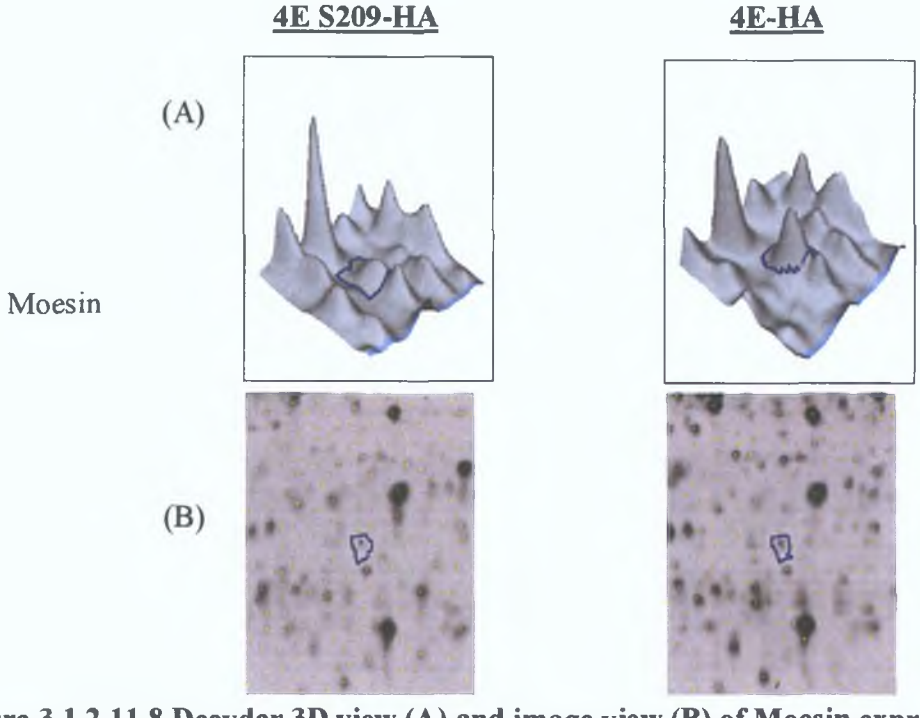


Figure 3.1.2.11.8 Decyder 3D view (A) and image view (B) of Moesin expression in 4E-HA and 4E S209-HA samples.

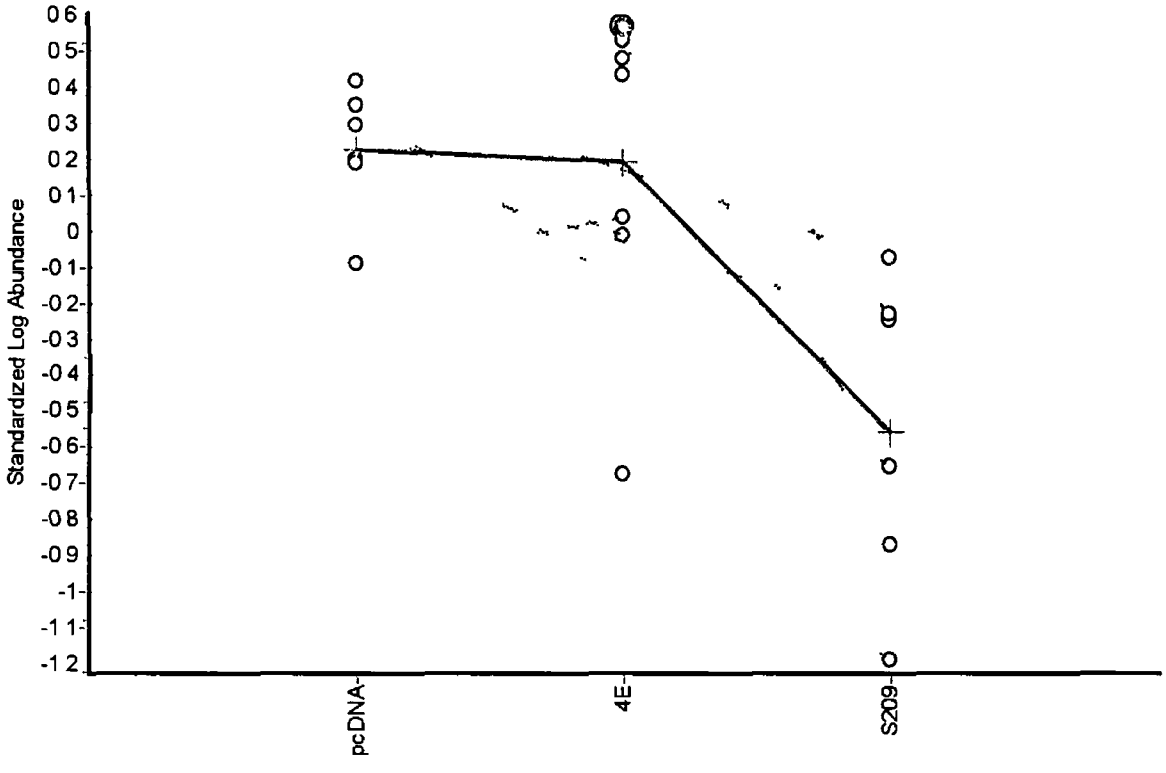


Figure 3 1 2 11 9 Decyder graph view of Cofilin expression in pcDNA, 4E-HA (4E) and 4E S209-HA (S209) samples

3 1 2 11 4 Differentially regulated proteins in 4E-HA clone 10 compared to 4E-HA clone 5

Comparison of 4E-HA overexpressing clone 10 to 4E-HA clone 5 showed lower expression of 14 proteins and higher expression of 8 proteins Of these 22 differentially expressed proteins 16 have been identified (Table 3 1 2 11 5)

Of the lower expression level proteins, six have been identified as cytoskeletal proteins (e g vimentin, Figure 3 1 2 11 10), three are metabolic proteins, one is involved in the processing of mRNAs (poly(rC)-binding protein 2) and one is involved in cellular signalling (Prohibitin)

Of the higher expression level proteins, one is involved in cytoskeleton regulation (CapG), one is involved in protein folding (Hsp 70kDa protein 5), one is involved in protein degradation (Proteasome activator PA28 beta) and one is a metabolic protein (Peroxiredoxin 2 isoform b)

Master No	Protein Name	Accession Number	4E-10 vs 4E-5 Fold Change (t-test score)	Reported Function
Cytoskeletal				
1264	Vimentin	gi|57471646|	-3 76 (0 006)	Cytoskeletal Protein
1261	Vimentin	gi|340219|	-3 26 (0 0016)	Cytoskeletal Protein
1573	Mutant Beta Actin	gi|28336|	-1 71 (0 00097)	Cytoskeletal Protein
1549	Mutant Beta Actin	gi|28336|	-1 49 (0 024)	Cytoskeletal Protein
1677	Chain A, ca2+ binding mimicry In The Crystal Structure Of The Eu3+-Bound Mutant Human Macrophage Capping Protein Cap G (CapG)	gi|21730367|	1 86 (0 027)	Actin Filament Capping Protein
1968	novel protein similar to annexin A2	gi|12314197|	-1 99 (0 0032)	Plasma membrane binding
1799	Annexin A1	gi|54696696|	-1 58 (0 028)	Plasma membrane binding
Protein Folding				
810	Hsp 70kDa protein 5 (BiP)	gi|16507237|	1 5 (0 029)	Protein Chaperone
mRNA Processing				
1654	poly(rC)-binding protein 2	gi|14141166|	-1 5 (0 021)	mRNA processing
Protein Degradation				
2312	Proteasome activator PA28 beta	gi|2136005|	1 58 (0 048)	Protein Degradation
Metabolism				
2514	IPP isomerase		-1 51 (0 046)	
1073	Glutaminase isoform C	gi|6002671|	-1 91 (0 0014)	Glutamine catabolism
989	FASN	gi|38196977|	-2 37 (0 00064)	Fatty acid biosynthesis
1071	Aldehyde Dehydrogenase 1	gi|2183299|	-2 18 (0 00018)	Oxidoreductase activity
2754	Peroxiredoxin 2 isoform b	gi|33188452|	1 46 (0 002)	Antioxidant activity
Cell Signalling				
2154	Prohibitin	gi|55646807|	-2 13 (0 0091)	
Unidentified				
1670	-	-	1 7 (0 0012)	-
2443	-	-	1 47 (0 0065)	-
2590	-	-	1 5 (0 0094)	-
1249	-	-	-2 17 (0 019)	-
560	-	-	-1 87 (0 032)	-
1541	-	-	1 84 (0 045)	

Table 3 1 2 11 5 Differentially Expressed Proteins in 4E-HA overexpressing clone 4E HA 5 compared to clone 4E-HA 10

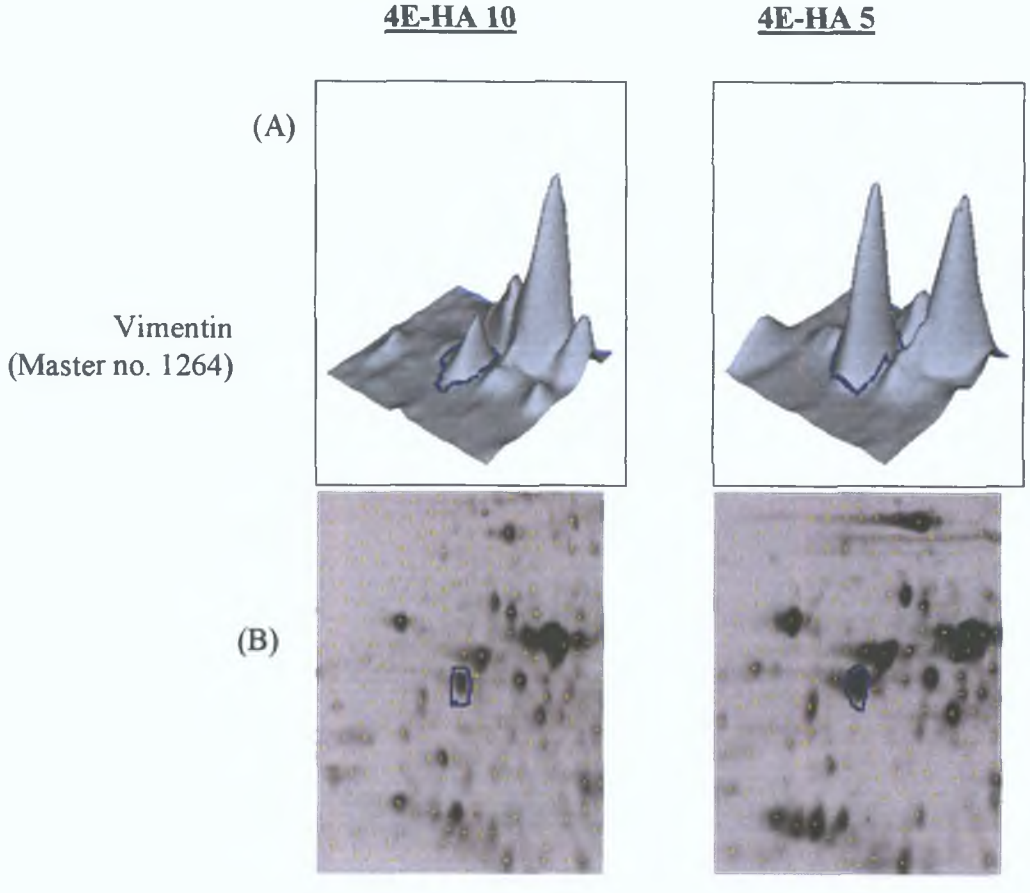


Figure 3.1.2.11.10 Decyder 2D view (A) and Image view (B) of protein spot identified as Vimentin (Master No. 1264).

3.1 2 11.5 UTRscan analysis of mRNAs of differentially regulated cytoskeletal proteins.

The mRNA sequences for some of the differentially regulated cytoskeletal proteins were analysed using the online mRNA analysis program UTRscan which detects regulatory nucleotide sequences present in mRNA UTRs (Mignone *et al*, 2005) The proteins analysed included cofilin, moesin, tropomyosin 3, EBP50, EPB41L2, ACTL6A and TBCA
(http //www ba itb cnr it/BIG/UTRScan/)

3 1 2 11 5 1 Cofilin (NM_005507 2)

```
-------------------------------------------------------
                  Pattern = 15-LOX-DICE
-------------------------------------------------------
       Found 1 matches in 1 sequences

gi|49472823|ref|NM_005507 2|  [908,924]  CCCTCCATCC CTTG ACG

-----> Checking repeats for  15-LOX-DICE  (min  2)
       Found 0 matches for pattern 15-LOX-DICE
```

3 1 2 11 5 2 Moesin (NM_002444 2)

```
------------------------------------------------------------
                    Pattern = 15-LOX-DICE
------------------------------------------------------------
       Found 3 matches in 1 sequences

gi|53729335|ref|NM_002444 2|  [421,435]  CCCCTGCTCT TT AAG
gi|53729335|ref|NM_002444 2|  [3256,3274]  CCCTTGCTCT CAACCC AGG
gi|53729335|ref|NM_002444 2|  [3435,3450]  CCCCACACCT GGA AAG

-----> Checking repeats for  15-LOX-DICE  (min  2)
       Found 0 matches for pattern 15-LOX-DICE

------------------------------------------------------------
                    Pattern = IRES
------------------------------------------------------------
       Found 1 matches in 1 sequences

gi|53729335|ref|NM_002444 2|  [3874,3981]  TTTGTG TACTTT TTGGG
TTTTTTAA AAATTGTT TTTGGAGG GGTTT ATGCT CAATC CATG TTCTA TTTCAG TGCCAA
TAAAA
TTTAG GAAGAC TTCAA AAAAAAAAAA


------------------------------------------------------------
                    Pattern = ADH_DRE
------------------------------------------------------------
       Found 1 matches in 1 sequences

gi|53729335|ref|NM_002444 2|  [2653,2660]  AAGGCTGA
```

3.1 2 11 5 3 Tropomyosın 3, varıant 1 (NM_152263 1)

```
-------------------------------------------------------
                  Pattern = 15-LOX-DICE
-------------------------------------------------------
       Found 3 matches ın 1 sequences

gı|22748618|ref|NM_152263 1|  [785,798]  CCACGCCCTC A ATG
gı|22748618|ref|NM_152263 1|  [971,990]  CCCCACCCCA AATTAAA ATG
gı|22748618|ref|NM_152263 1|  [1102,1120]  CCCCCTCCTCT GACTT ATG

-----> Checkıng repeats for  15-LOX-DICE  (mın  2)
       Found 0 matches for pattern 15-LOX-DICE

-------------------------------------------------------
                  Pattern = IRES
-------------------------------------------------------
       Found 1 matches ın 1 sequences

gı|22748618|ref|NM_152263 1|  [1179,1281]  TGATT CTACC AGAGTG A TGGAT
TTA GTACA GGTTACTC AGGA TAGTAATT TTAGT TATACT CCTCA AGCTG AACA AGATT
AAATTCCT TATTT CCAGGTTCTT
```

3.1 2 11 5 4 Tropomyosın 3, varıant 2 (NM_153649.2)

```
-------------------------------------------------------
                  Pattern = 15-LOX-DICE
-------------------------------------------------------
       Found 1 matches ın 1 sequences

gı|39725631|ref|NM_153649 2|  [1563,1579]  TCCTGCCTCC TTGA AAG

-----> Checkıng repeats for  15-LOX-DICE  (mın  2)
       Found 0 matches for pattern 15-LOX-DICE

-------------------------------------------------------
                  Pattern = Brd-Box
-------------------------------------------------------
       Found 1 matches ın 1 sequences

gı|39725631|ref|NM_153649 2|  [1387,1393]  AGCTTTA
```

3 1 2 11.5 5 EBP50 (NM_004252.1)

```
-------------------------------------------------------
                Pattern = 15-LOX-DICE
-------------------------------------------------------
       Found 2 matches in 1 sequences

gi|4759139|ref|NM_004252 1|  [1614,1633]  CCCTCCCTTC CTCCCCC ATG
gi|4759139|ref|NM_004252 1|  [1915,1932]  GCCCATCCCT GAGCC AGG

-----> Checking repeats for  15-LOX-DICE  (min  2)
       Found 0 matches for pattern 15-LOX-DICE

-------------------------------------------------------
                Pattern = K-Box
-------------------------------------------------------
       Found 1 matches in 1 sequences

gi|4759139|ref|NM_004252 1|  [1631,1638]  ATGTGATA
```

3 1 2 11 5 6 EPB41L2 (NM_001431 1)

```
-------------------------------------------------------
                Pattern = GY-Box
-------------------------------------------------------
       Found 1 matches in 1 sequences

gi|4503578|ref|NM_001431 1|  [155,161]  GTCTTCC
```

3 1 2 11 5.6 Actın-lıke 6A (ACTL6A), transcrıpt varıant 1 (NM_004301.2)

```
--------------------------------------------------------
                    Pattern = K-Box
--------------------------------------------------------
       Found 1 matches ın 1 sequences

gı|30089995|ref|NM_004301 2|  [1178,1185]  GTGTGATA

--------------------------------------------------------
                    Pattern = Brd-Box
--------------------------------------------------------
       Found 2 matches ın 1 sequences

gı|30089995|ref|NM_004301 2|  [1512,1518]  AGCTTTA
gı|30089995|ref|NM_004301 2|  [1601,1607]  AGCTTTA
```

3 1 2 11 5 7 TBCA, (NM_004607 1)

No regulatory elements found

3.1.2.12 Microarray analysis of stably transfected DLKP cells.

The results already presented show that overexpression of eIF4E in DLKP cells induced changes in the expression of growth and differentiation related proteins. The translational upregulation of gene expression by eIF4E may also have a knock on effect on regulation of gene transcription, which could contribute to changes in growth and differentiation in eIF4E overexpressing cells. We decided to analyse gene mRNA expression profiles of 4E-HA overexpressing cells using Affymetrix GeneChip microarrays (Fig. 3.1.2.12.1). The RNA quality of samples (Fig. 3.1.2.12.2) and fragmentation of biotin labelled cRNA (Fig. 3.1.2.12.3) were monitored for this experiment.

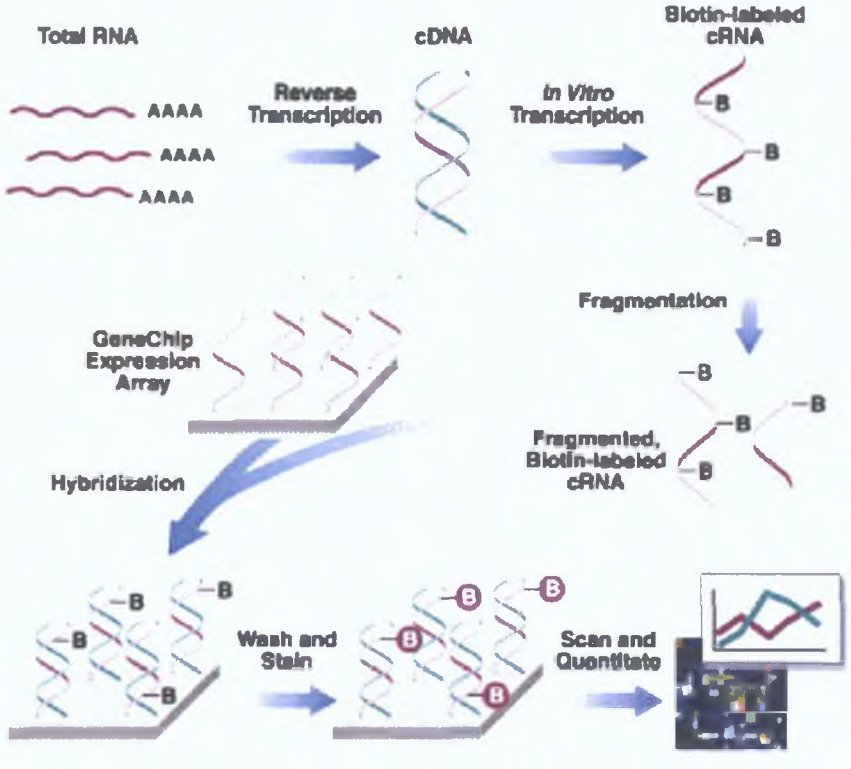


Figure 3.1.2.12.1 Affymetrix GeneChip gene expression assay. Labeled cRNA targets derived from the mRNA of an experimental sample are hybridized to nucleic acid probes attached to the solid support. By monitoring the amount of label associated with each DNA location, it is possible to infer the abundance of each mRNA species represented

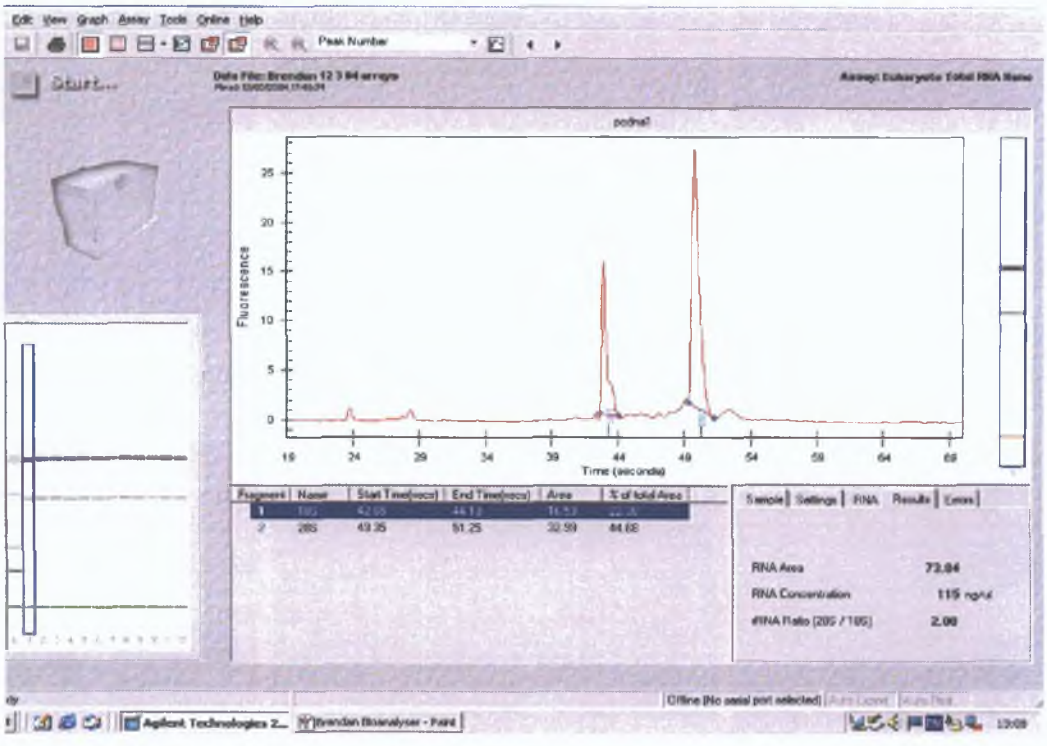


Figure 3.1.2.12.2 Analysis of RNA quality using Bioanalyser RNA chip. The two clear sharp peaks represent 18S and 20S ribosomal RNA. All samples tested show similar results indicating RNA was not degraded and no DNA contamination was present.

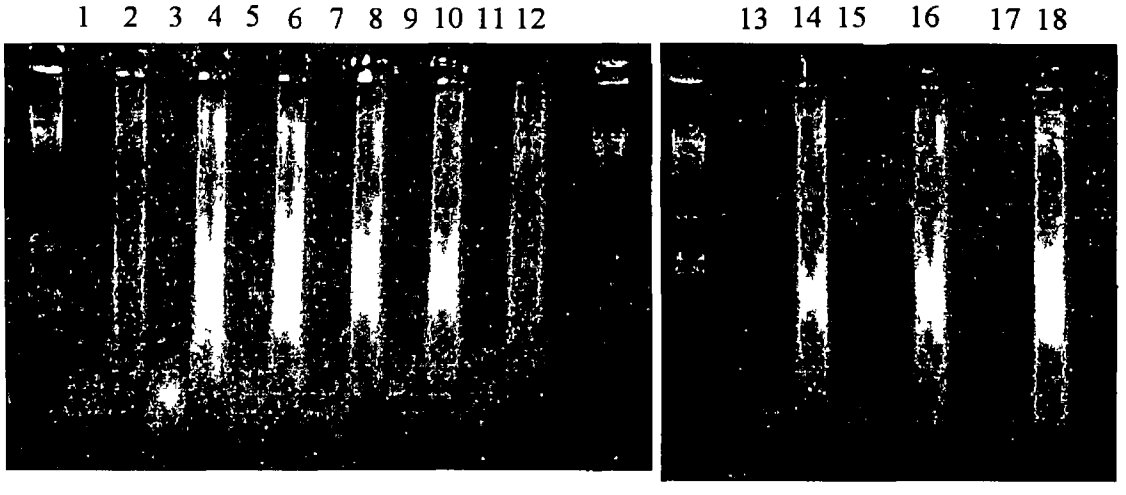


Figure 3 1.2 12 3 Monitoring of fragmented and unfragmented biotin labelled cRNA by agarose gel electrophoresis (Lane 1) pcDNA 2, sample 1 fragmented cRNA, (Lane 2) pcDNA 2, sample 1 unfragmented cRNA, (Lane 3) pcDNA 2, sample 2 fragmented cRNA, (Lane 4) pcDNA 2, sample 2 unfragmented cRNA, (Lane 5) pcDNA 2, sample 3 fragmented cRNA, (Lane 6) pcDNA 2, sample 3 unfragmented cRNA, (Lane 7) 4E-HA 5, sample 1 fragmented cRNA, (Lane 8) 4E-HA 5, sample 1 unfragmented cRNA, (Lane 9) 4E-HA 5, sample 2 fragmented cRNA, (Lane 10) 4E-HA 5, sample 2 unfragmented cRNA, (Lane 11) 4E-HA 5, sample 3 fragmented cRNA, (Lane 12) 4E-HA 5, sample 3 unfragmented cRNA, (Lane 13) 4E S209-HA 6, sample 1 fragmented cRNA, (Lane 14) 4E S209-HA 6, sample 1 unfragmented cRNA, (Lane 15) 4E S209-HA 6, sample 2 fragmented cRNA, (Lane 16) 4E S209-HA 6, sample 2 unfragmented cRNA, (Lane 17) 4E S209-HA 6, sample 3 fragmented cRNA, (Lane 18) 4E S209-HA 6, sample 3 unfragmented cRNA

3.1.2.12.1 Microarray expression data analysis

Affymetrix HGU133A oligonucleotide microarray chips were used to analyse mRNA expression levels of pcDNA-2, 4E S209-HA 6 and 4E-HA 5 cells.

HG-UI33A GeneChips are comprised of over 500,000 unique oligonucleotide features covering over 18,000 transcripts and variants, which, in turn, represent approximately 14,500 of the best characterised human genes.

pcDNA 2, 4E S209-HA 6 and 4E-HA 5 RNA samples were analysed in triplicate on Affymetrix HG-U133A GeneChips. All Q.C. parameters for each sample were found to be within acceptable limits (Fig. 3.1.2.12.4). Gene expression data was analysed in order to identify genes that are differentially expressed between these cell lines and hierarchical clustering was subsequently performed to group differentially expressed genes according to their expression pattern.

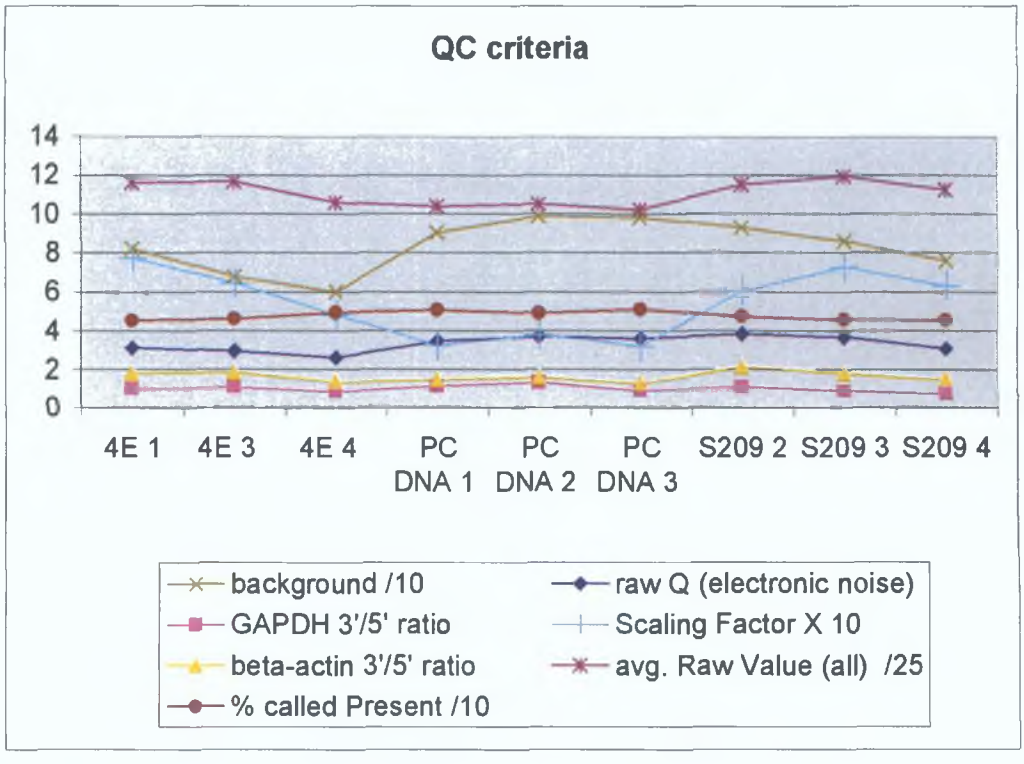


Figure 3.1.2.12.4 Analysis of Q.C. parameters for GeneChip microarrays

The heat map of clustered genes (Fig. 3.1.2.12.5) was used to select groups of genes which showed a common pattern of expression in the different samples analysed, e.g. group 1 contains genes which show decresed expression in both 4E-HA 5 and 4E S209-HA 6 cells compared to pcDNA 2 cells.

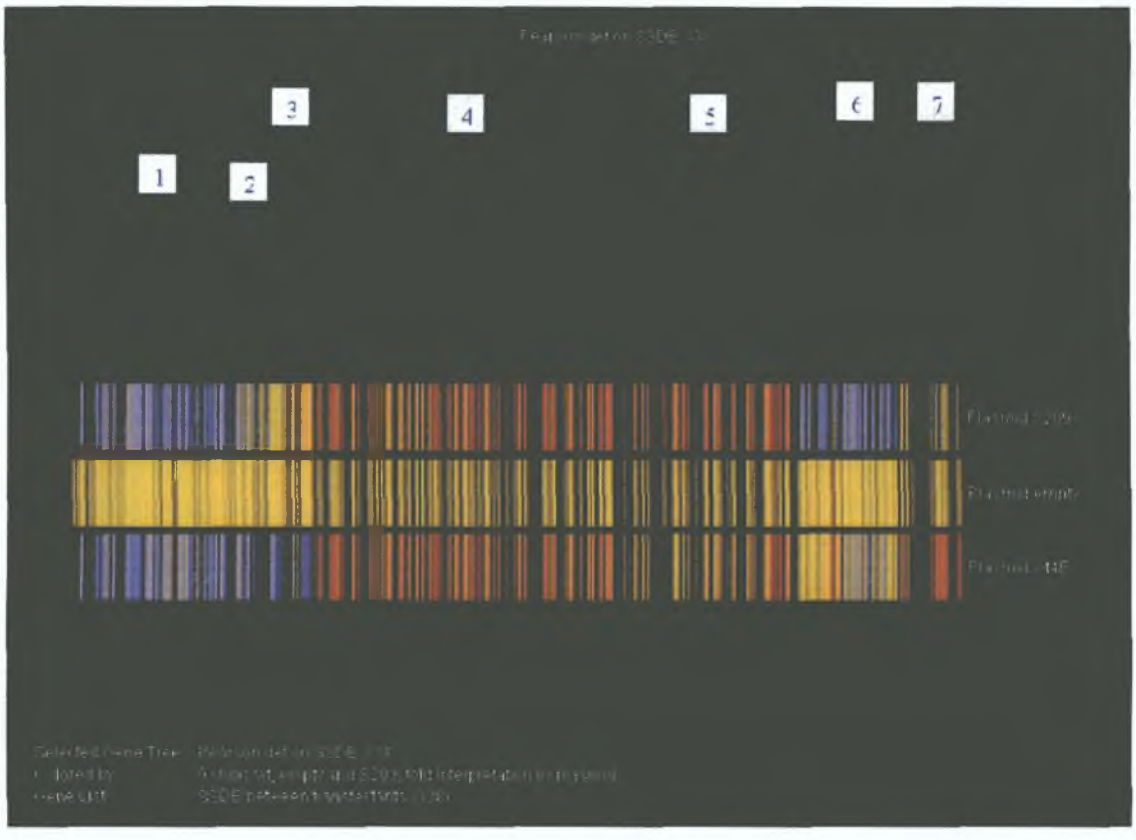


Figure 3.1.2.12.5 Heat Map representation of differentially expressed genes showing clustering of similarly expressed genes. Clusters labelled 1-7 were chosen for subsequent analysis. This heat map displays expression level of genes in the baseline/control sample (pcDNA 2) as yellow. Genes which show increased expression in the test samples (4E-HA 5, 4E S209-HA 6) are coloured red and genes which show decreased expression are coloured blue.

3 1 2 12 2 Analysis of differentially expressed genes

Various software tools were employed in the analysis of differentially expressed genes Probe set IDs for probe sets which were found to be differentially regulated where entered onto the NetAffx (hhtp //www affymetrix com) online database NetAffx is specifically designed for affymetrix data and provides up-to-date annotation information for each probe set, function information and links to online public databases

Bibliosphere (Genomatix, www genomatix de/go/bib) data-mining software was used to help identify gene-gene connections within lists of differentially expressed genes from microarray analysis Bibliosphere data mining software automatically detects co-citations of genes in PubMed literature abstracts Bibliosphere also automatically checks for alternative gene names in pubmed abstracts which proved to be a bottle-neck in manual analysis as many differentially expressed genes were known by multiple names in the literature Bibhosphere analysis data of gene-gene connections is displayed as a 3D interactive view of gene relationships (Fig 3 1 2 12 6)

EASE software (Hosack *et al* , 2003) was also used to help identify biological themes from lists of differentially regulated probe set IDs

Genes found to be differentially expressed in 4E-HA 5 and 4E S209-HA 6 cells compared to pcDNA 2 control cells were categorised and examined according to their function (e g transcription) or whether their expression is involved in regulating a certain type of cellular phenotype (e g invasiveness) Expression levels of genes involved in cellular invasion, tumor suppression, regulation of the actin cytoskeleton, transcription and integrin signalling/FAK interaction were found to be altered in 4E-HA and 4E S209-HA overexpressing cells

3 1 2 12 2 1 Invasion related gene expression

Previous experiments in this study have shown that 4E-HA 5 cells display increased *in vitro* invasiveness (Section 3 1 2 13) Analysis of differentially regulated gene lists revealed numerous differentially regulated genes which have previously been shown to regulate or are associated with cellular invasiveness (Table 3 1 2 12 1)

Table 3 1 2 12 1 Expression levels of differentially expressed invasion related genes Normalised and raw (in brackets) expression values are given for these genes *Genes

	pcDNA 2	4E S209-HA 6	4E-HA 5
TACSTD1	1 (18 2)	9 654 (175 7)	1 746 (31 77)
TPBG	1 (88 57)	0 324 (28 67)	1 204 (106 6)
MMP10	1 (681 2)	0 423 (288 4)	0 878 (597 9)
TFPI2*	1 (298 65)	0 006 (1 8665)	0 427 (127 615)

represented by more than one probe set, the average expression values are given for these genes

3 1 2 12 2 2 Tumor suppressor gene expression

Analysis of differentially expressed gene lists showed a large number of genes which are upregulated in 4E S209-HA 6 cells compared to pcDNA 2 and 4E-HA 5 cells are known to function as tumor suppressors (Table 3 1 2 12 2)

	pcDNA 2	4E S209-HA 6	4E-HA 5
ROBO1	1 (181 4)	0 513 (93 17)	0 389 (70 63)
SCRIB	1 (114 4)	0 442 (50 5)	0 497 (56 83)
THY1	1 (75 43)	0 302 (22 77)	0 285 (21 47)
C5orf13	1 (153 5)	0 92 (141 2)	0 243 (37 23)
CDKN1B	1 (98 03)	2 581 (253 6)	2 42 (237 2)
SFN*	**1 (61 234)**	**17.217 (1054.25)**	**8 883 (543 943)**
DAPK	**1 (22 5)**	**4 29 (96 53)**	**1 557 (35 03)**
TOB1	**1 (126 8)**	**2 163 (274.3)**	**1 526 (193.5)**
GAS2	**1 (1 8)**	**24 167 (43 5)**	**4 704 (8 467)**
PLAGL1	**1 (13 23)**	**6 283 (83 13)**	**2 207 (29 2)**
GPC3	**1 (17 8)**	**2 376 (42 3)**	**1 15 (20 47)**
DCN*	**1 (29 62)**	**10 03 (297)**	**0 833 (24 66)**
LUM	**1 (5 2)**	**62 73 (326 2)**	**0 256 (1 433)**
CYR61	1 (923)	0 371 (342 4)	0 565 (521 6)

Table 3 1 2 12 2 Expression levels of differentially expressed tumor suppressor genes Normalised and raw (in brackets) expression values are given for these genes Genes which are upregulated in 4E S209-HA 6 cells compared to pcDNA 2 and 4E-HA 5 cells are in bold type *Genes represented by more than one probe set, the average expression value is given for these genes

3 1 2 12 2.3 Cytoskeletal/actin related genes

A large number of genes involved in regulating the actin cytoskeleton or actin cytoskeleton protein interactions were represented in the differentially regulated genes from microarray analysis (Table 3 1 2 12 3) Analysis of F-actin in 4E-HA overexpressing cells showed alterations in F-actin structures in 4E-HA 5 cells (Section 3 1 2 13) Microarray analysis indicates transcriptional control of genes involved in regulating actin structures may play a role in mediating these changes

Other cytoskeletal protein genes such as the intermediate filament protein Vimentin (VIM) and genes involved in regulating microtubules (e g TBCD) were found to be differentially expressed

	pcDNA 2	4E S209-HA 6	4E-HA 5
FAT	1 (106)	0 22 (23 43)	0 14 (14 3)
DNM3	1 (36 77)	0 33 (12 27)	0 46 (16 8)
TAGLN2	1 (297 4)	0 40 (120)	0 39 (116 6)
TNNT1	1 (188 5)	0 54 (101 9)	0 43 (80 57)
FXYD5	1 (209 5)	0 32 (67 47)	0 45 (95 2)
PDLIM2	1 (149 4)	0 44 (65 13)	0 52 (77 6)
PDLIM4	1 (53 03)	0 71 (37 63)	0 24 (12 83)
CAPG	1 (163 4)	0 93 (151 7)	0 28 (45 27)
CSRP1	1 (43 9)	3 48 (152 9)	3 66 (160 8)
SLC9A3R1	1 (89 27)	1 55 (138 7)	2 30 (205)
VIM	1 (1,230 467)	1 97 (2,420 133)	2 36 (2,908 5)
MARCKS*	1 (11 519)	5 23 (60 235)	11 88 (136 85)
TBCD	1 (29 67)	1 48 (44)	2 13 (63 23)
NEBL*	1 (80 5)	1 51 (121 5)	2 38 (191 565)
LIM*	1 (104 185)	1 38 (143 785)	2 43 (252 65)
BASP1	1 (20 6)	3 62 (74 63)	0 94 (19 37)
ANK2*	1 (22 635)	8 37 (189 515)	1 21 (18 635)
SSPN*	1 (4 0335)	23 24 (93 75)	1 59 (6 4)
NAV3	1 (252 1)	0 03 (7 867)	0 85 (213 5)
ADD2	1 (259 1)	0 38 (97 77)	0 77 (199 3)
MIRAB13	1 (130 7)	0 49 (64 03)	0 64 (83 27)
SGCE	1 (63 8)	0 41 (26 03)	2 51 (160 4)
SGEF	1 (9 333)	1 14 (10 63)	2 2 (20 53)

Table 3 1 2 12 3 Expression levels of differentially expressed actin/cytoskeletal related genes Normalised and raw (in brackets) expression values are given for these genes *Genes represented by more than one probe set, the average expression values are given for these genes

3 1 2 12 2 4 Transcription factor gene expression

Transcription factors from differentially expressed gene lists were analysed in order to determine if any differentially expressed transcription factors might be responsible for changes in the expression levels of other differentially regulated genes which were detected These results are discussed in section 4 4 6

	pcDNA	4E S209-HA 6	4E-HA 5
TCF3	1 (179 6)	0 51 (91)	0 36 (65 17)
TIEG	1 (235 7)	0 29 (69 37)	0 48 (112 4)
NFIB*	1 (439 65)	0 98 (430 4)	0 44 (192 27)
TCF4*	1 (42 572)	7 94 (338 02)	5 57 (263 98)
FOXA1	1 (20 97)	2 89 (60 6)	2 34 (49 03)
JARID1A	1 (51 33)	1 89 (97 07)	2 02 (103 9)
ATF5	1 (35 8)	2 59 (92 87)	2 8 (100 2)
CART1	1 (51 4)	4 23 (217 4)	5 09 (261 8)
ZIC1	1 (136 3)	7 08 (965 1)	11 5 (1,567 93)
MSX1	1 (36 33)	13 49 (490 2)	10 31 (374 5)
ESR1	1 (18 53)	2 14 (39 63)	1 67 (30 93)
CREBL2	1 (44 4)	2 15 (95 315)	1 97 (87 5)
CSDA*	1 (234 4)	1 89 (443 8)	1 34 (313 685)
BHLHB3	1 (1 733)	63 07 (109 3)	5 00 (8 667)
ETV5	1 (99)	0 44 (43 43)	1 25 (124 2)
FOXD1	(156 1)	0 44 (68 3)	0 66 (103)

Table 3 1 2.12 4 Expression levels of differentially expressed transcription factor genes Normalised and raw (in brackets) expression values are given for these genes *Genes represented by more than one probe set, the average expression value is given for these genes

3.1.2.12.2.5 FAK/integrin signalling related gene expression

Microarray data was analysed for genes known to be involved in integrin signalling. A central protein in the integrin signalling pathway known as Focal Adhesion Kinase (FAK) (Mitra *et al.*, 2005) was downregulated in 4E-HA 5 and 4E S209-HA 6 cells compared to pcDNA 2 control cells (Table 3.1.2.12.5).

Analysis of differentially expressed gene lists for genes known to interact with FAK was aided by Bibliosphere software which automatically detects co-citations of genes in PubMed literature abstracts (Fig. 3.1.2.12.6).

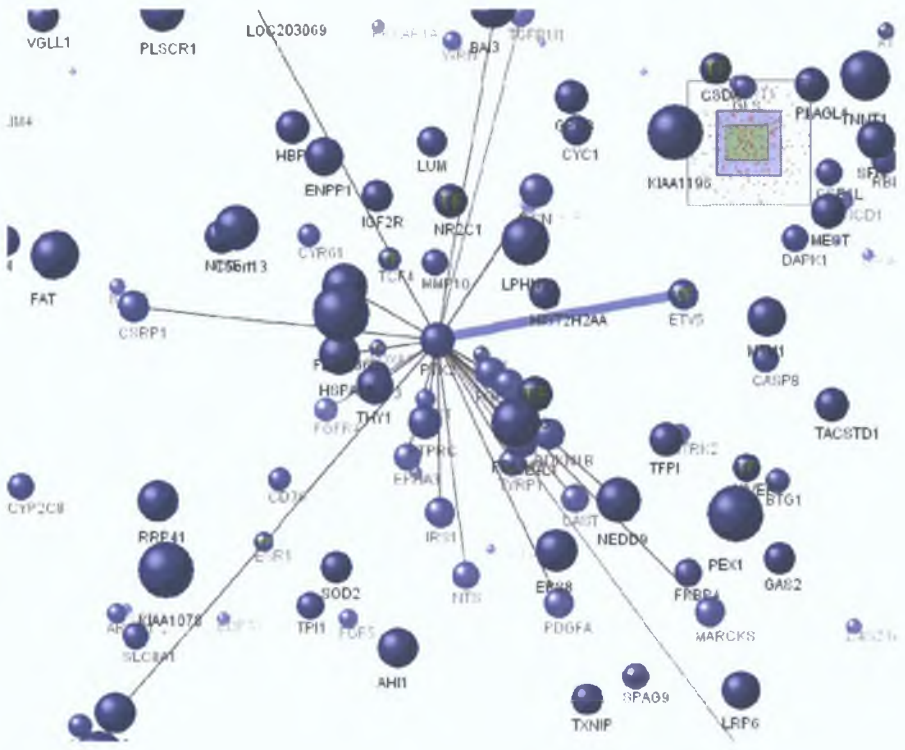


Figure 3.1.2.12.6 Bibliosphere analysis of FAK connections to differentially expressed genes.

	pcDNA 2	4E S209-HA 6	4E-HA 5
PTK2 (FAK)	1 (207 5)	0 51 (105 9)	0 378 (78 4)
TGFB1I1	1 (68 93)	0 76 (52 33)	0 447 (30 80)
EPHA3	1 (2 267)	189 15 (428 8)	181 78 (412 1)
NTS	1 (95 7)	34 66 (3316 8)	38 84 (3716 73)
CASP	1 (51 23)	1 943 (99 53)	1 45 (74 23)
NEDD9	1 (88 57)	0 32 (28 67)	1 20 (106 6)

Table 3 1 2 12 5 Expression levels of FAK related genes Normalised and raw (in brackets) expression values are given for these genes

3 1 2 12.2 6 Genes related to neuron guidance, tissue morphogenesis, cellular migration and invasion

Recent studies have shown that many proteins that have originally been identified in the nervous system as guidance cues for axons are also involved in regulating tissue morphogenesis, cellular migration and invasion (Hinck, 2004, Tamagnone and Comoglio, 2004) Microarray analysis of 4E-HA 5 and 4E S209-HA 6 cells in comparison to pcDNA 2 control cells identified a number of differentially regulated genes which fall into this category (Table 3 1 2 12 6)

	pcDNA 2	4E S209-HA 6	4E-HA 5
ROBO1	1 (181 4)	0 513 (93 17)	0 388 (70 63)
CRMP1	1 (152 9)	0 204 (31 17)	0 259 (39 63)
SLITRK3	1 (6 433)	15 42 (99 2)	20 301 (130 6)
EPHA3	1 (2 267)	189 15 (428 8)	181 78 (412 1)
SEMA3A	1 (33 17)	2 351 (77 97)	3 120 (103 5)
SEMA3C	1 (54 27)	2 04 (110 7)	1 623 (88 1)
DPYSL4 (CRMP3)	1 (61 03)	0 215 (13 1)	0 452 (27 57)

Table 3 1.2 12 6 Expression levels of genes related to neuron guidance, tissue morphogenesis, cellular migration and invasion Normalised and raw (in brackets) expression values are given for these genes

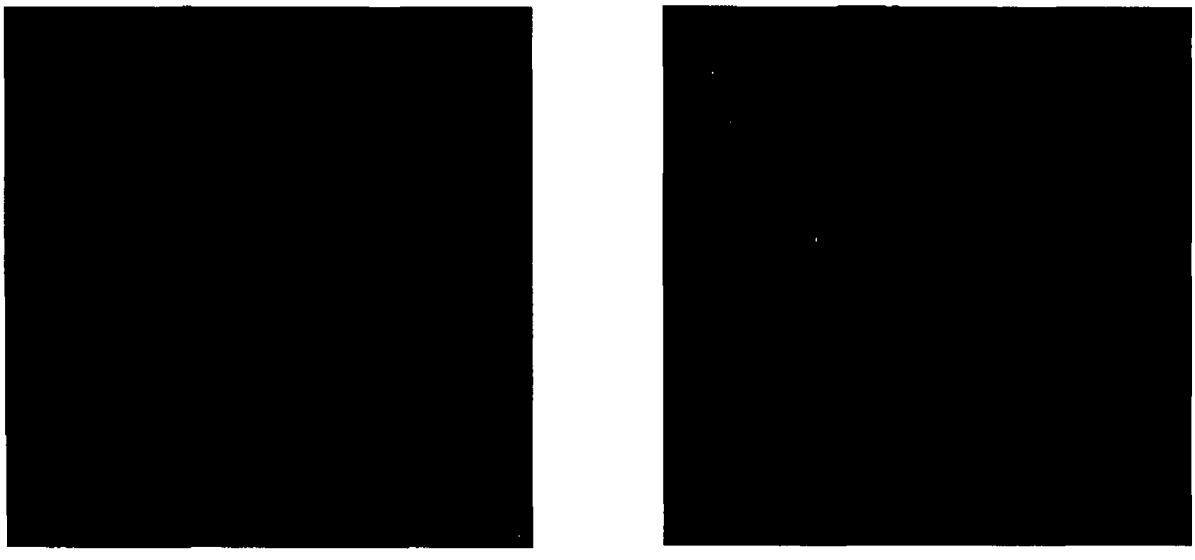
3 1.2.13 Analysis of F-Actin in 4E-HA Overxpressing Stably Transfected Clones

Results from both microarray and 2D-DIGE proteomic analysis indicate that a large number of genes and proteins that are differentially regulated in 4E-HA and 4E S209-HA cells are involved in regulating the actin cytoskeleton Organisation of actin structures in cells is involved in regulating the morphology and motility/invasiveness of cells (Lambrechts *et al*, 2004) We have analysed the F-actin (filamentous actin) structures in 4E-HA transfected cells using fluorescently labelled phalloidin (Molecular Probes) which binds and stabilises F-actin

F-actin staining in both pcDNA control and 4E S209-HA transfected cells was diffuse and little or no filamentous staining observed (Fig 3 1 2 13 1) In contrast 4E-HA 5 cells showed a more intense filamentous staining (Figs 3 1 2 13 2 and 3 1 2 13 3) Intense staining was seen in lone cells or cells at the edges of colonies The most intense staining was in these cells was observed in areas of cellular outgrowth (Fig 3 1 2 13 3) These results indicate actin cytoskeleton dynamics are altered in 4E-HA overexpressing cells

(A) pcDNA 2

(B) pcDNA 8



(C) 4E S209-HA 6

(D) 4E S209-HA 12

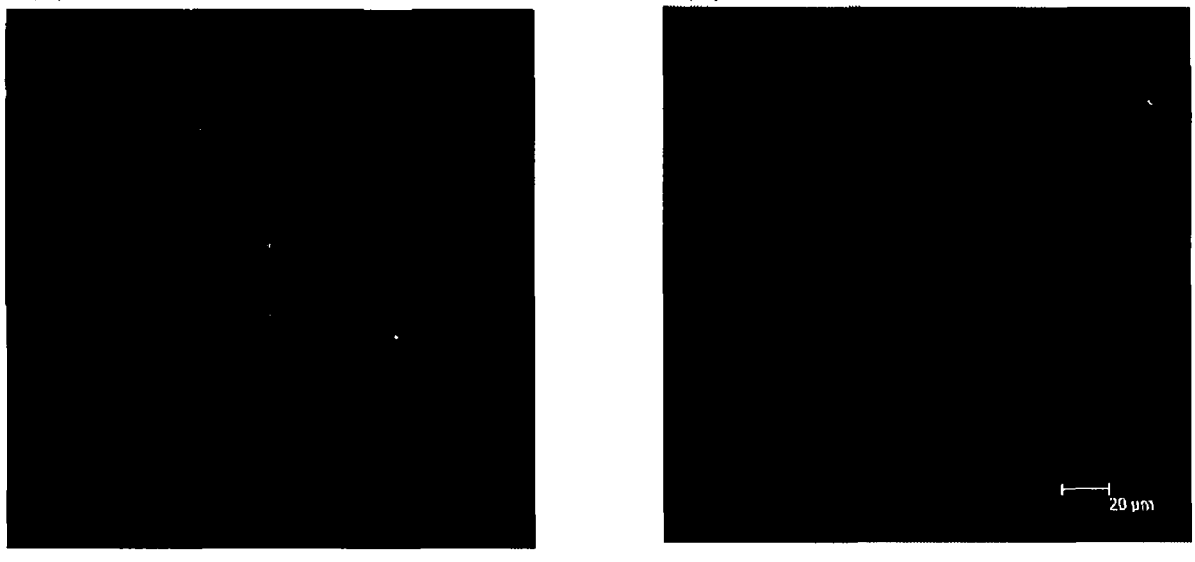
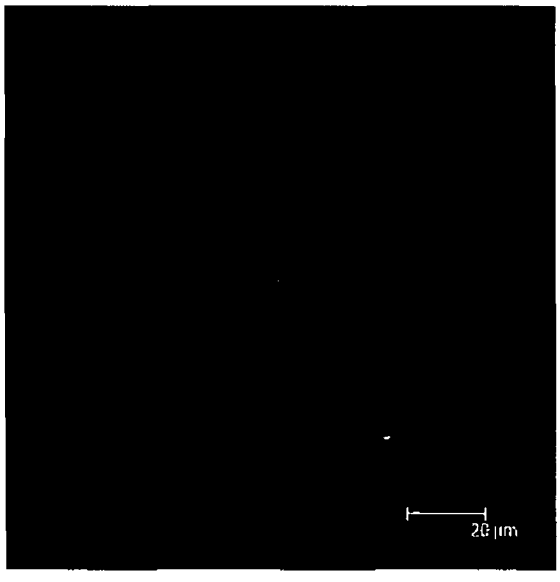
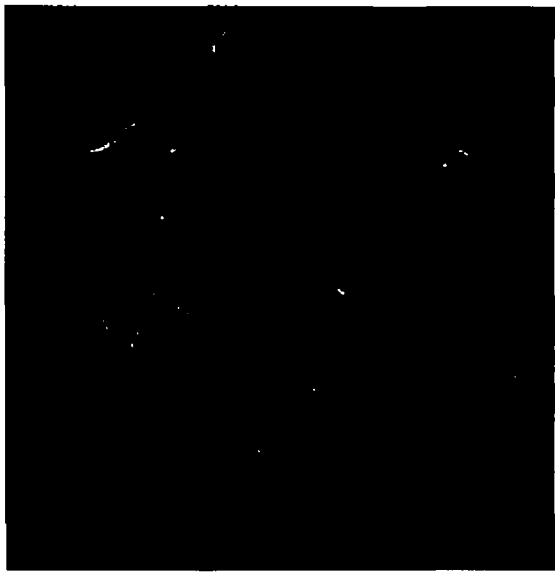


Figure 3 1 2 13.1 F-Actin fluorescent staining analysis of pcDNA control transfected and 4E S209-HA overexpressing cells

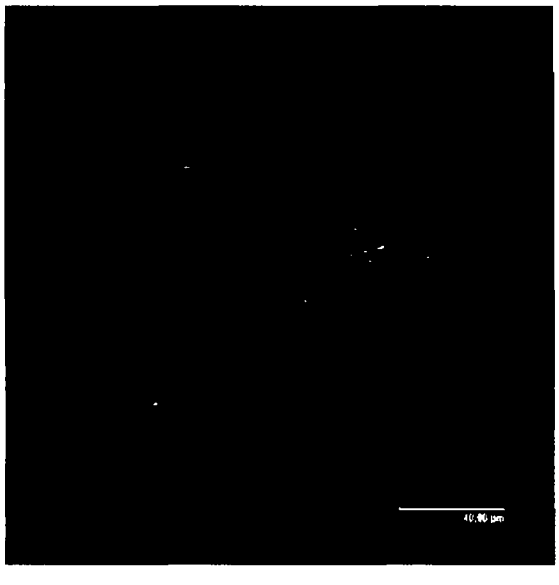
(A) 4E-HA 10



(B) 4E-HA 5



(C) 4E-HA 5



(D) 4E-HA 5

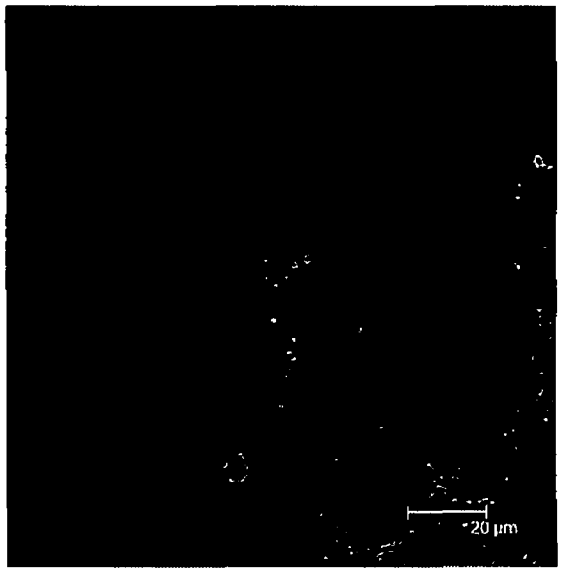


Figure 3 1 2 13 2 F-Actin fluorescent staining analysis 4E-HA 10 (A) cells display diffuse F-actin staining 4E-HA 5 (B and C) shows organisation of F-actin in filaments

(A) 4E-HA 5

(B) 4E-HA 5

Figure 3 1 2 13.3 F-Actin fluorescent staining analysis in 4E-HA 5 cells (A) Group of 4E-HA 5 cells containing stress fibers, (B) 4E-HA 5 cell at the edge of a colony displaying filopodial actin projections and a broad lammelipodial outgrowth

3.1.2.14 Focal Adhesion Kinase expression in 4E-HA overxpressing stably transfected clones

Focal Adhesion Kinase (FAK) is a major mediator of integrin dependent signalling and plays an important role in the regulation of cellular motility (Mitra *et al*, 2005) Microarray analysis showed that FAK mRNA expression was reduced in 4E-HA and 4E S209-HA cells (Table 3 1 2 12 5)

In order to determine if FAK protein levels were altered in 4E-HA transfected cells and examine the localisation of the protein, FAK expression was examined by Western blotting and also immunofluorescent labelling followed by confocal microscopy

Western blot analysis shows decreased expression of FAK in 4E-HA 5 and 4E S209-HA 6 cells in comparison to DLKP and pcDNA 2 cells (Fig 3 1 2 14 1) These results match the decreased expression level of FAK mRNA seen in these cells from microarray analysis (Table 3 1 2 12 5)

In pcDNA 2 control transfected cells, FAK protein is localised in patches (focal adhesions) that are distributed evenly throughout the cell (Fig 3 1 2 14 2) and does not appear to be concentrated in a particular area of the cell (e g peripheral regions) The 4E S209-HA 6 cells show an overall reduction in FAK expression with localisation of FAK in the periphery of cells at the edges of colonies (Fig 3 1 2 14 2 B) The 4E-HA 5 cells also display an overall decrease in FAK expression However FAK staining appears to be localised to the periphery of cells at areas of cellular outgrowth (Fig 3 1 2 14 2 C,D)

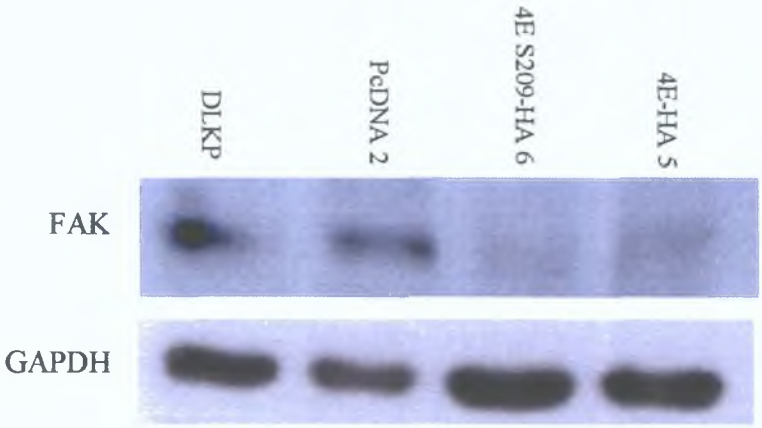


Figure 3.1.2.14.1 Western blot analysis of FAK expression in 4E-HA overexpressing cells. Expression of FAK protein is decreased in 4E S209-HA 6 and 4E-HA 5 cells compared to pcDNA 2 and DLKP cells.

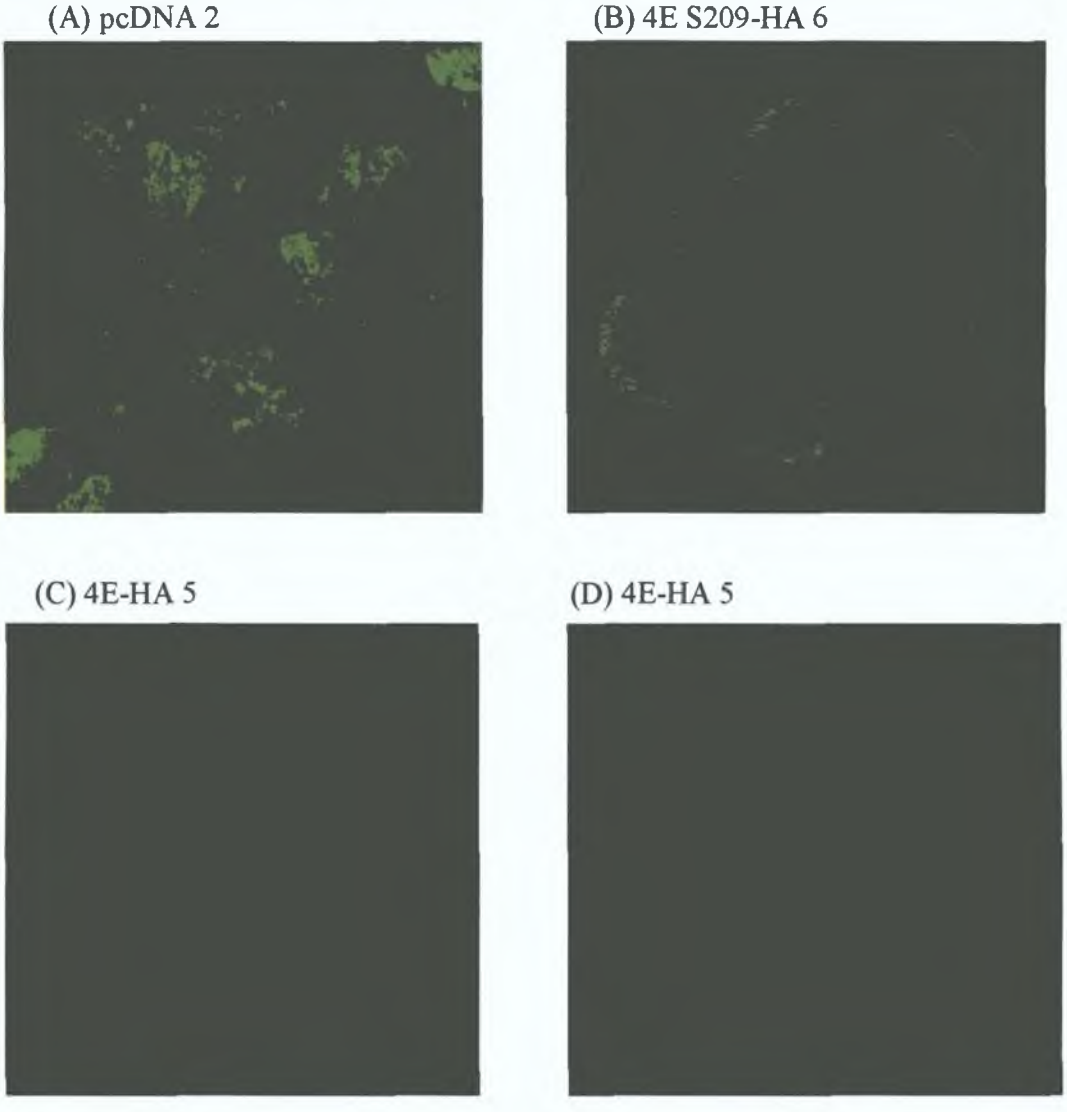


Figure 3.1.2.14.2 Immunofluorescence analysis of FAK expression. (A) In pcDNA 2 control cells FAK is highly expressed and is localised in patches that are distributed throughout the cell. **(B)** In 4E S209 overexpressing cells intense FAK staining is localised to the periphery of cells at the edge of colonies. FAK is localised to the periphery of 4E overexpressing cells **(C, D)** with high intensity in areas of cellular outgrowth.

3.1.3 Transfection of DLKP cells with eIF4E Antisense cDNA

As eIF4E has been implicated in the regulation of differentiation and growth in DLKP cells, it was decide to attempt to reduce eIF4E levels in DLKP cells by transfection with eIF4E antisense cDNA in order to analyse the effect of reduced eIF4E levels on growth and differentiation DLKP cells were stably transfected with antisense-eIF4E cDNA Clonal subpopulations of eIF4E-antisense cDNA transfected cells were generated by limiting dilution

Western blot analysis of eIF4E levels in clonal populations of DLKP cells stably transfected with eIF4E-antisense cDNA (Fig 3 1 3 1) showed no significant reduction of eIF4E levels in any of the clones analysed As no significant reduction in eIF4E levels were obtained in the clones generated these studies were discontinued

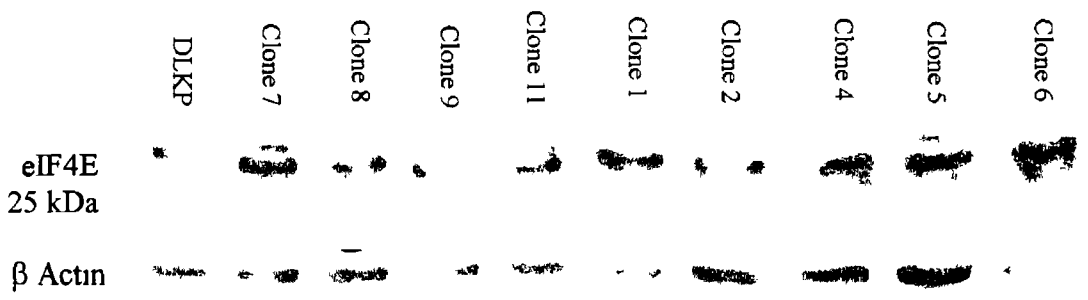


Figure 3 1 3 1 Western Blot analysis of eIF4E levels in eIF4E-antisense tranfected clones eIF4E levels do not appear to be reduced in eIF4E-antisense transfected clones

3.2 Ornithine Decaroxylase (ODC) Expression in BrdU treated DLKP cells.

Ornithine Decarboxylase is a key enzyme in the regulation of polyamine biosynthesis. Polyamine levels are known to be involved in the regulation of growth, differentiation and transformation of cells. Ornithine Decarboxylase (ODC) is known to be subject to translational regulation. Previous studies have shown that treatment of DLKP cells induces changes in the growth and differentiation of these cells in conjunction with increased expression and phosphorylation of the translation regulating protein eIF4E. It was therefore decided to examine the effect of BrdU treatment on the expression of ODC in these cells.

Immunocytochemical analysis shows increased expression of ODC in BrdU treated DLKP cells (Fig. 3.2.1)

(A) DLKP

(B) DLKP 7 Day BrdU

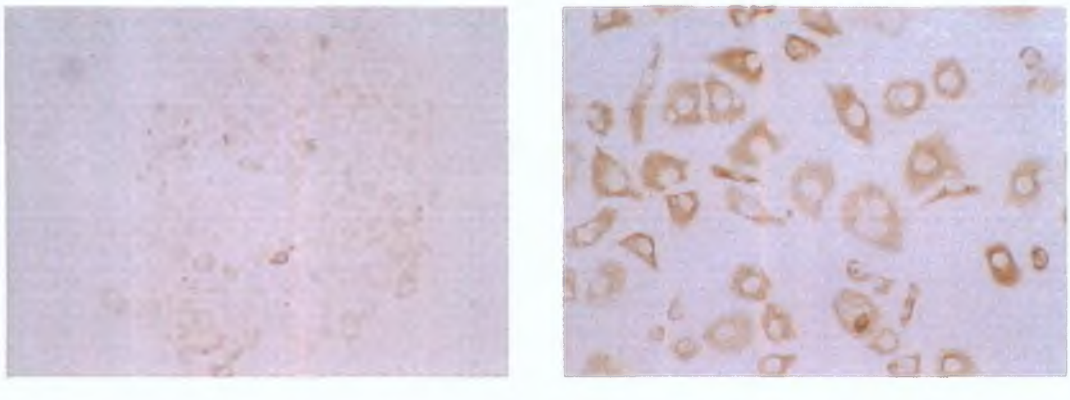


Figure 3.2.1 Immunocytochemical analysis of ODC expression in 7 day 10μM BrdU treated cells. Staining for ODC is increased BrdU treated DLKP cells (B). Magnification 20X.

3.3 C/EBP transcription factor expression in BrdU-treated DLKP cells.

The C/EBP family of transcription factors are important regulators of differentiation in a number of tissues and are involved in differentiation in lung cells (Cassel and Nord, 2003) C/EBPα and C/EBPβ protein expression is regulated in part at a translational level, with the expression of different sized isoforms of these proteins determined by the use of multiple translation initiation sites in the 5' UTRs of their mRNAs eIF4E is involved in the translational regulation of C/EBPα and C/EBPβ (Calkhoven *et al*, 2000) As post-transcriptional regulation of differentiation related gene expression has been observed in BrdU treated DLKP cells in conjunction with increased expression and phosphorylation of eIF4E, we decided to analyse the expression of C/EBPα and C/EBPβ in these cells

DLKP cells were treated with 10μM BrdU for 7 days and analysed by immunocytochemistry and Western blotting for C/EBPα and β protein expression

No changes in C/EBPα expression were detected in BrdU treated cells by immunocytochemical and Western blot analysis Immunocytochemical and Western blot analysis both show low expression of C/EBPα in both untreated and BrdU treated DLKP cells with no change in expression level (Figs 3 3 1 and 3 3 2)

Immunocytochemical analysis of C/EBPβ expresssion in BrdU treated DLKP cells shows a change in staining from predominantly cytoplasmic in untreated cells to predominantly nuclear in BrdU-treated cells indicating increased nuclear localisation of the protein (Fig 3 3 3) Western blot analysis of C/EBPβ expression in BrdU treated DLKP shows a reduction in the overall level of C/EBPβ protein present in these cells (Fig 3 3 4)

(A) DLKP Untreated

(B) DLKP BrdU Treated

Figure 3.3.1 Immunocytochemical analysis of C/EBPα expression in 7 day 10μM BrdU-treated cells. Staining for C/EBPα is weak in both untreated (A) and BrdU-treated (B) DLKP cells. Magnification 20X.

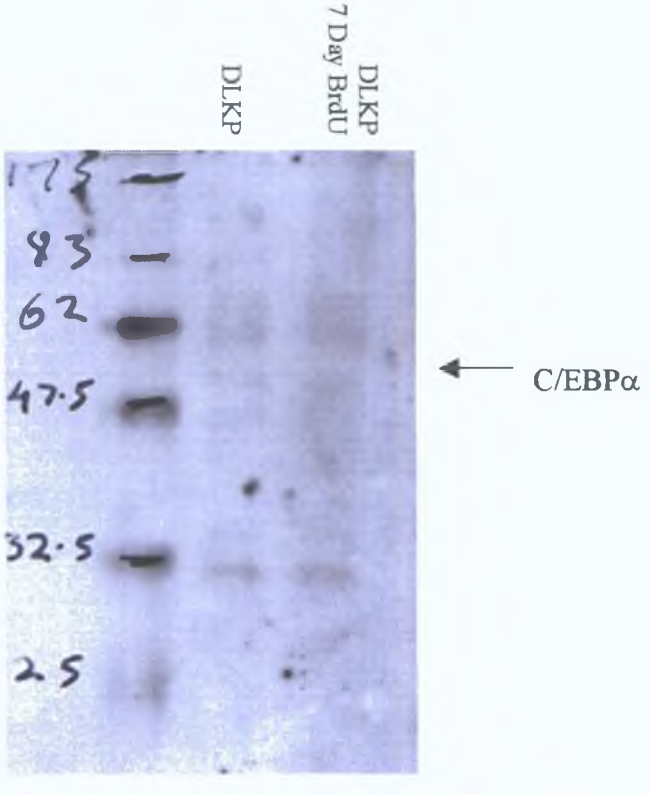


Figure 3.3.2 Western blot analysis of C/EBPα expression in 7 day 10μM BrdU treated DLKP cells. C/EBPα protein is not expressed in untreated or 7 day 10μM BrdU treated DLKP cells. Some faint non-specific bands are seen due to long exposure time.

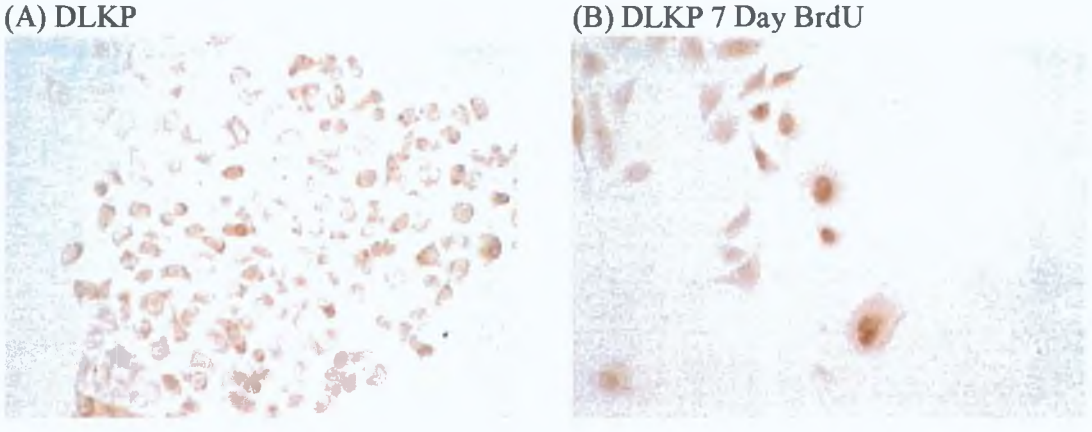


Figure 3.3.3 Immunocytochemical analysis of C/EBPβ expression in 7 day 10μM BrdU treated cells. Staining for C/EBPβ in untreated DLKP cells (A) is localised predominantly in the cytoplasm with little staining in the nucleus. BrdU treated DLKP (B) show decreased cyoplasmic staining and increased nuclear staining indicating increased nuclear localisation of the protein. Magnification 20X.

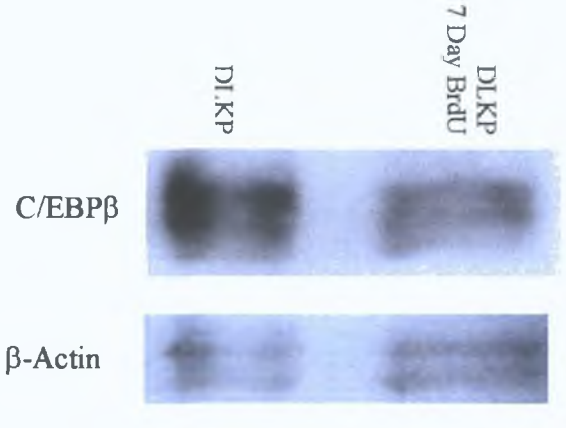


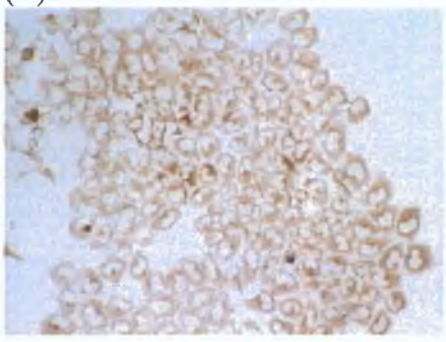
Figure 3.3.4 Western blot analysis of C/EBPβ expression in 7 day 10μM BrdU treated DLKP cells. Treatment of DLKP with BrdU causes a reduction in the overall level of C/EBPβ protein expression.

3.4 CBP/p300 expression in DLKP cells.

The expression of the transcriptional co-activators CBP and p300 were analysed in DLKP cells. These proteins are known to play a role in the expression of a number of lung differentiation related genes and are known to interact with a wide range of transcription factors.

Immunocytochemical and Western blot analysis showed strong expression of CBP and low p300 expression in DLKP (Fig. 3.4.1). Western blot analysis of CBP expression in BrdU-treated DLKP cells showed a decrease in the level of CBP protein expression in BrdU-treated cells (3.4.2).

(A) DLKP CBP



(B)DLKP p300

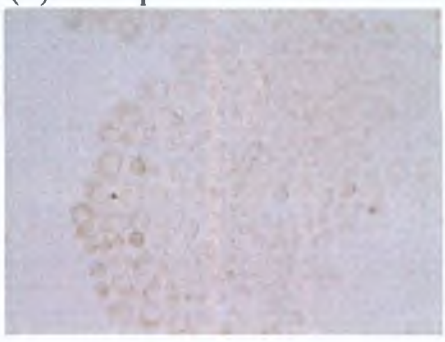


Figure 3.4.1 Immunocytochemical analysis of CBP and p300 expression in DLKP cells. DLKP cells show strong staining for CBP (A) and weak staining for p300 (B). Magnification 20X.

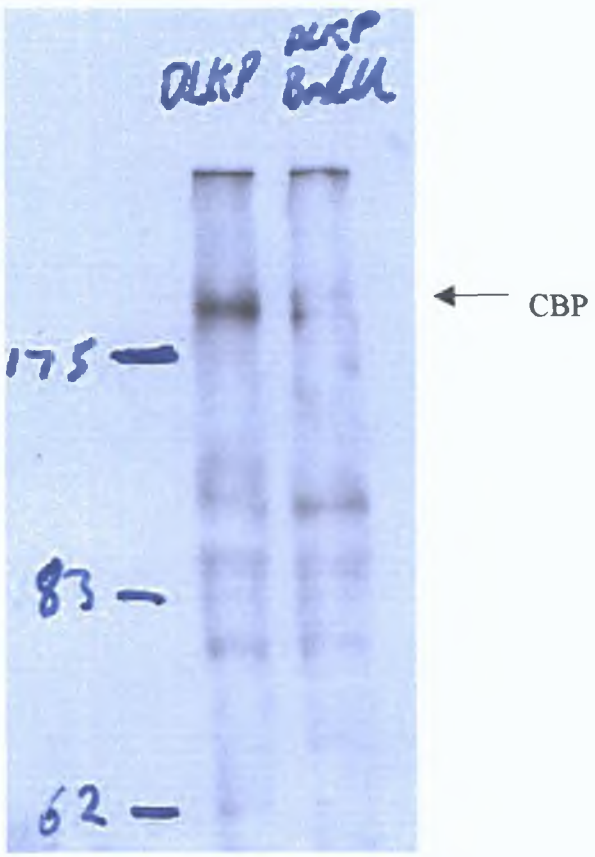


Figure 3.4.2 Western Blot analysis of CBP expression in BrdU-treated DLKP cells. BrdU-treated DLKP cells show a decrease in CBP protein expression.

3 5 Investigation into the ability of hormone-supplemented serum-free media to induce differentiation in DLKP cells.

Following on from previous studies in this laboratory (Finbar O'Sullivan PhD, 1999), we have investigated the ability of a complex hormone supplemented serum free media to induce differentiation in DLKP cells This was conducted in order to try to develop an alternative method of *in vitro* induction of differentiation to BrdU treatment using biologically active compounds The pathways by which these biologically active compounds induce differentiation (e g upregulation of eIF4E expression) could then be compared to BrdU treated cells to assess the physiological relevance of BrdU induced differentiation

DLKP cells were grown in a serum-free media (SFM) from stocks of DLKP cells which had previously been cultured in SFM in order to eliminate any interfering effects of growth factors and hormones present in serum-supplemented media (SSM) and increase the reliability and reproducibility of hormonal treatments which was a problem in previous studies

SFM consists of Ham's F12/DMEM (1 1) media, supplemented with the following compounds

Supplement	Concentration
Transferrin	5 0 µg/ml
Insulin	10 0 µg/ml
L-glutamine	2 mM
Fibronectin	5 0 µg/ml

The following components were then added to the SFM for hormonal supplemented media (HSM) treatments

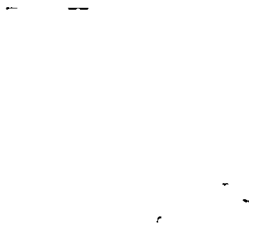
Components of HSM
Oestrogen 8 µg/ml
Hydrocortisone 3 µg/ml
Cholera Toxin 2 7 ng/ml
EGF 20 ng/ml
KGF 0 25 µg/ml

DLKP cells cultured in SFM were smaller and more circular than cells cultured in SSM Treatment with HSM caused cells to spread out and increase in size (Figs 3 5 1-3 5 7)

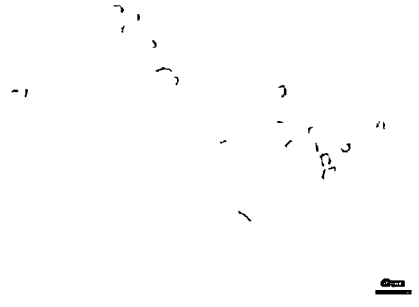
Immunocytochemical analysis of Keratin 8, 18, 19, β1 integrin and Ep-CAM protein expression was performed after 5 and 10 days of treatment

- DLKP cells grown in SFM and SFM+HSM showed strong keratin 8 staining after 5 days and weaker staining after 10 days (Fig 3 5 2)
- Keratin 18 expression was weakly positive in DLKP cells grown in SFM and SFM+HSM after 5 days and was similar to negative control levels after 10 days (Fig 3 5 3)
- Keratin 19 expressionwas not detected in DLKP cells grown in SFM and SFM+HSM after 5 and 10 days (Fig 3 5 4)
- β1 integrin staining was weakly positive in DLKP cells grown in SFM and SFM+HSM after 5 and 10 days (Fig 3 5 5)
- Ep-CAM staining was weakly positive in DLKP cells grown in SFM and SFM+HSM after 5 and 10 days (Fig 3 5 6)

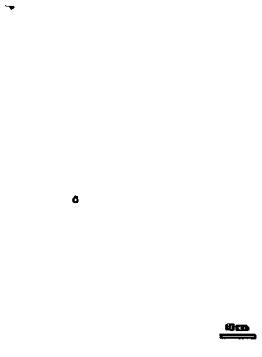
(A) SFM Day 5 Negative Control



(B) SFM+HSM Day 5 Negative Control



(C) SFM Day 10 Negative Control



(D) SFM+HSM Day 10 Control

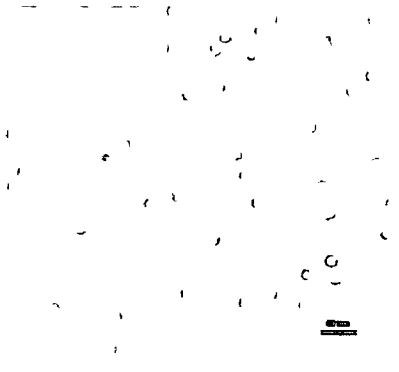
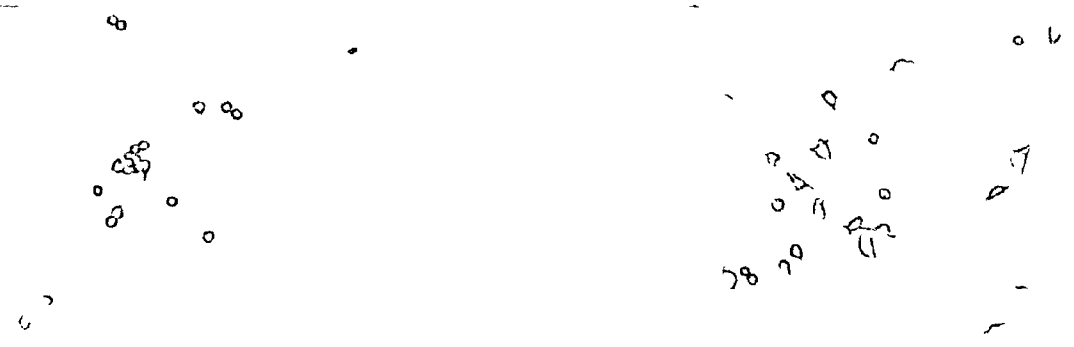


Figure 3 5 1 Negative controls for immunocytochemical analysis of DLKP cells grown in SFM and SFM+HSM Negative controls were treated as test samples without the addition of primary antibody

(A) SFM Day 5

(B) SFM+HSM Day 5



(C) SFM Day 10

(D) SFM+HSM Day 10

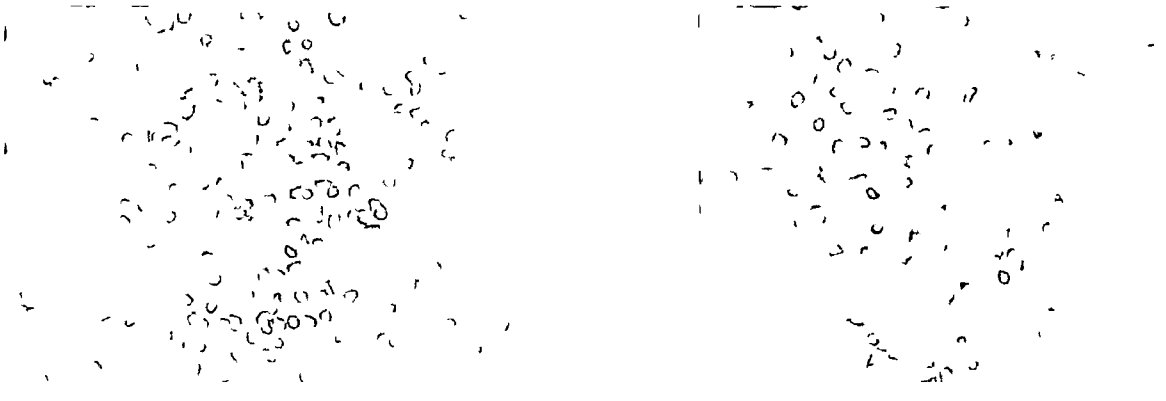
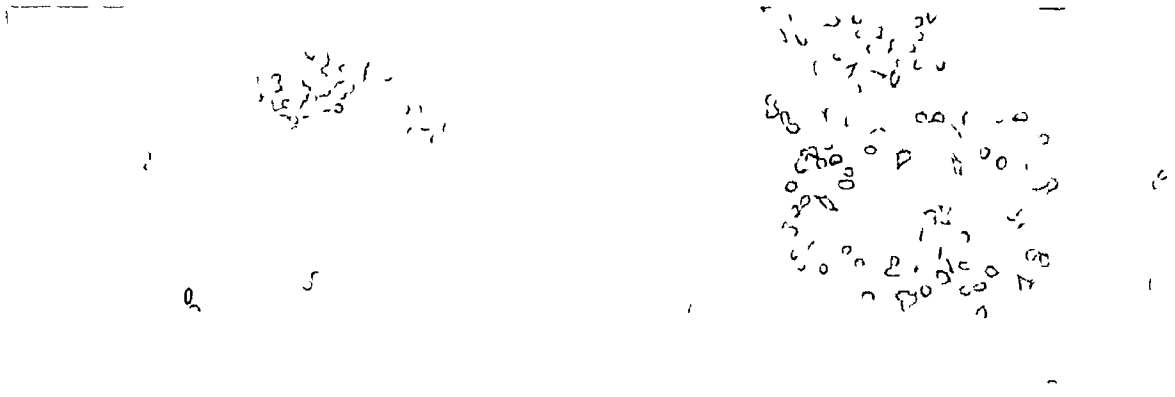


Figure 3 5 2 Immunocytochemical analysis of keratin 8 expression in DLKP cells grown in SFM and SFM supplemented with Hormones (SFM +HSM) DLKP cells grown in SFM (A) and SFM+HSM (B) show positive staining after 5 days After 10 days SFM (C) and SFM+HSM (D) cells show weak staining

(A) SFM Day 5 K18

(B) SFM+HSM Day 5 K18



(C) SFM Day 10 K18

(D) SFM+HSM Day 10 K18

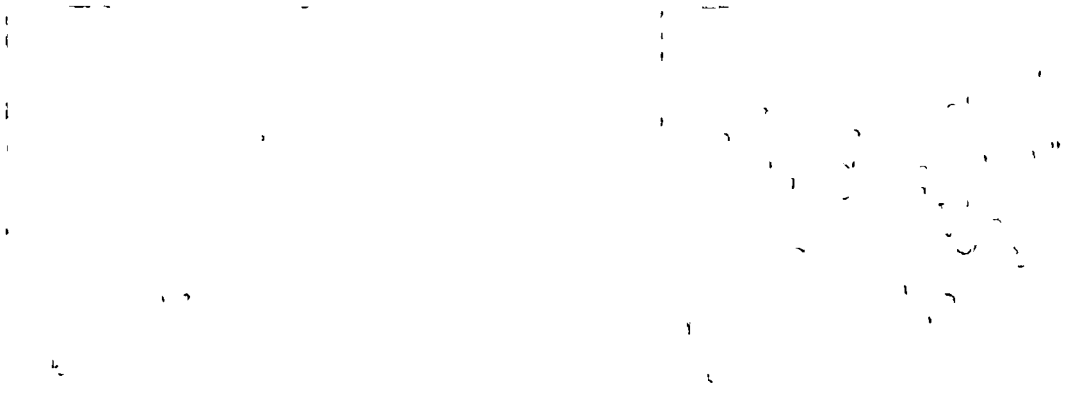


Figure 3 5 3 Immunocytochemical analysis of keratin 18 expression in DLKP cells grown in SFM and SFM supplemeted with hormones (SFM +HSM) DLKP cells grown in SFM (A) and SFM+HSM (B) show positive staining after 5 days After 10 days SFM (C) and SFM+HSM (D) cells show weak staining

(A) SFM Day 5 K19

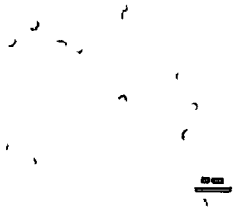
(B) SFM+HSM Day 5 K19

(C) SFM Day 10 K19

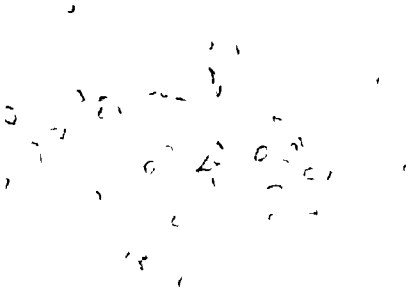
(D) SFM+HSM Day 10 K19

Figure 3 5 4 Immunocytochemical analysis of keratin 19 expression in DLKP cells grown in SFM and SFM supplemeted with Hormones (SFM +HSM) DLKP cells grown in SFM (A, C) and SFM+HSM (B, D) show no staining for keratin 19 after 5 and 10 days

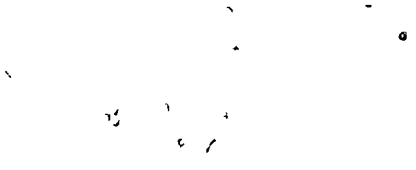
(A) SFM Day 5 β1 Integrin



(B) SFM+HSM Day 5 β1 Integrin



(C) SFM Day 10 β1 Integrin



(D) SFM+HSM Day 10 β1 Integrin

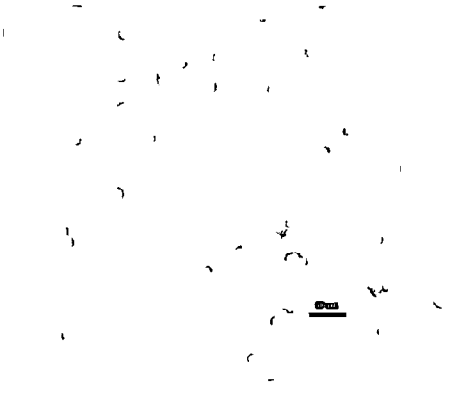
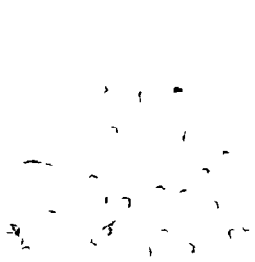
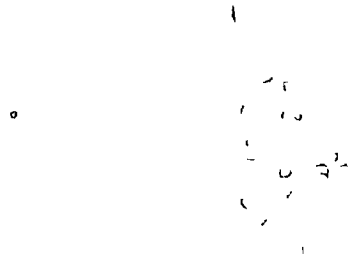


Figure 3 5 5 Immunocytochemical analysis of β1 integrin expression in DLKP cells grown in SFM and SFM supplemeted with hormones (SFM +HSM) DLKP cells grown in SFM (A, C) and SFM+HSM (B, D) show positive staining for β1 integrin after 5 and 10 days

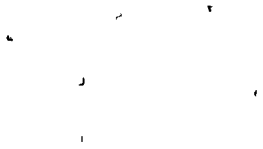
(A) SFM Day 5 Ep-CAM



(B) SFM+HSM Day 5 Ep-CAM



(C) SFM Day 10 Ep-CAM



(D) SFM+HSM Day 10 Ep-CAM

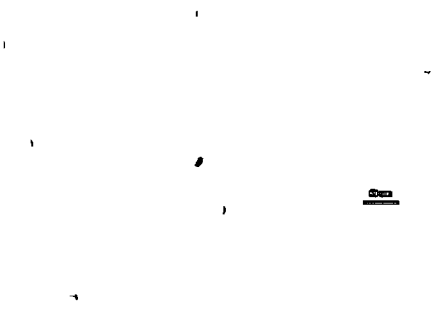


Figure 3 5 6 Immunocytochemical analysis of Ep-CAM expression in DLKP cells grown in SFM and SFM supplemeted with hormones (SFM +HSM). DLKP cells grown in SFM (A, C) and SFM+HSM (B, D) show weak staining for Ep-CAM after 5 and 10 days

4.0 Discussion

4.1 General Introduction

The lung cell line DLKP was isolated in our laboratory from a tumor histologically diagnosed as a poorly differentiated lung carcinoma (Law et al , 1992) It lacks many of the ultrastructural features associated with normal differentiated cells of the lung such as dense core granules or lamellar bodies (McBride *et al* , 1998) DLKP also fails to express many of the normal cytochemical markers associated with differentiated epithelial lung cells such as cytokeratin proteins and desmosomal protein The lack of differentiation-associated markers in DLKP and the induction of expression of some of these markers upon treatment with the differentiation modulating agent BrdU, suggests DLKP may behave to some extent like a lung stem cell line

Studies of changes in gene expression in DLKP cells following treatment with BrdU, revealed that increases in protein expression for certain growth and differentiation related genes were occurring due to post-transcriptional regulation This post-transcriptional regulation was found to occur in conjunction with increased expression and phosphorylation of the translation initiation factor eIF4E

These results suggest eIF4E may play a role in mediating changes in growth and differentiation related gene expression in BrdU treated DLKP cells

The expression of translation initiation factors is also known to be altered in a wide range of cancers and these factors are thought to cause changes in gene expression which contribute to the development and progression of cancer Recent studies have shown increased expression of eIF4E and other translation initiation factors in lung cancers suggesting a role for translational regulation of gene expression in the regulation of growth in these cells (Bauer *et al* , 2001, Bauer *et al* , 2002, Pincheira *et al* , 2001, Rosenwald *et al* , 2001, Seki *et al* , 2002)

It was therefore decided to examine the effects of overexpression of eIF4E in this cell line to determine if increased levels of this translation initiation factor could be responsible for mediating some of the changes seen in BrdU-treated DLKP cells and if increased eIF4E levels in these cells lead to changes in growth and gene expression which may be relevant to cancer progression

The role of the Ser209 phosphorylation site of eIF4E in regulating the translational control of gene expression has been a matter of debate recently, with research from different sources leading to differing views on the importance of the Ser209 phosphorylation site in regulating translation initiation (Scheper and Proud, 2002) A non-phosphorylatable eIF4E mutant lacking the Ser209 phosphorylation site was therefore also overexpressed in DLKP cells to help determine the role of phosphorylation of eIF4E in regulating changes in cellular growth and gene expression

4 2 Overexpression of eIF4E in DLKP cells

Initial experiments on overexpression of eIF4E in DLKP cells were conducted with DLKP cells stably transfected with eIF4E cDNA in a BK virus based episomal shuttle vector Immunocytochemical analysis showed an increase in staining for eIF4E indicating an increase in eIF4E protein levels in BK-4E cells (Fig 3 1 1 1) An initial immunocytochemical analysis of uncloned cell populations of transfected cells was conducted for ornithine decarboxylase and keratin 8 expression (Fig 3 1 1 2 and 3 1 1 3) Attempts were made at generating clonal populations of these cells by limiting dilution, however these attempts were unsuccessful and studies were discontinued with these cells

Further eIF4E overexpression experiments were performed using HA epitope tagged wild type (4E-HA) and S209 mutant eIF4E proteins (4E S209-HA) Western blot analysis of stable and transiently transfected cells with anti-HA antibody (Figs 3 1 2 1, 3 1 2 3, 3 1 2 2 1, 3 1 2 3 2, 3 1 2 3 3) showed expression of HA tagged eIF4E proteins at the expected molecular weight (32 5 kDa) with no expression in control pcDNA transfected cells (Fig 3 1 2 5 and 3 1 2 3 4)

Clonal populations of 4E-HA and 4E S209-HA overexpressing DLKP cells were successfully generated and clones overexpressing tranfected 4E-HA protein were selected (Figs 3 1 2 3 2-3 1 2 4) Two 4E-HA and two 4E S209-HA overexpressing clones were selected for analysis The 4E-HA overexpressing clone 5 was chosen as it expressed a high level of transfected protein Unfortunately no other 4E-HA or 4E S209-HA overexpressing clones analysed, matched the expression level of 4E-HA clone 5 The two 4E S209-HA clones chosen (6 and 12) expressed the highest levels of transfected protein of the 4E S209-HA clones analysed Clone 4E-HA 10 was chosen for further analysis as it expressed transfected 4E-HA at a similar level to the 4E S209-HA in the 4E S209-HA clones 6 and 12

Expression of 4E-HA and 4E S209-HA protein in DLKP cells results in an overall increase in eIF4E levels in these cells, allowing us to assess the role of increased eIF4E levels on the translational regulation of gene expression in these cells, as well as the role of the S209 phosphorylation site on translational regulation of gene expression

4 2 1 Morphology of eIF4E overexpressing cells

Analysis of the morphological features of 4E-HA and 4E S209-HA overexpressing cells was conducted in order to determine if any morphological changes were induced by overexpression of these proteins DLKP cells transfected with empty pcDNA vector possess an irregular morphology (Fig 3 1 2 5 1) The 4E S209-HA overexpressing clones possess a more smooth-edged rounded morphology with few cellular projections compared to pcDNA controls or 4E-HA overexpressing clones (Figs 3 1 2 5 1-3 1 2 5 3) The 4E-HA 10 cells possess an irregular morphology similar to pcDNA controls whereas 4E-HA 5 cells which express high levels of 4E-HA protein show large cells containing multiple cellular protrusions (Fig 3 1 2 5 3) It would appear therefore that overexpression of 4E S209-HA may alter the morphological features of DLKP cells restricting the formation of cellular protrusions This indicates that eIF4E phosphorylation may function in regulating the expression of genes involved in determining cellular morphology The presence of large cells containing numerous cellular protrusions in 4E-HA 5 cells (Fig 3 1 2 5 3) indicates that overexpression of high levels of wild type eIF4E may also increase expression of genes associated with these morphological features

4 2 2 Growth rate of eIF4E overexpressing cells

Analysis of the growth rate of the 4E-HA and 4E S209-HA overexpressing stably transfected DLKP clones 4E-HA 5 and 4E S209-HA 6 showed the growth rates of these cells were increased in comparison to pcDNA 2 control transfected cells (Fig 3 1 2 9 1) The growth curve shows a drop in the growth rate of 4E-HA 5 cells at day 6 This coincided with these cells achieving confluency, which suggests that the decreased growth rate at this time may be associated contact inhibition of growth The 4E S209-HA 6 cells continued to proliferate at day 6 These cells had not achieved confluency at this time-point due to the smaller size of these cells Overexpression of eIF4E has been shown to induce transformation and increase the growth rates of various cell types (DeBenedetti and Graff, 2004, Mamane *et al*, 2004) High levels of eIF4E expression are also found in many types of cancer indicating a role in tumor progression (DeBenedetti and Graff, 2004, Mamane *et al*, 2004) The overexpression of eIF4E in DLKP cells in this study caused increased cellular proliferation This would suggest that eIF4E may play a role in regulating proliferation in these cells and may also play a role in the regulation of growth in lung cancers in general The increased proliferation seen in 4E S209-HA cells indicates that eIF4E phosphorylation is not necessary for the increased proliferation mediated by increased eIF4E expression in these cells

4.2.3 Keratin expression in eIF4E overexpressing cells

4 2 3 1 Keratin 8 expression in eIF4E overexpressing cells

Keratin 8 expression was increased in BK-4E cells (Fig 3 1 1 3), 4E-HA transiently tranfected DLKP cells (Fig 3 1 2 8), 4E-HA stably transfected uncloned DLKP cells (Fig 3 1 2 2 2) and the stably transfected 4E-HA overexpressing clone 4E-HA 5 (Fig 3 1 2 6 1) Keratin 8 expression was not detected in 4E-HA clone 10 (Fig 3 1 2 6 1) The level of transfected 4E-HA protein in 4E-HA 10 cells was lower than in 4E-HA 5 cells indicating that higher 4E levels may be necessary to induce keratin 8 expression in these cells

Keratin 8 expression was not increased in transiently transfected 4E S209-HA cells (Fig 3 1 2 8) or stably transfected 4E S209-HA overexpressing clones (Fig 3 1 2 6 1) The level of transfected 4E S209-HA protein in these cells was at a similar level to 4E-HA protein in 4E-HA clone 10 which also did not express keratin 8 It therefore could not be determined if the lack of keratin 8 protein expression in these cells was due to the S209 phosphorylation site mutation or the lower level of transfected protein compared to 4E-HA clone 5

RT-PCR analysis of keratin 8 mRNA levels in stably transfected 4E-HA overexpressing clones showed no difference in keratin 8 mRNA levels between pcDNA control cells and 4E S209-HA and 4E-HA overexpressing cells examined (Fig 3 1 2 6 2) indicating that differences in keratin 8 protein expression were as a result of post-transcriptional/translational regulation Previous studies in our laboratory have shown treatment of DLKP cells with the differentiation modulating agent BrdU resulted in post-transcriptional/translational upregulation of keratin 8 expression in conjunction with increased eIF4E staining and phosphorylation Immunocytochemical analysis of keratin 8 expression in these cells showed intense filamentous staining in a proportion of BrdU treated DLKP cells after 7 days of treatment (McBride *et al*, 1999) In contrast, 4E-HA 5 cells show diffuse non-filamentous keratin 8 staining (Fig 3 1 2 6 1) It is possible that although 4E-HA overexpression may increase the levels of keratin 8 protein in these cells, further posttranslational processing may be necessary for keratin 8 to form the filamentous structures seen in BrdU-treated cells

Keratin 8 is considered to be a marker of simple epithelial cells and expression of keratin 8 expression in DLKP cells may be interpreted as an indication of differentiation from a poorly differentiated cell phenotype to an epithelial phenotype The DLKP cell line is considered to have stem cell-like properties due to its lack of differentiation markers and its ability to express such markers upon treatment with the differentiation modulator BrdU Stem cells in the adult lung are considered to act as cellular reservoirs that can be induced to proliferate, migrate and differentiate in order to replace damaged tissue after lung injury Upregulation of translation may play a role in this process by increasing expression of differentiation related proteins such as keratin 8 whilst also promoting the proliferation and migration of cells in order to replace damaged tissues

4 2 3 2 Keratin 18 and 19 expression in eIF4E overexpressing cells

Immunocytochemical analysis of keratins 18 and 19 expression in stably transfected 4E-HA and 4E S209-HA overexpressing DLKP clones revealed no staining for these proteins (Figs 3 1 2 6 3, 3 1 2 6 4) This indicates that these proteins are not subject to translational regulation through eIF4E protein expression levels Previous studies have shown these proteins are post-transcriptionally upregulated in BrdU treated DLKP cells (McBride *et al*, 1999, Meleady and Clynes, 2001) Increased expression of eIF4E may not be responsible for the increased expression of K18 and K19 upon BrdU treatment as no increase in their expression was detected in 4E overexpressing cells here

4.2.4 Integrin expression in eIF4E overexpressing DLKP cells

4 2 4 1 β1 integrin expression in eIF4E overexpressing DLKP cells

Treatment of DLKP cells with BrdU has previously been shown to cause a post-transcriptional increase in β1 integrin expression (Meleady and Clynes, 2000) It was therefore decided to analyse β1 integrin expression in 4E overexpressing cells to determine if increased 4E expression can cause this increase in β1 integrin levels Immunocytochemical analysis shows upregulation of β1 integrin expression in stably transfected uncloned 4E-HA overexpressing DLKP cells and the stably transfected 4E-HA overexpressing clone 4E-HA 5 although not in 4E-HA clone 10 (Fig 3 1 2 2 3, 3 1 2 7 1)

The level of transfected 4E-HA protein in 4E-HA 10 cells was lower than in 4E-HA 5 cells indicating that higher 4E levels may be necessary to induce β1 integrin expression in these cells

β1 integrin expression was not increased in transiently or stably transfected 4E S209-HA overexpressing clones (Figs 3 1 2 9, 3 1 2 7 1) The level of transfected 4E S209-HA protein in the stably transfected 4E S209-HA overexpressing clones was at a similar level to 4E-HA protein in 4E-HA clone 10 which also did not express β1 integrin It therefore could not be determined if the lack of β1 integrin expression in 4E S209-HA overexpressing clones was due to an effect on the S209 phosphorylation site mutation or the lower level of transfected protein compared to 4E-HA clone 5

Immunocytochemical analysis of β1 integrin expression in transiently transfected 4E-HA overexpressing DLKP cells shows no increase in β1 integrin expression (Fig 3 1 2 9) β1 integrin expression may not be increased by 4E-HA overexpression in transient tranfections due to the fact that these cells are almost confluent when they are transfected which may inhibit the expression of β1 integrin on the cell membrane

β1 integrin plays an important role in both normal lung development and also in lung cancer progression β1 integrin is expressed in lung epithelial cells during lung development and in normal adult epithelium (Coraux *et al*, 1998, Sheppard 2003)

Upregulation of β1 integrin subunits is also seen in epithelial repair following injury in lung (Sheppard 2003) Studies of α3 integrin deficient mice show α3β1 integrin plays a crucial role in lung organogenesis α3β1 integrin deficient mice die during the neonatal period and display abnormal branching morphogenesis of the lungs (Kreidberg *et al*, 1996) α3β1 integrin was also shown to be important for primary cultures of rat alveolar epithelial cells to adhere and form a confluent monolayer of cells (Lubman *et al*, 2000) The migration and tubular morphogenesis of human fetal tracheal epithelial cells in culture was prevented by incubation with anti-β1 integrin antibodies indicating the importance of β1 integrin in these processes (Coraux *et al*, 2000)

Various studies indicate β1 integrin also plays a role in lung cancer Binding of small cell lung cancer cells to ECM components via β1 integrin provides protection from chemotherapy induced apoptosis (Buttery *et al*, 2004) β1 integrin expression correlated with lymph node metastasis in non small cell lung cancer (NSCLC) (Han *et al*, 2003) Increased expression of α3β1 integrin was found to be responsible for brain metastasis in a NSCLC cell line (Yoshimasu, 2004) A recent study by Moro *et al* (2004) has also shown translational regulation of $\beta 1_A$ integrin expression in Prostate Carcinoma cells

Thus, the literature suggests that β1 integrin expression plays an important role in both normal lung epithelium and lung cancer cells and our results show that translational control of β1 integrin expression may be important in regulating the properties of both cell types

4 2 4 2 α Integrin subunit expression in eIF4E overexpressing DLKP cells

Immunocytochemical analysis of major pulmonary α integrin subunit-β1 integrin binding partners was conducted to determine if eIF4E overexpression induced increased expression of these proteins Immunocytochemical analysis of α1, α2 and α5 integrin showed no expression of these proteins in pcDNA controls, 4E-HA or 4E S209-HA overexpressing cells Previous studies had shown increased expression of α2 integrin in BrdU-treated DLKP cells (Meleady and Clynes, 2000) but this was due to transcriptional upregulation (Meleady and Clynes, 2000) Overexpression of eIF4E does not therefore appear to cause increased expression of this protein and transcriptional upregulation in BrdU treated cells is unlikely to be related to increased eIF4E expression

Immunocytochemical analysis of α3 integrin expression showed increased expression of α3 integrin in 4E-HA clones 5 and 10 (Fig 3 1 2 7 4) α3 integrin expression was undetected in 4E S209-HA overexpressing DLKP clones and pcDNA controls (Fig 3 1 2 7 4)

As mentioned with β1 integrin, α3β1 integrin plays a crucial role in lung organogenesis (Kreidberg *et al*, 1996) Other studies also confirm the importance of α3β1 integrin in regulating cellular adhesion and motility in development and wound repair in different cell types (Nguyen *et al*, 2001, Choma *et al*, 2004, Schmid, 2004) The association of α3β1 integrin with cellular motility correlates with its increased expression in 4E-HA 5 cells The 4E-HA 5 cells were found to be highly invasive which involves increased cellular motility

4 2 5 Ornithine Decarboxylase (ODC) expression in 4E overexpressing cells

Polyamines are ubiquitous cellular components that are involved in normal and neoplastic growth Polyamine biosynthesis is tightly regulated in mammalian cells by the activities of ornithine-decarboxylase (ODC) and S-adenosyl methionone decarboxylase ODC is a rate limiting enzyme for polyamine biosynthesis and is recognised as a proto-oncogene Overexpression of ODC causes transformation of NIH3T3 cells ODC mRNA contains a lengthy GC rich 5'UTR, rendering it poorly translated Overexpression of eIF4E in NIH 3T3 cells has been shown to increase ODC protein levels and depletion of eIF4E using anti-sense eIF4E suppressed ODC mRNA translation in eIF4E overexpressing cells (Mamame *et al*, 2004)

Overexpression of both 4E-HA and 4E S209-HA in DLKP cells caused increased expression of ODC as determined by immunocytochemical analysis (Fig 3 1 2 8) The relative levels of ODC in these cells is not known as attempts at Western blot analysis proved unsuccessful Western blot analysis showed large numbers of bands below the expected molecular weight This may have been due to protein degradation as ODC is known to have a short half-life and is subject to degradation by the 26S proteasome

As ODC is upregulated in both 4E-HA and 4E S209-HA overexpressing cells the phosphorylation of eIF4E on Ser209 may not be necessary for translational regulation of ODC protein expression by eIF4E

ODC expression is involved in regulating normal and neoplastic cellular growth (Graffe *et al*, 1997, Shantz and Pegg, 1999, Wallace and Fraser 2004) Translational regulation of ODC expression may play a significant role in lung cancer progression, and it would be of interest to analyse ODC expression in lung tumor samples to determine if ODC expression is increased and if so is it due to translational regulation

4 2 6 Analysis of in-vitro invasion of eIF4E overexpressing cells

The expression of the translation factor eIF4E has been shown to be associated with tumorigenesis, tumor invasiveness and metastasis (De Benedetti and Graff, 2004) For this reason, it was decided to analyse the effect of eIF4E overexpression on the ability of DLKP cells to invade through a reconstituted basement membrane (*in vitro* invasion assay)

In all, six cell lines were tested, two pcDNA control transfected clones (pcDNA 2, 8), two 4E S209-HA clones (4E S209-HA 6, 12) and two 4E-HA clones (4E-HA 5, 10) The 4E-HA clone 5 proved to be highly invasive in comparison to all other cell lines tested (Section 3 1 2 10) The 4E-HA 5 cells also express higher levels of transfected 4E-HA than the other cell lines and therefore this result correlates with previous studies in which expression of eIF4E is associated tumor invasiveness and metastasis (DeBenedetti and Graff 2004, Rosenwald, 2004) The 4E-HA clone 10 actually showed slightly lower invasiveness than both the pcDNA control cell lines and 4E S209-HA cell lines Differences in invasiveness were seen between pcDNA control clones tested and also between 4E S209-HA clones which express similar amounts of transfected 4E S209-HA protein The low level of invasiveness seen in 4E-HA clone 10 may be explained by the fact that this clone expresses a lower level of 4E-HA transfected protein than 4E-HA clone 5 which may not be sufficient to induce increased invasiveness in what may have been a clone which displayed low invasive potential to begin with 4E S209-HA overexpressing clones and pcDNA control clones expressed similar levels of invasiveness Some variation was seen between the pcDNA clones and 4E S209-HA clones though this may be attributable to the inherent clonal variation in DLKP cells

A study by Seki *et al* (2002) has previously shown that increased eIF4E expression levels correlate with more invasive subtypes of lung adenocarcinomas (Seki *et al*, 2002)

4.3 Proteomic analysis eIF4E overexpressing DLKP cells

In order to identify changes in protein expression as a result of overexpression of 4E-HA and 4E S209-HA in DLKP cells, it was decided analyse protein expression levels in these cells using two-dimensional difference gel electrophoresis (2-D DIGE)
Two dimensional electrophoresis separates proteins according to two independent properties in two discrete steps the first dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric point (pI), the second dimension step, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights (M_r, relative molecular weight) Thousands of different proteins can thus be separated and information such as the protein pI, the apparent molecular weight, and the amount of each protein is obtained

In this study, we have used the recently developed ETTAN DIGE (Amersham) 2-D electrophoresis system This method involves labelling different protein samples with three different charge- and size-matched fluorescent cyanine dyes which possess distinct excitation and emission spectra (Cy™2, Cy3 and Cy5) Different protein samples labelled with different fluorescent dyes can be run on the same gel, the properties of the fluorescent dyes ensure that resulting gel images will perfectly overlay The ability to compare different samples on the same gel avoids complications of gel-to-gel variation and enables more accurate and rapid analysis of differences between protein samples and reduces the number of gels that need to be run

Proteomic analysis was carried out on two 4E-HA transfected DLKP clones (4E-HA 5, 4E-HA 10), two 4E S209-HA clones (4E S209-HA 6, 4E S209-HA 12) and two pcDNA control plasmid transfected clones (pcDNA 2, pcDNA 8)
The main analysis of our results was performed by pooling the results from the two clones of each group together into three groups i e 4E-HA, 4E S209-HA and pcDNA The expression values of protein spots in each group were then compared against each other e g pcDNA vs 4E-HA, pcDNA vs 4E S209-HA, 4E-HA vs 4E S209-HA

We have also analysed differences in protein expression between the 4E-HA overexpressing clones 5 and 10 The 4E-HA clone 5 expresses high levels of transfected 4E-HA protein and is highly invasive whereas 4E-HA clone 10 expresses a lower level of transfected 4E-HA protein and is only mildly invasive Analysis of differences in protein expression between these two clones may further identify translationally regulated proteins and also proteins involved in regulating invasiveness

4.3.1 Analysis of differentially regulated proteins in a comparison between 4E-HA and 4E S209-HA overexpressing DLKP cells.

Analysis of differentially expressed proteins between 4E-HA overexpressing cells compared to 4E S209-HA overexpressing cells revealed changes in a number of different classes of protein (Table 4 3 1)

4E-HA vs 4E S209-HA			
4E-HA		**4E S209-HA**	
Increased expression	Decreased Expression	Increased Expression	Decreased Expression
Protein Chaperones	**Metabolism**	**Protein Degradation**	**Cytoskeletal**
Heat Shock Protein APG,	Aldehyde Dehydrogenase	PA28 alpha, PA28 beta, UCHL1, AHA1?	Cofilin, Moesin, EPB41L2, EBP-50, Tropomyosin 3, Tubulin Secific Chaperone A, Mutant beta actin, Actin-like 6A isoform 1
mRNA Processing		**Metabolism**	**Metabolism**
G3BP		DDAH1, Glyoxalase 1	Purine Nucleoside Phosphorylase
			mRNA Processing
			HNRPF, HNRPK

Table 4 3 1 Proteins differentially expressed between 4E-HA and 4E S209-HA overexpressing cells

4 3 1 1 Cytoskeletal Proteins

4 3 1 1 1 Cofilin

Expression of the actin regulatory protein cofilin was 5 38-fold lower in 4E S209-HA overexpressing cells compared to 4E-HA overexpressing cells

Cofilin is a small (19 kDa) ubiquitous protein that binds to both G- and F- actin, it has a higher affinity for ADP-bound subunits and enhances the rate of monomer dissociation from the pointed end of actin filaments In addition, cofilin can also sever actin filaments and thus directly generate free actin barbed ends The depolymerisation and severing activities of cofilin are thought to be due to its ability to bind cooperatively to F-actin and cause a twist in the actin filament, promoting the destabilisation of actin-actin interactions and thus fragmentation of the filament (DesMarais, 2005)
Cofilin has emerged as one of the protein families playing an essential role in actin dynamics at the plasma membrane during cell protrusion Although the activation of cofilin is required for cell motility, it was not clear until recently how the relative contributions of cofilin-mediated barbed end formation and subsequent polymerisation and cofilin-mediated actin depolymerisation are balanced during protrusion and cell motility Some considered that the cofilin-mediated depolymerisation of actin provides the actin monomers necessary for ongoing filament assembly whereas others maintained that the severing of actin filaments by cofilin generates the free barbed ends for actin polymerisation essential for motility (Des Marais, 2005)

It is now thought that synergy between cofilin and the Arp2/3 complex contributes to barbed end generation and cellular protrusion The Arp2/3 complex consists of seven polypeptides and is found at actin filament Y-branches in the submembrane array Owing to its *in vitro* ability to generate new filament branches in a pre-existing actin filament network, it is believed to be a major contributor to barbed-end generation and cellular protrusion (DesMarais, 2005)
The Arp2/3 complex and cofilin are present together in the dendritic arrays of actin filaments at the leading edge of motile cells (Des Marais *et al*, 2005) Both the

Arp2/3 complex and cofilin contribute to barbed-end generation at the leading edge, since function blocking antibodies directed against either protein significantly decrease barbed-end generation and cell protrusion (Des Marais *et al*, 2005) The severing activity of cofilin can increase the nucleation activity of the Arp 2/3 complex This occurs because cofilin creates free barbed ends that nucleate the growth of new actin filaments which are preferred sites for the ATP binding of the Arp2/3 complex, compared with the older, ADP containing filaments The availability of new actin filaments increases the nucleation activity of the Arp 2/3 complex and biases its branching activity towards the barbed end of the mother filament In this manner, cofilin plays a major role in regulating the formation of actin based cellular protrusions and also plays a related role in controlling the direction of cellular motility By utilizing a chemically engineered, light sensitive phosphocofilin mimic, Ghosh *et al* (2004) demonstrated that activated cofilin polymerises actin, generates cellular protrusions and can determine the direction of cellular migration Overexpression of cofilin was also found to enhance the motility of glioblastoma tumour cells in a concentration dependent fashion (Yap *et al*, 2005)

4 3 1 1 2 Moesin

Expression of Moesin was 1 71-fold lower in 4E S209-HA overexpressing cells compared to 4E-HA overexpressing cells

Moesin is a member of a family of proteins collectively known as ERM (ezrin-radixin-moesin) proteins which are involved in linking the cytoskeleton to the plasma membrane

The ERM proteins are structured into three functional domains an N-terminal FERM (four point one, ERM) domain, an extended coiled-coil region and a short C-terminal domain ERM proteins are negatively regulated by an intramolecular interaction between the amino- and carboxy- terminal domains that masks at least some sites of protein interaction Activation therefore requires separation of the two domains (Bretscher *et al*, 2002) ERM proteins contain an F-actin binding site within their carboxy terminal 30 residues which is masked in the isolated dormant monomer The

amino terminal FERM domain interacts with membrane proteins (Bretscher *et al*, 2002)

ERM proteins provide a regulated linkage from filamentous (F)-actin in the cortex to membrane proteins on the surface of cells Regulated attachment of membrane proteins to F-actin is essential for many fundamental processes, including the determination of cell shape and surface structures, cell adhesion, motility, cytokinesis, phagocytosis and integration of membrane transport with signaling pathways (Bretscher *et al*, 2002)
ERM proteins associate with specific membrane associated proteins, either directly or through adapter molecules such as EBP50 (ERM-phosphoprotein of 50 kDa)

4 3 1.1 3 EPB41L2 (4 1G)

Expression of EPB41L2 was 1 98-fold lower in 4E S209-HA overexpressing cells compared to 4E-HA overexpressing cells

EPB41L2 (erythrocyte membrane protein band 4 1-like 2) is a member of the band 4 1 superfamily of proteins which also contains the ERM family of membrane-cytoskeleton linker proteins The protein 4 1 superfamily encompasses a group of structural proteins that play important roles in membrane biophysical processes through their interactions with actin, members of the spectrin family and the cytoplasmic domain of integral membrane proteins The prototypical member of this family is the major 80-kDa protein 4 1R isoform found in red blood cells, where it is a key component of the erythroid membrane skeleton that underlies and mechanically supports the plasma membrane The 4 1R gene (EPB41) is noteworthy for elaborate alternative pre-mRNA splicing pathways by which it encodes tissue specific protein isoforms Mutations in this gene result in membrane mechanical defects and morphological abnormalities characteristic of the red cell disorder hereditary elliptocytosis EBP41L2 is a widely expressed homologue of EBP41 This gene encodes a protein that is highly homologous to the prototypical 4 1R in three key structural domains the membrane binding domain, the spectrin-actin binding domain, and the conserved C-terminal domain

Members of the Protein 4 1 superfamily are characterized by the presence of a conserved FERM (Four 1 protein, Ezrin, Radixin, Moesin) domain at the N-terminus of the molecule and in many cases, a spectrin/actin binding domain (SABD) The main function of these proteins is linking cell surface glycoproteins to the actin cytoskeleton thereby providing structural stabilization of the cell membrane (Sun *et al*, 2002)

4 3 1 1 4 EBP-50 (Solute Carrier Family 9, isoform 3 regulator 1)

Expression of the protein EBP-50 was 2 24-fold lower in 4E S209-HA overexpressing cells compared to 4E-HA overexpressing cells

ERM proteins provide a regulated linkage from filamentous (F)-actin in the cortex to membrane proteins on the surface of cells Regulated attachment of membrane proteins to F-actin is essential for many fundamental processes, including the determination of cell shape and surface structures, cell adhesion, motility, cytokinesis, phagocytosis and integration of membrane transport with signaling pathways (Bretscher *et al*, 2002)
ERM proteins associate with specific membrane associated proteins, either directly or through adapter molecules such as EBP-50 (ERM-phosphoprotein of 50 kDa) EBP-50 is 358-residue adapter molecule that has two PDZ domains and a C-terminal ERM binding (EB) region EBP-50 binds to numerous membrane proteins through its PDZ domains and binds to the FERM domain of ERM proteins through its EB region Through these interactions EBP-50 functions as a scaffolding protein, linking the actin cytoskeleton to the plasma membrane (Bretscher *et al*, 2002) The interaction of plasma membrane proteins with the underlying cytoskeleton facilitated by ERM proteins and EBP-50 plays an important role in regulating cell shape, adhesion, motility and other plasma membrane processes including endocytosis and exocytosis (Bretscher *et al*, 2002)

4 3 1 1 5 Tropomyosin 3

Expression of tropomyosin 3 was 1 5-fold lower in 4E S209-HA overexpressing cells compared to 4E-HA overexpressing cells

The binding of tropomyosin to actin filaments prevents them from being depolymerised or severed by cofilin Tropomyosin also prevents the Arp2/3 complex from binding to filaments to initiate branches Thus, tropomyosin may be able to restrict spatially the activities of cofilin and the Arp2/3 complex *in vivo* to certain populations of actin filaments in certain compartments of the cell
In carcinoma cells, the dynamic nucleation zone at the leading edge of the lamellipod is enriched in cofilin and the Arp2/3 complex but tropomyosin is depleted from this region of the cell and is present mainly on actin filaments in the cell body and on stress fibers (Des Marais *et al*, 2002) This allows the establishment of functionally distinct actin compartments in the cells, with rapid generation of actin barbed ends in the cofilin and Arp2/3 complex-rich leading edge compartment and very little barbed end formation in the tropomyosin rich cell body

4 3 1 1 6 Tubulin Specific Chaperone A (TBCA)

Expression of TBCA was 1 86-fold lower in 4E S209-HA cells compared to 4E-HA overexpressing cells

Microtubules are polarized polymers of α/β tubulin participating in essential cell functions A multistep process involving distinct molecular chaperones and cofactors produces new tubulin heterodimers competent to polymerise Tubulin specific chaperone A (TBCA) interacts with β tubulin in a quasi-native state behaving as a molecular chaperone (Lewis *et al*, 1997)

A recent study by Nolasco *et al* (2005) used siRNA to silence TBCA expression in HeLa and MCF-7 mammalian cell lines It was found that TBCA was essential for cell viability and its knockdown produced a decrease in the amount of soluble tubulin,

modifications in microtubules and G1 cell cycle arrest In MCF-7 cells, cell death was preceded by a change in cell shape resembling differentiation (Nolasco *et al*, 2005) Knockdown of TBCA expression also caused changes in the actin cytoskeleton in HeLa cells (Nolasco *et al*, 2005) These results suggest that TBCA expression and activity can have a profound effect on the microtubule cytoskeleton and normal cell function

4 3.1 1 7 β-Actin

Expression of a protein identified as β-Actin was 2 08-fold lower in 4E S209-HA overexpressing cells compared to 4E-HA overexpressing cells

In vertebrates 3 main groups of actin isoforms, alpha, beta and gamma have been identified The alpha actins are found in muscle tissues and are a major constituent of the contractile apparatus The beta and gamma actins coexist in most cell types as components of the cytoskeleton and as mediators of internal cell motility

4 3 1.1 8 Mutant Beta Actin

Expression of a protein identified as mutant beta actin was 1 61-fold lower in 4E S209-HA overexpressing cells compared to 4E-HA overexpressing cells

The differences between mutant beta actin identified here and normal beta actin are two mutations in the coding region that substitute two amino acid residues ($Val^{139}\rightarrow$Met, $Ala^{295}\rightarrow$Asp) Mutant beta actin when expressed in cells in the absence of normal beta actin confers resistance to cytochalasin which is a toxin which binds actin filaments preventing polymerization (Ohmori *et al*, 1992) Whether this mutation affects the normal function of beta actin is unknown

4 3 1 1 9 Actin-like 6A isoform 1 (BAF53)

Expression of Actin-like 6A isoform 1 was 1 76-fold lower in 4E S209-HA cells compared 4E-HA overexpressing cells

The gene Actin-like 6A isoform 1 (BAF53) encodes a family member of actin-related proteins (ARPs), which share significant amino acid sequence identity to conventional actins BAF53 is a mammalian nuclear Arp that is an integral component of many chromatin modifying complexes Chromatin modifying complexes activate or repress transcription of many genes by altering chromatin structure in an autonomous or concerted manner ATP-driven chromatin remodeling complexes such as the mammalian SWI/SNF and PBAF complexes have BAF53 and β-actin as their components Though many BAF53-containing chromatin modifying complexes have been identified little is known of the role of BAF53/β-actin in the complexes (Lee *et al*, 2003)

4 3 1 1 10 Overview of cytoskeletal proteins differentially regulated in 4E S209-HA cells compared to 4E-HA cells

The largest category of differentially regulated proteins in this comparison were cytoskeletal and cytoskeleton regulatory proteins The majority of these proteins were actin cytoskeleton regulatory proteins The expression levels of each of these proteins was lower in 4E S209-HA cells compared to 4E-HA cells The largest difference in protein expression seen was in the actin regulatory protein cofilin whose expression level was 5 38-fold lower in 4E S209-HA cells compared to 4E-HA cells As discussed in section 4 3 1 1 1 cofilin possesses actin severing and depolymerisation activity and acting in synergy with the Arp2/3 complex plays an important role in regulating actin dynamics at the plasma membrane in cellular protrusions and motility (Des Marais, 2005) The actin binding proteins Moesin and EPB41L2 and the scaffold protein EBP-50 all function in linking the actin cytoskeleton to the plasma membrane

(Sections 4 3 1 1 2-4 3 1 1 4) The expression of these three proteins was also lower in 4E S209-HA cells in comparison to 4E-HA cells

The actin cytoskeleton plays an important role in regulating cellular morphology and motility (Section 1 4) The decreased expression of cofilin in 4E S209-HA cells compared to 4E-HA overexpressing cells correlates with differences in cellular morphology seen in these cells (Section 3 1 2 5) The 4E S209-HA cells exhibit a smooth edged rounded morphology with very few cellular protrusions in contrast to 4E-HA overexpressing cells which contain numerous cellular protrusion particularly 4E-HA clone 5 cells which express high levels of transfected 4E-HA Cofilin is considered to play an essential role in actin dynamics at the cell membrane during cellular protrusion (DesMarais, 2005), the more than 5-fold decrease in its expression levels in 4E S209-HA cells in comparison to 4E-HA cells would suggest its decreased expression plays an important role in mediating this morphological phenotype The binding of F-actin structures to the plasma membrane also plays an important role in regulating cellular morphology (Bretscher *et al*, 2002) The proteins Moesin, EPB41L2 and EBP-50 all function in this role and their expression levels were all decreased in 4E S209-HA cells compared to 4E-HA cells This suggests that the linkage of the actin cytoskeleton to plasma membranes may be impaired in these cells and combined with decreased cofilin expression may prevent the formation of protrusive structures in these cells Confocal microscopy analysis of actin cytoskeletal structures using fluorescently labelled phalloidin showed no actin staining at the plasma membrane in 4E S209-HA cells (Section 3 1 2 13) This was in contrast to staining seen 4E-HA overexpressing cells particularly 4E-HA 5 cells (Section 3 1 2 13)

The expression of a normal and mutant isoform of β-actin protein was also lower in 4E S209-HA cells compared to 4E-HA cells (Table 3 1 2 11 4) A lower level of actin protein in 4E S209-HA cells may contribute to the morphological and invasive phenotype of these cells also

Expression of the actin binding protein Tropomyosin 3 was also reduced in 4E S209-HA overexpressing cells in comparison to 4E-HA overexpressing cells Binding of tropomyosin to actin filaments prevents them from being depolymerised and severed by cofilin Tropomyosin can also prevent the Arp2/3complex from binding to

filaments to initiate branches (Des Marais, 2002) Tropomyosin therefore has a stabilising effect on actin filaments within cells Analysis of cellular localisation of tropomyosin by DesMarais (2002) showed that tropomyosin localises to stress fibers and actin filaments in the cell body but is absent from the leading edge of cells where cofilin and Arp2/3 complexes function (Des Marais, 2002) Confocal microscopic analysis of actin cytoskeletal structures using fluorescently labelled phalloidin showed that 4E S209-HA overexpressing cells did not display any stress fibers or other actin filaments within the cell body (Fig 3 1 2 13 1) The 4E-HA overexpressing cells, particularly 4E-HA clone 5, clearly showed the presence of these structures (Fig 3 1 2 13 2-3) The reduced expression of Tropomyosin 3 in the 4E S209-HA cells may decrease the stability of actin fibers in these cells

Expression of a member of the Arp family, Actin-like 6A isoform 1 (A K A BAF53) was also lower (1 76-fold) in 4E S209-HA cells compared to 4E-HA cells and may cause differences in transcriptional regulation as it is considered to be an integral component of chromatin modifying complexes which can alter transcriptional regulation by modifying access of transcriptional regulatory factors to DNA (Lee *et al*, 2003)

Regulation of the Microtubule cytoskeleton may also be affected in 4E S209-HA cells as expression of the β-tubulin chaperone, tubulin specific chaperone A (TBCA) was 1 86-fold lower in these cells compared to 4E-HA cells Studies have shown that lowering TBCA expression levels can have a profound effect on the microtubule cytoskeleton and normal cell function (Nolasco *et al*, 2005) The decreased expression of TBCA in 4E S209-HA cells may therefore cause changes in the microtubule cytoskeleton which affect the morphological phenotype and growth of these cells

The down regulation of actin cytoskeleton regulatory proteins in 4E S209-HA cells is also likely to impact on the invasive capacity of these cells The actin cytoskeleton is considered as the cellular engine which drives cell motility (Lambrechts *et al*, 2004) The aberrant cell migration that characterises tumor invasion and metastasis is associated with deregulation of the actin system (Lambrechts *et al*, 2004) The 4E-

HA 5 cells were highly invasive in comparison to both 4E S209-HA overexpressing clones whereas 4E-HA 10 cells were slightly less invasive than the 4E S209-HA overexpressing clones (Section 3 1 2 10) The higher level of cofilin and other actin regulatory proteins in 4E-HA overexpressing cells is likely to contribute to the high invasiveness of 4E-HA 5 cells Cofilin expression in all 4E-HA 5 cell samples was on average over two fold higher than in 4E-HA 10 cells in our proteomic analysis The t-test score for cofilin expression comparison between these cells though was greater than 0 05 and therefore did not pass our statistical analysis specifications although this result would suggest that cofilin expression may be linked to eIF4E levels and invasiveness in these cells

The expression of the cytoskeletal proteins cofilin, moesm, tropomyosin 3 and Tubulin Specific Chaperone A were all decreased in 4E S209-HA cells in comparison to pcDNA control cells The lower expression level of these proteins in 4E S209-HA cells compared to 4E-HA cells is therefore due to a decrease in their expression in 4E S209-HA cells rather than an increase in 4E-HA cells

4 3 1 1 10 1 Actin related cytoskeletal protein expression regulation

The differential expression of actin cytoskeletal proteins seen in this study begs the question, why might this class of protein be particularly affected by disruption of phosphorylation at the eIF4E S209 phosphorylation site? A clue as to why this may be, could be the fact that a large proportion of these proteins function in the periphery of the cell For example Moesin, EBP-50 and EPB41L2 all function in linking actin cytoskeletal structures to the plasma membrane and are therefore located at the very edge of the cytoplasm Cofilin functions at the leading edge of motile cells as a key regulator of actin dynamics (DesMarais *et al*, 2005) These proteins are therefore utilized in particular areas of the cell One mechanism for localizing a protein within a particular area of a cell is through localized translation

There are numerous examples of localized translation of actin cytoskeleton proteins, the best characterized of which being β-actin mRNA (Shav-Tal and Singer, 2000) In migrating fibroblasts, β-actin mRNA is localized to the leading edge of the cells

(Lawrence and Singer, 1986). This correlates with the elevated levels of β-actin protein required in lammelipodia which depend on the rapid polymerization of actin for cell movement (Condeelis and Singer, 2005). In neuronal cells, β-actin mRNA is localized to neuronal growth cones and dendrites (Shav-Tal and Singer, 2005).
As discussed in section 4.4.1.1.1, the Arp2/3 complex is one of the major modulators of actin polymerisation in cell protrusions. The Arp2/3 complex consists of seven polypeptides and is found at actin filament Y-branches in the submembrane array (DesMarais *et al.*, 2005). A recent study by Mingle *et al.* (2005) has shown that the seven mRNAs that encode Arp2/3 subunits are all localised to areas of cellular protrusion in fibroblast cells. As mentioned previously, recent studies have also shown that Arp2/3 and Cofilin act synergistically in local actin polymerisation responses upon cell stimulation and play a central role in regulating the direction of cell motility (DesMarais *et al.*, 2005).
Transcripts for the actin regulatory GTPase RhoA are localised to developing axons and growth cones in neuronal cells (Wu *et al.*, 2005).

There is therefore a growing body of evidence which suggests that localised translation of actins and actin regulatory proteins plays an important role in regulating cellular morphology and motility.

A study by Topisirovic *et al.* (2004) was conducted, in which, both wild type eIF4E and a non-phosphorylatable eIF4E-S209 mutant similar to the one used in this study were overexpressed in NIH3T3 cells. The ability of wild type eIF4E and eIF4E S209 mutant to cause transformation of these NIH3T3 cells was then examined by anchorage dependent foci formation assay. The transformation of NIH3T3 cells was greater in wild type eIF4E overexpressing cells compared to eIF4E S209 mutant cells. The eIF4E S209 phosphorylation site mutation therefore abrogated the ability eIF4E to transform these cells (Topisirovic *et al.*, 2004). The ability of wild type eIF4E and eIF4E S209 mutant overexpression to increase nucleo-cytoplasmic transport of cyclin D1 mRNA was also examined and it was found that mutation of the S209 phosphorylation site also abrogated the ability of eIF4E to increase nucleo-cytoplasmic mRNA transport (Topisirovic *et al.*, 2004). The phosphorylation of the

S209 site therefore appears to play a role in cellular transformation through regulation of mRNA transportation

The transportation of mRNAs is regulated by the binding of proteins to nucleotide sequences in their 3'UTRs β-actin mRNA localization is regulated by ZBPs (zipcode binding proteins) which bind to 'zipcode' sequences immediately downstream of the ORF (Shav-Tal and Singer, 2005) ZBP1 binds to β-actin mRNA in the nucleus and travels in cytoplasmic granules to the leading edge (Shav-Tal and Singer, 2005) Translation is thought to be inhibited, perhaps by ZBP1, until the mRNA reaches the lammelipodia

There are numerous examples of proteins which bind mRNAs and are involved in regulating their transport and localized translation including examples where interaction with eIF4E is required (Huang and Richter, 2004, Shav-Tal and Singer, 2005) A recent study by Culikovic *et al* (2005) has identified an ~100 nucleotide sequence in the 3'UTR of cyclin D1 mRNA referred to as an eIF4E sensitivity element (4E-SE) which is responsible for eIF4E mediated mRNA transport (Culikovic *et al*, 2005) It may be the case that phosphorylation of eIF4E plays a role in stabilizing interactions between eIF4E and other proteins which bind to elements such as the 4E-SE which regulate mRNA transport

We therefore searched the literature to see if the differentially regulated proteins identified in this study have been reported to be regulated by mRNA transport/localization A recent study by Willis *et al* (2005) utilizing proteomics technology and RT-PCR to analyse the axonal localization of proteins and mRNA in injury-conditioned adult dorsal root ganglion (DRG) neurons identified numerous cytoskeletal proteins whose mRNA was also present in axonal preparations (Willis *et al*, 2005) Among the proteins identified were beta-actin, tropomyosin 3 and cofilin (Willis *et al*, 2005), these proteins were also identified in our study as proteins differentially regulated between 4E-HA and 4E S209-HA overexpressing DLKP cells In a subsequent follow on study by Aranda-Abreu *et al* (2005) the 3'UTRs of the axonally located mRNAs where analysed for similarity to a well characterized U-rich 3'UTR sequence responsible for localization of the axonally located Tau protein mRNA A U-rich sequence highly similar to the Tau mRNA localisation was detected

in the 3'UTR of cofilin mRNA (Aranda-Abreu *et al*, 2005) The cofilin mRNA 3'UTR has therefore already been shown to contain sequences which are likely to regulate its cellular localization

In order to further investigate the possibility that mRNA 3'UTR elements are responsible for regulating the expression of differentially expressed proteins between 4E-HA and 4E S209-HA cells, we analysed the mRNA sequences of differentially expressed cytoskeletal proteins using the online mRNA analysis program UTRscan UTRscan looks for UTR functional elements by searching through user submitted sequence data for patterns defined in the UTRsite collection (Mignone *et al*, 2005) The mRNA sequences of the cytoskeletal proteins Cofilin, Moesin, EBP50, EPB41L2, Tropomyosin 3 and Actin Like 6 isoform A were submitted for analysis The presence of one or more 15-Lipoxygenase Differentiation Control Element (15-LOX DICE) translation regulatory elements was detected in the majority of the mRNA transcripts submitted (Cofilin x1, EBP50 x2, Moesin x3, Tropomyosin 3 x3) The 15-LOX-DICE element was therefore a common feature among transcripts analysed The cytidine-rich 15-LOX DICE, is a multifunctional cis-element found in the 3'-UTR of numerous eukaryotic mRNAs The 15 LOX-DICE element binds KH domain proteins of the type hnRNP E and K, thus mediating mRNA stabilization and translational control (Reimann *et al*, 2002) The exact mechanism of translation silencing by 15-LOX-DICE is unknown but it is known that it is at the level of translation initiation indicating interaction of 15-LOX-DICE binding proteins such as hnRNP K and hnRNP E with translation initiation proteins at the mRNA 5' end (Fig 4 3 1) (Ostareck, 2001)

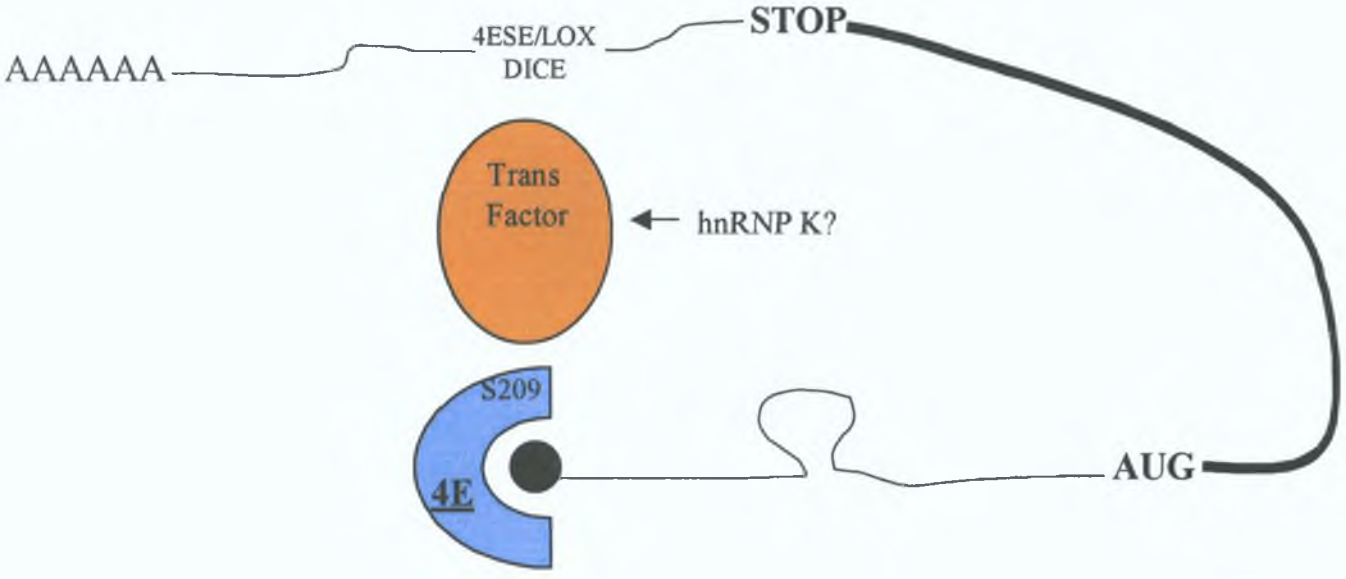


Figure 4.3.1 Model of possible interaction between eIF4E and proteins binding to 3'UTR elements such as 4ESE and 15-LOX-DICE regulating mRNA transport and localization. Phosphorylation of eIF4E may play a role in regulating eIF4E-trans factor interaction or eIF4E/trans factor complex-mRNA interaction.

Experimental evidence that the 15-LOX-DICE element is involved in regulation of cofilin is seen in a study by Klimek-Tomczak *et al.* (2004) which detected cofilin RNA among RNA co-immunoprecipitated with hnRNP K. HnRNP K may therefore be involved in translational repression of the 15-LOX DICE element containing mRNAs identified in our study. If hnRNP K is involved in repressing translation of these mRNAs for the purpose of mRNA transportation /translational localization, an activation mechanism would be necessary to allow translation to proceed once an appropriate destination is reached.

hnRNP K binds selectively to the SH3 domains of tyrosine kinases, Src, Fyn, Lyn and Lck (Bomsztyk, 2004). A study by Ostareck Lederer *et al.* (2002) showed that interaction between hnRNP K and c-Src leads to c-Src activation and tyrosine phosphorylation of hnRNP K. c-Src-mediated phosphorylation reversibly inhibited

the binding of hnRNP K to the 15-LOX DICE mRNA 3'UTR element *in vitro* and specifically derepressed the translation of 15-LOX DICE bearing reporter mRNAs *in vivo* (Ostareck Lederer *et al*, 2002) Src functions as an integrin signaling protein as part of focal adhesion complexes and directly interacts with focal adhesion kinase (Section 1 3 1 1) Src therefore functions in peripheral cellular regions where the differentially expressed proteins identified with 15 LOX-DICE bearing mRNAs also function (Cofilin, Moesin, EBP50, EPB41L2, Tropomysosin 3) A recent study by Hoog *et al* (2004) used specialized mass spectrometry methods to identify proteins interacting with focal adhesion proteins Numerous RNA binding and ribosomal proteins were identified including hnRNP K (Hoog *et al*, 2004) Subsequent confocal microcopic analysis of spreading cells showed hnRNP K protein localized to focal adhesion associated sites termed spreading initiation centers (SICs) (Hoog *et al*, 2004) Focal Adhesion Kinase (FAK) was also present in spreading initiation centers (Hoog *et al*, 2004) These results would indicate that Focal Adhesion Complex sites or SICs may be areas where hnRNP K localizes to activate translation of bound mRNAs A model for this translation regulatory mechanism is outlined in Figure 4 3 2 (on following page)

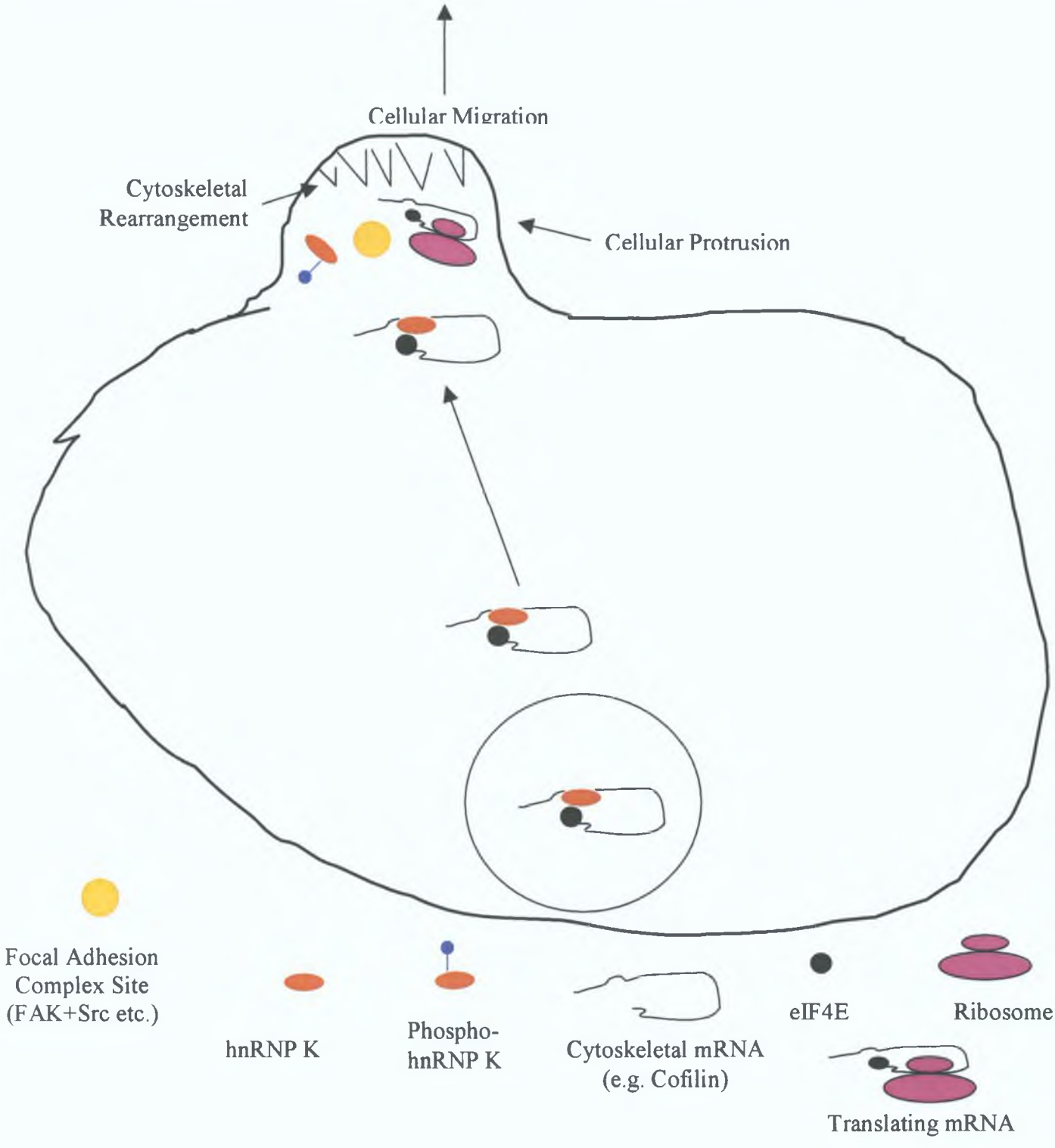


Figure 4.3.2 Model of mRNA transport/translation localization of differentially expressed cytsokeletal proteins identified in proteomic analysis. Cytoskeletal mRNA (e.g. Cofilin) with eIF4E bound to 5'-cap interacts with hnRNP K bound to 15 LOX DICE element in 3'UTR causing translational repression. This mRNP complex is transported to Focal Adhesion Complex where phopsphorylation of hnRNP K by Src causes release of hnRNP K and translational activation. This allows translation to occur in the vicinity of the focal adhesion complex resulting in cytoskeletal rearrangement, morphological change and migration.

An explanation for the decreased expression of cytoskeletal proteins with mRNAs bearing 15 LOX-DICE sequences in 4E S209-HA overexpressing cells compared to 4E-HA overexpressing cells may be that phosphorylation of eIF4E plays a role in regulating interaction of eIF4E with hnRNP K eIF4E phosphorylation may also affect the interaction of a hnRNP K-eIF4E complex with the cap structure Either of these scenarios could result in disruption of mRNA transport or translational activation following silencing The exact details of this mechanism will be the subject of future research but we can conclude from this study that it is highly probable that eIF4E plays a role in regulating the transport and/or translation of a new class of mRNAs in conjunction with 15 LOX DICE element binding proteins such as hnRNP K This novel mRNA regulation model is particularly relevant to the regulation of genes involved in regulating actin cytoskeleton dynamics, cellular morphology and migration and therefore is important to the field of cancer invasion/metastasis It is also noteworthy that hnRNP K has recently been identified as a regulator of eIF4E transcription and binds to an element critical for promoter function known as the 4E basal element (4EBE) further implicating hnRNP K in regulation of gene expression in conjunction with eIF4E (Lynch *et al*, 2005)

4.3.1.2 mRNA Processing Proteins

4 3 1 2 1 HnRNP F

Heterogeneous nuclear ribonucleoproteins (hnRNPs/HNRPs) constitute a set of polypeptides that bind heterogeneous nuclear RNA (hnRNA), the transcripts produced by RNA polymerase II and precursors to mRNAs This family of proteins is involved in the processing of RNA molecules from transcription to translation, including splicing, transportation, degradation and translation of RNAs

Expression of two proteins identified as hnRNP F were 1 84 (Master No 1296) and 1 73 (Master No 1299) fold lower in 4E S209-HA overexpressing cells compared to 4E-HA overexpressing cells

HnRNP F is best characterized in its role as a regulator of pre-mRNA splicing HnRNP F binds preferentially to CBC-RNA (Cap Binding Complex-RNA) complexes rather than naked RNA Depletion of hnRNP F from HeLa cell nuclear extract was found to decrease the efficiency of pre mRNA splicing (Gamberi *et al*, 1997) HnRNP F is involved in regulating the splicing of Bcl-x Bcl-x is a member of the Bcl-2 family of proteins that are key regulators of apoptosis The Bcl-x pre-mRNA is alternatively spliced to yield Bcl-x_S and Bcl-x_L, two isoforms that have been associated, respectively, with the promotion and the prevention of apoptosis A 30-nucleotide G-rich element (B2G) which is responsible for regulating mRNA splicing of Bcl-x was found to bind to hnRNP F The addition of hnRNP F to a HeLa extract improved the production of the Bcl-x_S variant Consistent with the *in vitro* results, small interfering RNA targeting hnRNP F and H decreased the Bcl-x_S/Bcl-x_L ratio of plasmid-derived and endogenously produced Bcl-x transcripts These results show a positive role for the hnRNP F proteins in the production of the proapoptotic regulator Bcl-x_S (Garneau *et al*, 2005)

4 3 1 2 2 HnRNP K

Expression of a protein identified as hnRNP K was 1 56-fold lower in 4E S209-HA overexpressing cells compared to 4E-HA overexpressing cells

HnRNP K is a member of the heterogeneous nuclear ribonucleoprotein family discussed in section 4 4 1 2 1 hnRNP K protein has been found not only in the nucleus but also in the cytoplasm and mitochondria and is implicated in chromatin remodeling, transcription, splicing and translation processes hnRNP K protein contains multiple modules that on the one hand, bind kinases while on the other hand recruit chromatin, transcription, splicing and translation factors These protein mediated interactions are regulated by signaling cascades These observations are consistent with hnRNP K protein acting as a docking platform to integrate signaling cascades by facilitating cross talk between kinases and factors that mediate nucleic acid directed processes (Bomsztyk *et al* , 2004) hnRNP K binds to CU rich repetitive stretches known as DICE (differential control elements) elements in mRNA 3'UTRs and can block translation initiation by blocking the recruitment of the 60S ribosomal subunit and the formation of the translation competent 80S ribosome (Bomsztyk *et al* , 2004) A recent study by Lynch *et al* (2005) has found that hnRNP K binds to a polypyrimidine element in the promoter sequence of eIF4E which they termed the 4EBE (eIF4E basal element), which functions as a basal promoter element hnRNP K was shown to regulate eIF4E levels and also increased translation initiation, cell division and promoted neoplastic transformation in an eIF4E dependent manner (Lynch *et al* , 2005) Overexpression of hnRNP K in this study caused an increase in the rate of global translation levels (Lynch *et al* , 2005)

4 3 1 2 3 G3BP

Expression of the RasGAP associated endoribonuclease G3BP was 2 36-fold lower in 4E S209-HA overexpressing cells in comparison to 4E-HA overexpressing cells G3BP is involved in regulating mRNA fate through degradation and sequestration in cellular bodies known as stress granules (Tourriere *et al* , 2003, Kedersha *et al* , 2005)

4 3 1.2 4 Overview of mRNA Processing proteins

The expression levels of two members of the heterogeneous nuclear ribonucleoprotein family, hnRNP F and hnRNP K and also the mRNA regulatory protein G3BP were all lower in 4E S209-HA overexpressing cells in comparison to 4E-HA overexpressing cells

As hnRNP F expression is associated with the production of pro-apoptotic Bcl-x_s (Garneau *et al*, 2005), decreased expression of hnRNP F in 4E S209-HA cells may contribute to the survival of these cells Decreased expression of hnRNP F may also affect mRNA splicing of other genes

The lower level of hnRNP K in 4E S209-HA cells compared to 4E-HA cells may have a widespread effect on gene expression hnRNPK activity is implicated in the regulation of transcription, RNA processing and translation and may therefore affect the expression of a large number of genes The fact that hnRNP K is involved in regulating transcription of eIF4E is also of interest

The expression level of hnRNP F protein is decreased in 4E S209-HA cells in comparison to pcDNA control cells The lower expression level of this protein in 4E S209-HA cells compared to 4E-HA cells is therefore due to a decrease in its expression in 4E S209-HA cells rather than an increase in 4E-HA cells On the other hand expression of G3BP is increased in 4E-HA cells in comparison to pcDNA control cells The lower expression level of this protein in 4E S209-HA cells compared to 4E-HA cells is therefore due to an increase in expression in 4E-HA cells rather than a decrease in 4E S209-HA cells The increased expression of G3BP in 4E-HA cells is discussed further in section 4 3 2 2

The lower level of RNA processing proteins in 4E-S209 HA overexpressing cells compared to 4E-HA overexpressing cells may result in altered gene expression due to their role in mRNA splicing, transport, degradation, translation and also in the case of hnRNP K, transcription Whether the lower expression of this class of protein in 4E S209-HA cells is a direct result of reduced 4E phosphorylation is unclear A recent study by Topisirovic *et al* (2004) analysing the ability of an eIF4E S209 mutant to transform NIH3T3 cells discovered that mutation of the eIF4E S209 phosphorylation site reduced eIF4E dependent nucleo-cytoplasmic mRNA transport (Topisirovic *et al*, 2004) A proposed explanation for this reduction in eIF4E dependent nucleo-

cytoplasmic mRNA transport was the disruption of interactions between eIF4E and other proteins in the nucleus involved in regulating nucleo-cytoplasmic mRNA transport or alterations in the binding of other ribonucleoproteins to mRNAs in the nucleus due to the altered affinity of non-phosphorylated eIF4E for the cap structure (Topisirovic *et al*, 2004) There is therefore, a possible connection between 4E phosphorylation and RNA binding proteins involved in nucleo-cytoplasmic transport Whether the altered expression of the RNA binding proteins seen here is connected to this process remains to be seen

4.3 1.3 Protein Chaperones

Expression of two proteins identified as Heat shock protein APG1 were 2 25 and 2 3-fold higher in 4E-HA overexpressing cells than 4E S209-HA cells
Heat shock protein APG is a member of the HSP110 family of heat shock proteins and possesses protein chaperone activity (Matsumori *et al*, 2002)

The heat shock protein chaperones interact with diverse protein substrates to assist in their folding and have a critical role during cell stress to prevent the appearance of folding intermediates that lead to misfolded or otherwise damaged molecules Consequently, heat shock protein chaperones assist in the recovery from stress by repairing damaged proteins (protein refolding), thus restoring protein homeostasis and promoting cell survival A major characteristic of tumor cells is their resistance to cell death Increased expression of heat shock proteins has been detected in a number of cancers (Jolly and Morimoto, 2000) It is considered that increased expression of heat shock proteins may confer a survival advantage on cancer cells eIF4E has been classified as an oncogene due to its ability to transform cells and its increased expression in a number of cancers (Mamane *et al*, 2004, Rosenwald, 2004) Increased expression of heat shock protein chaperones in eIF4E overexpressing cells may be necessary for folding of proteins whose levels are increased as a result of translational upregulation

The increased expression of heat shock protein APG1 in 4E-HA overexpressing cells in comparison to 4E S209-HA overexpressing cells may be an indication of higher protein production, or increased production of a subset of proteins requiring protein chaperone activity for correct folding

4.3.1.4 Protein Degradation proteins

The expression of the proteasome activator proteins PA28 alpha and PA28 beta were 1 85-fold and 2 07-fold higher respectively in 4E S209-HA overexpressing cells in comparison to 4E-HA overexpressing cells Expression of Ubiquitin carboxy terminal hydrolase L1 was 1 5-fold higher in 4E S209-HA overexpressing cells in comparison to 4E-HA overexpressing cells

These proteins are upregulated in 4E S209-HA cells in comparison to pcDNA control cells and the higher levels seen 4E S209-HA cells in comparison to 4E-HA cells are therefore due their increased expression in 4E S209-HA cells rather than decreased expression 4E-HA cells

This category of proteins were the only proteins that showed increased expression in 4E S209-HA cells apart from the nucleotide metabolism protein Purine Nucleoside Phosphorylase It would seem therefore that an increase in non-phosphorylated eIF4E levels has an effect on protein degradation pathways Increased protein degradation in 4E S209-HA cells may cause decreased expression of other proteins in the cells

The increased expression of protein degradation proteins in 4E-S209-HA cells is discussed further in Section 4 3 3 1

4.3.1.5 Metabolic Proteins

Expression of five proteins identified as Aldehyde Dehydrogenease 1 and a protein identified as Purine Nucleoside Phosphorylase were lower in 4E-HA overexpressing cells compared to 4E S209-HA overexpressing cells Aldehyde dehydrogenase 1 expression was decreased in 4E-HA cells in comparison to pcDNA controls indicating the lower expression seen here is due to reduced expression in 4E-HA cells rather than increased expression in 4E S209-HA cells On the other hand, Purine Nucleoside Phosphorylase expression is increased in 4E S209-HA cells in comparison to pcDNA controls indicating its lower expression in 4E-HA cells seen here is due to increased expression in 4E S209-HA cells rather than decreased expression in 4E-HA cells

Expression of a protein identified as Glyoxalase 1 is lower in 4E S209-HA overexpressing cells compared to 4E-HA overexpressing cells Glyoxalase 1 expression was reduced in 4E S209-HA cells in comparison to pcDNA controls indicating its lower expression in 4E-S209 HA cells seen here is due to decreased expression in 4E S209-HA cells rather than increased expression in 4E-HA cells

The relevance of decreased expression of Aldehyde dehydrogenase in 4E-HA cells is discussed in section 4 3 2 1, and the increased expression of Purine Nucleoside Phosphorylase and decreased expression of Glyoxalase 1 in 4E S209-HA cells in section 4 3 3 5

4 3 2 Differentially expressed proteins in 4E-HA overexpressing cells compared to pcDNA controls

Analysis of differentially expressed proteins between 4E-HA overexpressing cells compared to pcDNA controls revealed changes in a number of different classes of proteins (Table 4 3 2)

pcDNA vs 4E-HA	
4E-HA Increased Expression	**4E-HA Decreased Expression**
Protein Folding	**Metabolism**
Heat Shock Protein APG, Chaperonin (Hsp60)	Aldehyde Dehydrogenase
mRNA Processing	
G3BP	

Table 4 3 2 Proteins differentially expressed between 4E-HA and 4E S209-HA overexpressing cells

4 3 2 1 Aldehyde Dehydrogenase

Five proteins identified as Aldehyde Dehygrogenase 1 enzymes were downregulated in 4E-HA overexpressing cells compared to pcDNA controls, with fold changes ranging from a 1 59-fold to a 2 11-fold decrease (Table 3 1 2 11 2) Previous 2D electrophoresis analysis of Aldehyde Dehydrogenase enzymes has shown separation of multiple variants of aldehyde dehydrogenase enzymes by this method (Park *et al*, 2002)

Aldehyde Dehydrogenases (ALDH) are considered as general detoxifying enzymes which eliminate toxic biogenic and xenobiotic aldehydes Adehyde dehydrogenase 1 (ALDH1) is a cytosolic enzyme ubiquitously distributed in various tissues The enzyme has a high activity for the oxidation of both all-trans- and 9-cis-retinal and it may play a role in the formation of retinoic acid, which is a potent modulator for gene expression and tissue differentiation (Yoshida *et al*, 1998) Retinoic acid is known to

play an important role in the development and differentiation of the lung (Ross, 2004) and reduced expression of aldehyde dehydrogenase may therefore have an effect on the growth and differentiation of lung cells through it's regulation of retinoic acid Aldehyde Dehydrogenase also plays an important role in metabolizing acetaldehyde Acetaldehyde is a known carcinogenic aldehyde that is found in cigarette and car exhaust smoke and is also a product of ethanol metabolism Acetaldehyde plays an important role in the pathogenesis of tissue injury that results from alcohol or cigarette consumption As acetaldehyde is a substrate for ALDH, high levels of ALDH could protect against the toxicity of acetaldehyde and vice-versa (Moreb *et al*, 2005)

4 3 2 2 G3BP

The RasGAP-associated endoribonuclease G3BP was 1 86-fold upregulated in 4E-HA cells compared to pcDNA control cells

G3BP is an RNA binding protein known to bind to the SH3 domain of the Ras GTPase activating protein (RasGAP) Recent studies have shown G3BP is involved in the assembly of the mRNA regulatory bodies known as stress granules (Tourniere *et al*, 2003, Kedersha *et al*, 2005) Stress granules (SGs) are cellular bodies where mRNAs and associated proteins are involved in regulating the sequestration and degradation of mRNAs under conditions of cellular stress Stress granules are described as sites of translationally inactive protein synthesis machinery Stress granules form in the cytoplasm in response to various toxic agents and are believed to play a critical role in the regulation of mRNA translation during stress A study by Tourniere *et al* (2003), showed that G3BP is recruited to SGs in cells exposed to arsenite (Tourniere *et al*, 2003)

In a recent study by Kedersha *et al* (2005), G3BP was transfected into DU145 cells in order to induce the formation of stress granules to allow for analysis of their constituent proteins This study showed that stress granules induced by increased expression of G3BP contained eIF4E and other translation initiation factors such as eIF4G, eIF3 and Poly(A) binding protein They also showed the induction of related cellular bodies known as processing bodies in G3BP overexpressing cells which are involved in mRNA degradation Processing bodies were also found to contain eIF4E protein (Kedersha *et al* (2005)

In stressed cells, Stress Granules form as a result of eIF2α phosphorylation which causes a situation where the level of eIF2-GTP-$tRNA_i^{Met}$ ternary complex becomes limiting for translation initiation

In normal cellular conditions, eIF4E levels are considered to be a rate limiting factor and the level of available eIF4E is regulated by it being bound by 4E-BP repressor proteins It may be possible that in eIF4E overexpressing cells, such as those used in our study, the raising of eIF4E levels results in a situation whereby eIF2-GTP-$tRNA_i^{Met}$ ternary complex levels become limiting regardless of eIF2α phosphorylation This would lead to the assembly of eIF2/eIF5-deficient preinitiation complexes which subsequently are routed to stress granules as in stressed cells The increased expression of G3BP in 4E overexpressing cells could therefore be in response to this situation as G3BP has been shown to be involved in stress granule formation and may therefore affect the ability of 4E overexpression to cause change in gene expression levels via regulation of mRNA translation

It would be of interest to analyse the localization of G3BP and translation initiation factors within 4E-HA overexpressing cells to determine if they are present in stress granules

4 3.2 3 Protein Chaperones

Two heat shock proteins possessing protein chaperone activity were upregulated in 4E-HA overexpressing cells in comparison to pcDNA control cells Heat shock protein APG was upregulated 1 9-fold and chaperonin (Hsp60) was upregulated 1 51-fold Heat shock protein APG is a member of the HSP110 family of heat shock proteins (Matsumori *et al*, 2002, Nonoguchi *et al*, 1999) Chaperonin is known commonly as a mitochondrial protein in mammalian cells and is involved in assisting in the correct folding of mitochondrial proteins (Barazi *et al*, 2002)

The heat shock protein chaperones interact with diverse protein substrates to assist in their folding and have a critical role during cell stress to prevent the appearance of folding intermediates that lead to misfolded or otherwise damaged molecules Consequently, heat shock protein chaperones assist in the recovery from stress by repairing damaged proteins (protein refolding), thus restoring protein homeostasis and promoting cell survival
A major characteristic of tumor cells is their resistance to cell death Increased expression of heat shock proteins has been detected in a number of cancers (Jolly and Morimoto, 2000) It is considered that increased expression of heat shock proteins may confer a survival advantage on cancer cells eIF4E has been classified as an oncogene due to its ability to transform cells and its increased expression in a number of cancers Increased expression of heat shock protein chaperones in eIF4E overexpressing cells may be necessary for folding of proteins whose levels are increased as a result of translational upregulation

Recent studies have shown that chaperonin may also function outside the mitochondria A study by Barazi *et al* (2002) showed that chaperonin protein on the cell surface was involved in the activation of $\alpha 3\beta 1$ integrin (Barazi *et al*, 2002) Increased expression of $\alpha 3$ and $\beta 1$ integrin was detected in 4E-HA 5 cells (Section 3 1 2 7) It is possible that chaperonin expression may therefore play a role in modulating integrin activity on 4E-HA 5 cells if expressed on the cell surface in these cells

4.3.3 Differentially expressed proteins in 4E S209-HA overexpressing cells compared to pcDNA controls.

Analysis of differentially expressed proteins between 4E S209-HA overexpressing cells compared to pcDNA controls revealed changes in a number of different classes of proteins (Table 4 3 3)

pcDNA vs 4E S209-HA	
4E S209-HA Increased Expression	**4E S209-HA Decreased Expression**
Protein Degradation	**Cytoskeletal**
PA28 alpha, PA28 beta, UCHL1, AHA1?	Cofilin, Moesin Tropomyosin 3, Tubulin Secific Chaperone A
Protein Folding	**mRNA Processing**
AHA1	HNRPF
Metabolism	**Metabolism**
Purine Nucleoside Phosphorylase	DDAH1, Glyoxalase 1

Table 4.3 3 Proteins differentially expressed between pcDNA controls and 4E S209-HA overexpressing cells

4 3 3 1 Protein Degradation Proteins

4 3 3 1 1 PA28 Alpha and PA28 Beta

The expression of the proteasome activator proteins PA28 alpha and PA28 beta was 1 85-fold and 2 07-fold higher respectively in 4E S209-HA overexpressing cells in comparison to pcDNA control cells

Proteasomes perform the majority of proteolysis that occurs in the cytosol and nucleus of eukaryotic cells and thereby, perform crucial roles in cellular regulation and homeostasis Isolated proteasomes are inactive because substrates cannot access the proteolytic sites PA28 proteins are activators that bind to proteasomes and stimulate the hydrolysis of peptides (Reichsteiner and Hill, 2005)

The 20S proteasome is a barrel shaped assembly of 28 protein subunits that possess three distinct proteolytic active sites with different specifities Together, the three active sites, present in the two central rings of β subunits, hydrolyse almost all peptide bonds, having trouble only with those bonds that follow glycine and proline The elimination of
inappropriate substrates is prevented by sequestration of active sites within the hollow structure of the 20S proteasome Substrates access the central catalytic chamber through axial ports in the end rings of α subunits, although in the absence of activators these channels are closed and activity is repressed
Proteasomes are activated by protein complexes that bind to the end of α subunits The best known activator is PA700 (proteasome activator MW 700, also known as 19S or regulatory complex (RC)), which has been conserved from yeast to humans and binds to the 20S proteasome to form the 26S proteasome PA700 is the only proteasome activator that is known to stimulate degradation of protein substrates, which it generally recognizes by a polyubiquitination modification and which it processes by an ATP-dependent mechanism Thus PA700 is though to mediate most of the biological effects of the proteasome by facilitating substrate degradation

In contrast to the evolutionarily conserved protein complex PA700, PA28 (also known as 11S or REG), has been shown to bind specifically to and activate 20S proteasomes against model peptide substrates but does not recognize ubiquinated proteins or use ATP It is possible that PA28 functions normally in mixed complexes known as hybrid proteasomes in which the 20S proteasome is bound at one end by PA700 and at the other end by PA28
PA28 family members, which are found in higher eukaryotes but are absent from yeasts exist as homo- or heteromeric complexes of seven ~28-kDa subunits There are three PA28 homologs called α, β, and γ The α and β subunits form a heteroheptamer whereas γ forms a homoheptamer The biological roles of PA28 proteins are understood less well than those of PA700 although their biochemical activities and evolutionary conservation implies that they have important roles in cellular physiology and several important functions have been proposed
Although PA28α and β subunits are expressed in many organs, they are particularly abundant in immune tissues PA28αβ proteins are mainly found in the cytoplasm and

are induced by interferon and infection The best characterized function of PA28 proteasomal activators are in cellular immunity where they are known to be involved in the generation of peptides for presentation on class I molecules and subsequent recognition by cytotoxic T lymphocytes (Reichsteiner and Hill, 2005)
Although the main role of PA28 α and β appear to be in cellular immunity, studies have also shown that expression of PA28 α and β proteasome activators are also increased in conditions which appear unrelated to immune response In cases such as these it is thought that there is more generation of hybrid 26S proteasomes, thereby increasing proteolytic efficiency (Tanahashi *et al*, 2000)

4 3 3 1 2 UCHL1

The expression of the ubiquitin carboxy terminal hydrolase L1 (UCHL1) was increased 1 91-fold in 4E S209-HA cells in comparison to pcDNA controls

The ubiquitin proteasome system is a major pathway for selective protein degradation Ubiquitin attaches to the target proteins and forms a polyubiquitin chain and the ubiquitinated proteins are recognized and degraded by a multi-subunit protease complex, called the proteasome Ubiquitin carboxy terminal hydrolases recycle ubiquitin from ubiquitin/protein complexes or polyubiquitin chains by cleaving the amide linkage neighbouring the C-terminal glycine of ubiquitin (Liu *et al*, 2002)
UCHL1 hydrolase activity is thought to be important for cytoplasmic protein degradation, recycling free ubiquitin by cleaving ubiquitinated peptides that are the products of proteasomal degradation of polyubiquitinated proteins Association of UCHL1 with ubiquitin also plays a role in maintaining ubiquitin levels by inhibiting its degradation (Osaka *et al*, 2003)
UCHL1 is expressed at high levels in the neural and neuroendocrine systems UCHL1 is one of the major proteins of the brain, constituting 1-5% of total soluble brain protein UCHL1 expression has been associated with cancer progression and also with the development of Parkinsons disease (Liu *et al*, 2002) It is not known if increased expression of UCHL1 is a cause or a result of cancer progression

Increased expression of UCHL1 in 4E S209-HA cells may contribute to increased protein degradation in these cells UCHL1 is also considered to be a marker of neuroendocrine differentiation in lung cancer cell lines (Castro *et al*, 2000) The

increased expression of UCHL1 and the proteasomal activators PA28 alpha and beta may suggest a general upregulation of protein degradation in 4E S209-HA cells It would be of interest to determine if these results are replicated in other cell lines overexpressing 4E S209-HA

4.3.3.2 Chaperone Activity

4.3 3 2 1 AHA1

Expression of a protein identified as AHA 1 was increased 1 98-fold in 4E S209-HA overexpressing cells in comparison to pcDNA controls

AHA1 is an activator of the highly expressed heat shock protein Hsp90 and is induced under stressful conditions such as heat shock Hsp90 is an highly conserved and essential stress protein that is present in all eukaryotic cells Despite being a heat shock protein, hsp90 is one of the most abundant proteins in non heat shocked cells (1-2% of cytosolic protein), where it performs housekeeping functions controlling the activity, turnover and trafficking of a variety of proteins Most of the hsp90-regulated proteins that have been discovered are involved in signal transduction (Panaretou *et al*, 2002)
Rather than acting at an early stage of folding, Hsp90 binds client proteins in a substantially folded form and facilitates their association with cofactors or other proteins required for full activity Hsp90-dependent activation of CPs (client proteins) *in vivo* involves a plethora of co-chaperones, which associate with the Hsp90-based complex at different stages of the activation process (Panaretou *et al*, 2002)
The biological activity of Hsp90 depends on its ability to bind and hydrolyze ATP AHA1 stimulates the inherent ATPase cycle of Hsp90, which is essential for its chaperone activity *in vivo* (Panaretou *et al*, 2002)

In addition to its chaperone activity, Hsp90, like the proteasomal activators PA28α and β, is also implicated in protein degradation and MHC class I antigen processing (Yamano *et al*, 2002) Hsp90 directly associates with the 20S proteasome and can

influence the enzyme activity, in a study by Imai *et al* (2003) functional loss of Hsp90 in yeast caused dissociation of the 26S proteasome The dissociated constituents then reassembled in a Hsp90-dependent fashion both *in vivo* and *in vitro* This process was found to require ATP-hydrolysis by Hsp90 (Imai *et al*, 2003) Therefore Hsp90 and its ATP hydrolysis activity which is regulated by AHA1 may play a role in proteasomal activity and assembly Increased expression of AHA1 in 4E S209-HA cells may therefore play a role in regulating proteasomal protein degradation and MHC class I antigen processing As Hsp90 is known to function as a chaperone for signal transducing proteins increased expression of the Hsp90 activating protein AHA1 may therefore contribute to the survival and growth of 4E S209-HA overexpressing cells

4.3.3.3 Increased Protein Degradation and MHC I antigen presentation in 4E S209-HA cells due to defective translation?

A model proposed some years ago suggests that a large proportion of peptides which are processed for antigen presentation on MHC class I molecules are derived from degradation of defective ribosomal products (DRiPs) rather than from the degradation of full length proteins or 'old' proteins that are no longer useful to the cell (Yewdell *et al*, 1996) Subsequent studies have provided evidence to support this theory Production of DRiPs occurs through mistranslation of proteins in various ways including, (a) alternative initiation or pre-termination of translation, (b) through ribosomal slipping in which one part of the peptide is derived from one open reading frame (ORF) and the other half from another ORF and (c) they have also been shown to generated from the 3'UTR both in and out of frame with the main ORF and/or with a leucine as the initiation codon (Fahreaus, 2005) A study by Schubert *et al* (2000) showed that DRiPs constitute upwards of 30% of newly synthesized proteins and also that the maturation of MHC class I molecules is correlated directly with the production of DRiPs and their degradation by proteasomes (Schubert *et al*, 2000) These studies show that there is a link between aberrant protein translation, protein degradation and the MHC I antigen processing pathway

The exact role of 4E S209 phosphorylation in translation initiation is still a matter for debate Studies have shown that 4E S209 phosphorylation decreases the affinity of 4E for the 5' cap structure (Section 1 1 2 2 2) In their review of the role of 4E S209 phosphorylation Scheper and Proud (2002) proposed a number of possible models explaining the role 4E S209 phosphorylation may play in translation initiation It is proposed in these models that 4E and its associated translation factors may remain attached to the 5' end cap structure until phosphorylation of 4E occurs at a certain point in the translation initiation process e g association of the 40S ribosomal subunit or AUG initiation codon recognition The phosphorylation of 4E at this point would allow the release of the translation initiation factors from the cap structure to allow scanning away from the 5' end of the RNA or release of the translation factors for further initiation events Lack of phosphorylation of 4E could therefore disrupt the translation initiation process by preventing the release of translation initiation complexes from the 5' end of mRNA at the correct point in the translation initiation process

As mentioned previously, production of DRiPs occurs through mistranslation of proteins in various ways such as alternative initiation or pre-termination of translation and ribosomal slipping (Fahreaus, 2005) It may be the case that in 4E S209-HA overexpressing cells, the rate of occurrence of events such as these is increased due to the release of translation initiation complexes from 5'end cap structures being inhibited during the initiation process as phosphorylation of 4E fails to occur

Increased expression of PA28 α and β and also UCHL1 in 4E S209-HA cells may occur as a result of increased production of 'DRiPs' due to abnormalities in protein translation caused by the 4E-S209 phosphorylation site being absent from the transfected protein These DRiPs would require increased levels of ubiquitin to direct them to proteasomal degradation and presentation on MHC class I molecules Increased UCHL1 levels may provide the necessary rise in ubiquitin through its ability to prevent degradation of ubiquitin and its reprocessing of ubiquitin from previously ubiquitinated proteins (Liu *et al*, 2002, Osaka *et al*, 2003) Increased levels of PA28 α and β may be necessary to activate proteasomal degradation and processing of DRiPs for presentation on the MHC class I molecules Previous studies

have shown that the maturation of MHC class I molecules is correlated directly with the production of DRiPs and their degradation by proteasomes (Schubert *et al*, 2000) Increased expression of AHA1 may have a role to play in this process as an activator of Hsp90 Hsp90 has been shown to interact with proteasomes and can compensate for loss of PA28 in antigen presentation in certain cellular situations (Yamano *et al*, 2002) Increased expression of AHA1 may therefore assist in proteasomal degradation and antigen presentation through its activation of Hsp90

In summary, increased expression of PA28 α and β, UCHL1 and AHA1 have a common link in proteasomal degradation of proteins and antigen presentation on MHC class I cells This process is considered by many to be directly connected to mistranslation of proteins (DRiPs) The lack of phosphorylation of eIF4E in 4E S209-HA overexpressing cells may therefore be causing an increase in production of DRiPs and a subsequent increase in proteasomal degradation and antigen processing

4.3.3.4 mRNA Processing

4 3 3 4 1 HnRNP F

Expression of hnRNP F was decreased 1 98-fold in 4E S209-HA overexpressing cells in comparison to pcDNA controls HnRNP-F and its decreased expression in 4E S209-HA cells is discussed in Section 4 3 1 2 1

4.3.3 5 Metabolic Proteins

4 3 3 5 1 Purine Nucleoside Phosphorylase

Expression of Purine nucleoside phosphorylase (PNP) was increased 2 19-fold in 4E S209-HA overexpressing cells in comparison to pcDNA controls
PNP is a key enzyme in the purine-salvage pathway, which allows cells to utilize preformed bases and nucleosides in order to synthesize nucleotides (Bzowska *et al*, 2000) Increased levels of PNP have been detected in certain types of cancers (Roberts *et al*, 2004) indicating increased PNP levels may confer a growth advantage to these cells The increased expression of PNP in 4E S209-HA cells may therefore confer a growth advantage on these cells Analysis of the growth rate of 4E S209-HA 6 in comparison to 4E-HA 5 and pcDNA 2 cells showed that the growth rates of both 4E overexpressing cells were increased in comparison to the pcDNA controls (Section 3 1 2 9)

4 3 3 5 2 DDAH1

Expression of DDAH1 was decreased 1 73-fold in 4E S209-HA overexpressing cells in comparison to pcDNA controls
Dimethylarginine dimethylaminohydrolase regulates cellular methylarginine concentrations, which in turn inhibit nitric oxide synthase Nitric oxide is an important signaling molecule and regulator of angiogenesis Factors that regulate nitric oxide synthesis are therefore important targets in the control of tumor progression

In a study by Kostouro *et al* (2004), overexpression of DDAH in a glioma tumor cell line resulted in increased tumor growth They found that increased DDAH expression caused hypoxia in tumors which resulted in activation of angiogenesis Expression of DDAH therefore appears to play a role in regulating tumor oxygenation Decreased expression of DDAH1 in 4E S209-HA overexpressing cells could therefore play a role in regulating the oxygenation of these cells in an *in-vivo* situation although its role *in vitro* may not be significant

4 3 3 5 3 Glyoxalase 1

Expression of Glyoxalase 1 was decreased 1 46-fold in 4E S209-HA overexpressing cells in comparison to pcDNA controls

Glyoxalase 1 is an essential component of the pathway leading to the detoxification of methylglyoxal, a side product of glycolysis Accumulation of methylglyoxal causes DNA modification and protein cross-links and thus initiates the activation of apoptosis (Thornalley, 2003a)

Overexpression of glyoxalase I was found in drug-resistant tumour cells and may be an example of an undesirable effect of the enzymatic protection against DNA glycation (Thornalley, 2003b) Experimental overexpression of glyoxalase I conferred resistance to drug-induced apoptosis Glyoxalase I-mediated drug resistance was found in human leukaemia and lung carcinoma cells (Thornalley, 2003b) Elevated levels of glyoxalase expression have been reported in lung cancer cell lines and glyoxalase activity is associated with resistance to apoptosis inducing anti-cancer agents (Sakamoto *et al*, 2001)

Decreased expression of Glyoxalase 1 in 4E S209-HA cells may therefore increase the susceptibility of these cells to apoptosis induced by anti-cancer drugs

4.3.4 Differentially expressed proteins in a comparison of 4E-HA clone 5 and 4E-HA clone 10 cells

Analysis of differentially expressed proteins between 4E-HA clone 5 and 4E-HA clone 10 cells revealed changes in a number of different classes of proteins (Table 4 3 4)

4E-HA 5 vs 4E-HA 10	
4E-HA 5 High Expression	**4E-HA 5 Low expression**
Cytoskeletal	**Cytoskeletal**
Vimentin, Mutant Beta Actin, Annexin A1, Annexin A2	CapG
mRNA Processing	**Protein Folding**
poly(rC)-binding protein 2	Hsp 70kDa protein 5 (BiP)
Cell Signalling	**Protein Degradation**
Prohibitin	PA28 beta
Metabolism	**Metabolism**
FASN, Glutaminase isoform C, Aldehyde Dehydrogenase 1, IPP isomerase	Peroxiredoxin 2 isoform b,

Table 4 3 4 Proteins differentially expressed between 4E-HA clone 5 and 4E-HA clone 10 cells

4 3 4 1 Prohibitin

Prohibitin expression was 2 13-fold higher in 4E-HA 5 cells compared to 4E-HA 10 cells Recent studies by Rajalingam *et al* (2005), have shown prohibitin plays an important role in the activation of the Ras-Raf signalling pathway and can regulate epithelial cell migration (Rajalingam *et al*, 2005) In this study, knockdown of prohibitin expression eliminated activation of c-Raf by Ras and caused major changes in the morphology, migration and adhesion of epithelial cells (Rajalingam *et al*, 2005) The 4E-HA 5 and 4E-HA 10 cells show different morphological features which correlate with these results The 4E-HA 5 cells show numerous large cells with multiple cellular protrusions which aren't present in the 4E-HA 10 cell population (Section 4 2 1) The high level of invasiveness of 4E-HA 5 cells in comparison to 4E-

HA 10 cells also indicates increased motility of these cells Previous studies have also shown prohibitin to be overexpressed in carcinomas (Asamoto and Cohen, 1994, Wang *et al*, 2004)

4 3 4 2 Vimentin

Two protein spots with identified as vimentin were 3 76-fold (master no 1264) and 3 26-fold (master no 1261) more highly expressed in 4E-HA 5 cells compared to 4E-HA 10 cells

Vimentin is an intermediate filament cytoskeletal protein whose expression is characteristic of cells of mesenchymal origin e g fibroblasts However, evidence has accumulated which shows that atypical expression of vimentin in epithelial cancer cells is associated with invasiveness and metastasis potential for a variety of cancers including hepatocellular carcinoma (Hu *et al*, 2004), prostate carcinoma (Lang *et al*, 2002 Singh *et al*, 2003), breast carcinoma (Hendnx *et al*, 1997) and cervical carcinoma (Gilles *et al*, 1996)

Analysis of vimentin expression in migrating MCF10A breast carcinoma cells using an *in vitro* wound assay model revealed induction of vimentin mRNA and protein in migrating cells (Gilles *et al*, 1999) Reduction of vimentin expression using antisense cDNA caused a reduction in the migration of these cells also (Gilles *et al*, 1999) These studies show that vimentin is strongly associated with cellular motility and invasiveness We have previously shown that 4E-HA 5 cells are highly invasive compared to 4E-HA 10 cells (Section 3 1 2 10) The increased expression of vimentin in 4E-HA 5 cells may therefore contribute to the invasiveness of these cells

Microarray analysis detected an increase in vimentin mRNA expression in 4E-HA 5 and 4E-S209 6 cells in comparison to pcDNA 2 cells (Section 3 1 2 12 2 3) The increase in vimentin mRNA expression in these cells was matched by the vimentin protein levels detected in these cells This suggets that changes in vimentin protein expression are due to transcriptional regulation in these cells

4.3 4 3 CapG

A protein spot identified as the actin regulatory protein CapG (master no 1677) was downregulated 1 86-fold in 4E-HA 5 cells compared to 4E-HA 10 cells
CapG is a member of the gelsolin/villin family of actin-regulatory proteins Unlike other members of this family though, CapG caps the barbed ends of actin filaments, but does not sever them (Mishra *et al*, 1994)
Dynamic shifts in the concentration and length of actin filaments provide the force and structure for nonmuscle cell motility Many actin-binding proteins exist to temporally and spatially regulate actin filament assembly A key site for the regulation of actin filament assembly is the fast growing or barbed actin filament ends In living cells, the number of barbed ends available for the addition of actin monomers is likely to determine where new forces for directional cell movement are generated Proteins capable of blocking exchange at the barbed end can prevent indiscriminate growth of actin filaments and control where new actin filaments are assembled The gelsolin/villin family of actin regulatory proteins, of which CapG is a member can serve this function (Witke et al, 2001) For this reason CapG expression is likely to affect the regulation of cellular motility and invasion Decreased expression of CapG in 4E-HA 5 cells may contribute to the increased invasiveness of these cells by reducing the capping of barbed ends and allowing increased assembly of actin filaments in areas of cellular outgrowth thereby increasing cellular motility Analysis of F-actin structures in these cells using fluorescent labeling and confocal microscopy also revealed strong staining for actin structures in 4E-HA 5 cells which was not detected in 4E-HA 10 cells

Microarray analysis detected a decrease in CapG mRNA expression in 4E-HA 5 cells in comparison to 4E-S209 6 cells and pcDNA 2 cells (Section Section 3 1 2 12 2 3) This indicates that the lower level of CapG protein in 4E-HA 5 cells may be due to transcriptional regulation

4.3.4.4 Mutant Beta Actin

Two protein spots which were identified as mutant beta actin were 1 71-fold (master no 1573) and 1 49-fold (master no 1549) more highly expressed in 4E-HA 5 cells compared to 4E-HA 10 cells This is the same isoform of beta actin as described in section 4 3 1 1 8

The increased expression of this form of beta actin in 4E-HA 5 cells compared to 4E-HA 10 cells may contribute to differences in morphology and invasiveness observed in these cells

4 3 4 5 Annexins

Annexins are a family of closely related calcium and membrane binding proteins expressed in most eukaryotic cell types Annexins are proposed to act as membrane-cytoskeleton and membrane-membrane linkers They undergo Ca^{2+}-dependent binding to phospholipids that are preferentially located on the cytosolic face of the plasma membrane The central biochemical characteristic of annexins is their Ca^{2+}-regulated binding to the periphery of membranes containing acidic phospholipids This could allow them to organize the interface between the cytoplasm (or cytoskeleton) and the cytoplasmic face of cellular membranes (Rescher and Gerke, 2004) Annexins have been implicated in Ca^{2+}-regulated exocytotic events, certain aspects of endocytosis and stabilization of specific domains of organelle membranes and the plasma membrane (Rescher and Gerke, 2004)

4 3 4 5 1 Annexin A1

Expression of annexin A1 was1 58-fold higher in 4E-HA 5 cells compared to 4E-HA 10 cells

Annexin A1 binds to F-actin and also interacts with profilin, a G-actin binding protein and regulator of actin polymerization Complex formation between annexin A1 and profilin modifies the profilin effect on actin polymerization Because of the partially overlapping intracellular localization of the two proteins, it has been speculated that the annexin A1-profilin interaction participates in regulating the membrane-associated cytoskeleton (Gerke and Moss, 2002, Hayes *et al*, 2004)

The role of annexin A1 in membrane interaction with actin structures and regulation of the membrane associated cytoskeleton could contribute to differences in invasiveness, morphology and F-actin staining The 4E-HA 5 cells display increased invasiveness compared to 4E-HA 10 cells which is associated with actin based cellular motility Analysis of F-Actin structures in these cells also showed intense F-actin staining at cell membranes in 4E-HA 5 cells (Section 3 1 2 13)

4 3 4.5 2 Novel Protein Similar to Annexin A2

Annexin A2 (pseudogene 2) protein was increased 1 99-fold in 4E-HA 5 cells compared to 4E-HA 10 cells

Annexin A2 is an F-actin binding annexin that also has a Ca^{2+}-dependent filament bundling activity Annexin A2 protein is associated with actin structures and annexin A2 along with its binding partners can interact directly with F-actin (Rescher and Gerke, 2004, Hayes *et al* , 2004) Annexin A2 is associated with dynamic actin structures In particular, those actin structures associated with cellular membranes during e g phagocytosis, pinocytosis and cell migration, contain annexin A2 and probably require the protein (Hayes *et al* , 2004)

The association of annexin A2 and membrane interaction with dynamic actin structures involved in processes such as migration correlates with its increased expression in 4E-HA 5 cells compared to 4E-HA 10 cells The 4E-HA 5 cells display increased invasiveness compared to 4E-HA 10 cells, which is associated with actin based cellular motility Analysis of F-actin structures in these cells also showed intense F-actin staining at cell membranes in 4E-HA 5 cells (Section 3 1 2 13)

4 3 4 6 FASN

Fatty acid synthase (FAS) protein expression was 2 37-fold higher in 4E-HA 5 cells compared to 4E-HA 10 cells

Tumors overexpressing FAS, the enzyme responsible for de novo synthesis of fatty acids, display aggressive biologic behavior compared to those tumors with normal FAS levels, suggesting that FAS overexpression confers a selective growth advantage (Baron *et al*, 2004)
Once considered largely an anabolic-energy-storage pathway, FAS has become a novel target pathway for chemotherapy development (Francis and Kuhajda, 2000)

The 4E-HA 5 cells which express higher levels of FAS are also more invasive than 4E-HA 10 cells, this correlates with the aggressive behaviour reported for tumors which overexpress this protein

4 3 4 7 Glutaminase isoform C

Glutaminase isoform C protein expression was 1 91-fold higher in 4E-HA 5 cells compared to 4E-HA 10 cells

The mitochondrial enzyme glutaminase catalyzes the hydrolysis of glutamine (Gln) to glutamate and ammonia Through this reaction and the subsequent conversion to α-ketoglutarate, glutamine serves as a major source of tricarboxylic acid cycle intermediates and ultimately provides a large fraction of cellular energy and reducing equivalents In culture, most mammalian cells depend on Gln for their survival and proliferation and tumor cells have been identified as particularly avid consumers of Gln How and why Gln influences cell survival and proliferation and the determinants of Gln utilization rates are not precisely known However, it is believed that the majority of Gln utilization by tumor cells is driven by its enzymatic hydrolysis via glutaminase (Medina, 2001)

Experimental evidence supports the correlation of glutaminase activity with the extent of malignant proliferation Glutaminase reaches a maximum of expression and activity immediately before the maximum proliferation rate (Medina, 2001) In a study by

Lobo *et al* (2000) knockdown of glutaminase expression in a tumor cell line using antisense technology caused major morphological changes in these cells, reduced their growth rates and caused loss of tumorigenicity

The higher expression of glutaminase isoform C in 4E-HA 5 cells correlates with increased glutaminase expression seen in tumorigenic cells The 4E-HA 5 cells are highly invasive compared to 4E-HA 10 cells which indicates these cells are more likely to be tumorigenic Increased glutaminase expression may contribute to the invasive phenotype of these cells

4 3.4.8 Peroxiredoxin 2 isoform b

Peroxiredoxin 2 isoform b protein expression was 1 46-fold higher in 4E-HA 10 cells compared to 4E-HA 5 cells

This gene encodes a member of the peroxiredoxin family of antioxidant enzymes, which reduce hydrogen peroxide and alkyl hydroperoxides The encoded protein is thought to play an antioxidant protective role in cells (Shen and Nathan, 2002)

The lower level of peroxiredoxon 2 isoform b protein in 4E-HA 5 cells compared to 4E-HA 10 cells may therefore render these cells more susceptible to oxidant injury

4 3 4 9 poly(rC)-binding protein 2 (PCBP2), (hnRNP E2)

poly(rC)-binding protein 2 expression was 1 50-fold higher in 4E-HA 5 cells compared to 4E-HA 10 cells

Poly(C)-binding proteins (PCBPs) constitute a family of nucleic acid-binding proteins that play important roles in a wide spectrum of regulatory mechanisms The diverse functions of PCBPs are dependent on the ability of the PCBPs to recognize poly(C) sequences with high affinity and specificity PCBPs contain three copies of KH (hnRNP K homology) domains, which are responsible for binding nucleic acids Interaction of PCBPs with the UTRs of mRNAs have been shown to regulate the stability of these molecules and can also regulate their translational activation and repression (Waggoner and Liebhaber, 2003, Du *et al*, 2004) Waggoner and Liebhaber identified one hundred and sixty mRNAs which interact with poly(rC)-binding protein 2 indicating a role for this protein in post-transcriptional regulation of the expression of a wide number of genes (Waggoner and Liebhaber, 2003) This

study also showed that poly(rC)-binding protein 2 protein interacted with its own mRNA, which suggests autoregulatory control of its gene expression (Waggoner and Liebhaber, 2003)

The higher expression level of poly(rC)-binding protein 2 in 4E-HA 5 cells may affect post-transcriptional/translational control of gene expression in these cells

4 3 4 10 Hsp 70kDa protein 5 (BiP/GRP78)

Hsp 70kDa protein 5 (BiP) expression was 1 50-fold higher in 4E-HA 10 cells compared to 4E-HA 5 cells

BiP/GRP78 functions as an endoplasmic reticulum protein chaperone and aids in the production of properly folded proteins and protein complexes and prevents the accumulation of misfolded proteins and protein aggregation BiP/GRP78 is also a major regulator of the stress response in the endoplasmic reticulum GRP78 binds to all three ER stress sensors (PERK, IRE 1, ATF 6) through its peptide binding domains and keeps them in an inactive conformation The peptide binding domain also serves as the binding region for misfolded proteins When misfolded proteins accumulate in the cell, they bind to GRP78 and disrupt its interaction with these proximal stress sensors While free IRE 1 and PERK homodimerise and undergo autophosphorylation and activation, ATF6 transits to the Golgi for proteolytic activation (Rao and Bredesen, 2004) Increased expression of GRP78 is induced by the ATF 6 transcription factor in response to cell stress (Rao and Bredesen, 2004)

The higher level of expression of GRP78/BiP in 4E-HA 10 cells in comparison to 4E-HA 5 cells may occur as a result of cell stress response in these cells There may also be higher constitutive level of GRP78/BiP in these cells which may aid in protein production in the endoplasmic reticulum

4.4 Microarray analysis of eIF4E overexpressing cells

Microarray analysis was performed on eIF4E overexpressing cells to determine if translational changes induced by eIF4E overexpression were causing a downstream effect on gene transcription The 4E-HA clone 5, 4E S209-HA clone 6 and pcDNA 2 cells were analysed in triplicate using affymetrix HG-U133A genechips which contain oligonucleotide probesets for 14,500 of the best characterised human genes The results from this experiment were analysed using Genespring statistical analysis software Using Genespring software we selected genes that showed a two-fold up or down regulation between the different transfected DLKP clones tested The genes selected using these parameters underwent further statistical analysis for selection of significantly changed genes Genes that passed this statistical analysis were then clustered into groups which showed similar expression levels in the three cell lines tested (Figure 3 1 2 12 5) The genes represented in these clusters were then analysed and genes of interest were categorised according to cellular function

The overexpression of 4E-HA and 4E S209-HA resulted in altered transcriptional regulation of large numbers of genes involved various cellular processes This indicates that changes that occur as a result of eIF4E-mediated translational regulation of protein expression have a knock-on effect on the transcriptional regulation of certain genes It would be of interest to determine if the changes in transcriptional regulation of gene expression seen in these cells are replicated in other lung carcinoma cell lines and cells from other tissues upon overexpression of eIF4E

4.4.1 Actin cytoskeleton-related genes

A large number of genes known to interact with and regulate the actin cytoskeleton were found to be differentially regulated in 4E-HA 5 and 4E S209-HA 6 cells compared to pcDNA 2 controls (Table 3 1 2 12 3) This corresponds with differences in F-actin staining seen in 4E-HA 5 and 4E S209-HA 6 cells compared to pcDNA 2 control cells when analysed with fluorescently labelled phalloidin (Section 3 1 2 13) The 4E-HA 5 cells exhibited actin structures such as lammelipodia and filopodia associated with migratory cells which were not present in 4E S209-HA 6 and pcDNA 2 cells Regulation of actin cytoskeletal dynamics is known to play an important role in normal and pathological cell motility (Lambrechts *et al*, 2004) The changes seen in the actin cytoskeleton of 4E-HA 5 and 4E S209-HA 6 cells are likely to play a role in regulating the invasiveness of these cells The effects of eIF4E and translational control in regulating the actin cytoskeleton in invasive and metastatic cancer should be an interesting area for future studies A number of these genes are discussed below

4.4 1 1 TNNT1 (Troponin T)

Microarray analysis showed TNNT1 mRNA expression was down regulated in 4E-HA 5 and 4E S209-HA 6 cells compared to pcDNA 2 control cells (Section 3 1 2 12 2 3)

The asymmetric extended comma-shaped molecule of troponin T provides contacts between troponin components, tropomyosin and actin The C-terminal globular domain of troponin T interacts with tropomyosin (in the vicinity of Cys-190), troponin components and actin (Filatov *et al*, 1999) This molecule therefore plays a role in regulating the organisation of the actin cytoskeleton It is likely that decreased expression of TNNT1 in 4E-HA 5 and 4E S209-HA 6 cells may contribute to the organisation of actin structures in these cells

4 4 1 2 CAPG

Microarray analysis showed CAPG mRNA expression was down-regulated in 4E-HA 5 cells compared to 4E S209-HA 6 and pcDNA 2 control cells (Section 3 1 2 12 2 3)

Dynamic shifts in the concentration and length of actin filaments provide the force and structure for nonmuscle cell motility Many actin-binding proteins exist to temporally and spatially regulate actin filament assembly A key site for the regulation of actin filament assembly is the fast growing or barbed actin filament ends In living cells the number of barbed ends available for the addition of actin monomers is likely to determine where new forces for directional cell movement are generated Proteins capable of blocking exchange at the barbed end can prevent indiscriminate growth of actin filaments and control where new actin filaments are assembled The gelsolin/villin family of actin regulatory proteins, of which CapG is a member can serve this function (Witke et al, 2001)

The decreased expression of CAPG in 4E-HA 5 cells may therefore contribute to the expression of F-actin structures in these cells Decreased CAPG expression may cause dysregulation of actin filament assembly in 4E-HA 5 cells and therefore contribute to increased motility and invasiveness

4 4 1 3 MARCKS

Microarray analysis showed MARCKS mRNA expression was increased in 4E-HA 5 (11 88-fold) and 4E S209-HA 6 (5 23-fold) cells compared to pcDNA 2 control cells (Section 3 1 2 12 2 3)

The protein encoded by this gene is a substrate for protein kinase C It is localized to the plasma membrane and is an actin filament crosslinking protein Phosphorylation by protein kinase C or binding to calcium-calmodulin inhibits its association with actin and with the plasma membrane, leading to its presence in the cytoplasm The protein is thought to be involved in cell motility, phagocytosis, membrane trafficking and mitogenesis (Disatnick *et al*, 2004, Stumpo *et al*, 1989)

MARCKS mRNA expression was increased 11 88-fold in 4E-HA 5 cells, the increased expression of this gene product may contribute to expression of actin structures in these cells and contribute to increased invasiveness

4 4 1 4 ADD2 (Adducin 2, beta)

Microarray analysis showed Adducin mRNA expression was decreased in 4E S209-HA 6 cells compared to 4E-HA 5 and pcDNA 2 control cells (Section 3 1 2 12 2 3) Adducins are heteromeric proteins composed of different subunits referred to as adducin alpha, beta and gamma The three subunits are encoded by distinct genes and belong to a family of membrane skeletal proteins involved in the assembly of spectrin-actin network in erythrocytes and at sites of cell-cell contact in epithelial tissues Adducin forms ternary complexes between spectrin and actin and promotes association of spectrin with actin filaments Adducin exhibits the highest affinity for complexes between spectrin and the fast-growing ends of actin filaments (Bennet and Baines, 2001, Matsuoka *et al* , 2000)

The relative activities of adducin for actin filament ends and sides in the presence and absence of spectrin suggest that the preferred role of adducin in cells is to form a complex with the fast-growing ends of actin filaments that recruits spectrin and prevents addition or loss of actin subunits Adducin thus is an actin-capping protein that recruits other proteins to actin filament ends and could represent a new class of assembly factor with the function of integrating actin into other cell structures (Bennet and Baines, 2001)

Recent observations suggest a role for adducin in cell motility and as a target for regulation by Rho-dependent and Ca^{2+}-dependent pathways Prominent physiological sites of regulation of adducin include dendritic spines of hippocampal neurons, platelets and growth cones of axons (Matsuoka *et al* , 2000)

Decreased expression of ADD2 in 4E S209-HA 6 cells may play a role in the organisation of actin cytoskeletal structures in these cells

4.4.2 Integrin signalling/focal adhesion proteins/FAK related genes

Microarray analysis revealed the differential expression of a number of integrin signalling/focal adhesion proteins/FAK related genes (Table 4 4 2)

	pcDNA 2	4E S209-HA 6	4E-HA 5
PTK2 (FAK)	1 (207 5)	0 51 (105 9)	0 378 (78 4)
TGFB1I1 (Hic-5)	1 (68 93)	0 76 (52 33)	0 447 (30 80)
EPHA3	1 (2 267)	189 15 (428 8)	181 78 (412 1)
NTS	1 (95 7)	34 66 (3316 8)	38 84 (3716 73)
CASP	1 (51 23)	1 943 (99 53)	1 45 (74 23)
NEDD9 (HEF-1)	1 (88 57)	0 32 (28 67)	1 20 (106 6)

Table 4 4 2 Microarray analysis of expression levels of FAK related genes Normalised and raw (in brackets) expression values are given for these genes

4 4 2 1 FAK

Microarray analysis showed decreased expression of FAK mRNA in 4E-HA 5 and 4E-S209-HA 6 cells compared to pcDNA 2 control cells (Table 3 1 2 12 2 5) Western blot analysis of FAK protein expression also shows decreased expression of FAK protein in 4E-HA 5 and 4E S209-HA 6 cells in comparison to DLKP and pcDNA 2 cells (Fig 3 1 2 14 1) This result matches the decreased expression level of FAK mRNA seen in these cells from microarray analysis and therefore provides verification for that result

Immunofluorescent analysis of FAK expression in stably transfected 4E-HA overexpressing clones showed a dramatic alteration in the level and localisation of FAK protein within these cells In pcDNA control transfected DLKP cells, strong FAK immunofluorescence was distributed in patches evenly throughout the cell (Fig 3 1 2 14 2) In 4E-HA 5 cells FAK immunflourescence was decreased overall and showed localisation to areas of cellular outgrowth at the edges of cells (Fig 3 1 2 14 2) The 4E S209-HA 6 cells also showed an overall decrease in FAK immunofluorescence and localisation at the edge of colonies (Fig 3 1 2 14 2) The

decrease in FAK expression in eIF4E overexpressing cells was likely due to decreased transcription as microarray analysis showed decreased FAK mRNA expression in 4E-HA 5 and 4E S209-HA 6 cells compared to pcDNA 2 cells (Table 3 1 2 12 2 5) Focal adhesion kinase (FAK) plays a prominent role in integrin signalling FAK activation, demonstrated by an increase in phosphorylation of Tyr397 as well as other sites in the protein, is best understood in the context of the engagement of integrins at the cell surface Activation of FAK results in recruitment of a number of SH2-domain and SH3-domain containing proteins, which mediate signalling to several downstream pathways FAK-dependent activation of these pathways has been implicated in a diverse array of cellular processes including cell migration, invasion, growth factor signalling, cell cycle progression and cell survival (Parsons, 2003, Schlaepfer *et al*, 2004) The 4E-HA 5 cells show increased *in vitro* invasiveness It is possible FAK expression and localisation may be an important factor in this result It will be important in future studies to determine the activation status of FAK within these cells using phosphospecific antibodies It is possible that increased expression of β1 integrin in 4E-HA 5 cells may result in increased FAK activation and play a role in the increased invasiveness of these cells Analysis of FAK in A549 lung carcinoma cells revealed that inhibition of FAK expression or function resulted in decreased MMP-9 secretion and the inhibition of *in vitro* A549 lung adenocarcinoma cell invasion through reconstituted basement membrane (Huack *et al*, 2001) FAK tyrosine phosphorylation and the co-localisation of v-Src with FAK and β1 integrin at invadopodia cell projections was shown to promote cell invasion in fibroblasts (Huack *et al*, 2002) A study of squamous cell carcinoma cells showed that recruitment of FAK and paxillin to β1 integrin promoted cancer cell migration and invasion via the mitogen activated protein kinase pathway (Crowe and Ohannessian, 2004) These and other studies suggest that β1 integrin and FAK are important regulators of cellular migration and invasion

The results for FAK expression show that the level of FAK expression and its localisation are altered in 4E-HA 5 overexpressing cells compared to pcDNA 2 control cells The importance of FAK in regulation of cell signalling and cellular behaviour would suggest this may have a major effect on the phenotype of these cells Further analysis of FAK activation using phospho-specific antibodies should allow us to gain further insights into the role of FAK in these cells

4.4.2 2 Hic-5 (TGFB1I1) and HEF 1 (NEDD9)

Hic 5 (TGFB1I1) and HEF 1 (NEDD9) are proteins homologous to paxillin and p130Cas respectively, which are two of the most important proteins known to interact with FAK in focal adhesion complexes Paxillin is considered to be important for the localisation of FAK to focal adhesions and signalling to FAK from β-integrin cytoplasmic domains via its interaction with talin Upon activation, FAK combines with Src family kinases, which then phosphorylate paxillin and p130Cas Both of these molecules serve as scaffolds for the recruitment of various adaptors and signalling intermediates (Giancotti, 1999)

Microarray analysis showed Hic-5 mRNA expression was down regulated in 4E-HA 5 cells compared to pcDNA 2 and 4E S209-HA 6 cells (Section 3 1 2 12 2 5) Hic-5 is a paxillin homologue localised to focal adhesion complexes but has distinct functional features from paxillin Unlike paxilhn, Hic-5 is a negative regulator of cell growth Hic-5 inhibits cell spreading via competition with paxillin for FAK and subsequent prevention of downstream signalling transduction Expression of antisense Hic-5 is shown to increase cell spreading It is hypothesised that Hic-5 could compete for common interaction factors with paxillin and antagonise the signal pathways that involve Paxillin This competitive effect between paxillin and Hic-5 through interaction with FAK suggests that the counterbalance of paxillin and Hic-5 expression may be a novel mechanism regulating integrin mediated signal transduction and the resultant cytoskeletal reorganisation (Nishiya *et al* , 2001, Yuminamochi *et al* , 2003) Decreased levels of this protein in 4E-HA 5 cells may have an important effect on regulation of FAK signalling and interactions It may also play a role in the localisation of FAK protein in these cells

Microarray analysis showed NEDD9/HEF1 mRNA expression is downregulated in 4E S209-HA 6 compared to 4E-HA 5 cells and pcDNA 2 cells HEF1 (NEDD9) possesses a similar protein sequence and domain structure to the prototypical member of the Cas family p130Cas, with both proteins containing an ammo terminal SH3 domain, multiple potential SH2 binding sites in the central substrate domain and a

carboxy terminal dimerisation molecule Ligation of β1 integrins in hematopoietic or lymphocytic cells causes tyrosine phosphorylation of HEF1 HEF1 is a substrate for several tyrosine kinases including FAK, RAFTK, and Src family members In adherent cells, HEF1 localises to focal adhesions where it may modulate adherence based signalling Studies suggest that phosphorylated HEF1 can function as a downstream effector of FAK to promote integrin-dependent cell motility (O'Neill *et al*, 2000, Zhenge and McKeown-Longo, 2002) Decreased HEF1 expression in 4E S209-HA 6 cells is likely to have an important effect on regulation of FAK signalling

4 4 2 3 EphA3

Microarray analysis showed that mRNA levels for the receptor tyrosine kinase EphA3 was highly upregulated in both 4E-HA 5 (428 8-fold) and 4E S209-HA 6 (412 1-fold) cells compared to pcDNA 2 control cells (Section 3 1 2 12 2 5) The cytoplasmic domain of this class of EphA receptors is known to interact with FAK (Murai and Pasquale, 2003) and therefore may play a role in the localisation and activation of FAK in these cells

4 4 2 4 Neurotensin

Microarray analysis showed that mRNA levels for the neuropeptide Neurotensin was also highly upregulated in both 4E-HA 5 (34 66-fold) and 4E S209-HA 6 (38 84-fold) cells compared to pcDNA 2 control cells (Section 3 1 2 12 2 5) Neurotensin is a neuropeptide which alters the growth of cancers cells and stimulates growth and colony formation of small cell lung cancer cells (Leyton *et al*, 2002, Sethi and Rozengurt, 1991) A study by Leyton *et al* (2002) showed that treatment of the large cell lung carcinoma cell line NCI-H1299 with neurotensin caused tyrosine phosphorylation of focal adhesion kinase This effect was inhibited by treatment of the cells with the neurotensin receptor antagonist SR48692 These studies suggest that increased expression of neurotensin in 4E-HA 5 and 4E S209-HA 6 cells may increase activation of FAK signalling in these cells

4 4 2 5 Casp (Calpastatin)

Microarray analysis showed that mRNA levels for the calpain inhibitor calpastatin were upregulated in 4E S209-HA 6 cells and also at a lower level 4E-HA 5 cells compared to pcDNA 2 control cells (Section 3 1 2 12 2 5)

The Calpains are a well-conserved family of intracellular calcium dependent cysteine proteases *In vivo*, calpain activity is tightly regulated by its highly specific endogenous inhibitor **calpastatin** Calpains cleave numerous cellular proteins, including components of focal adhesions, cell-cycle regulatory proteins and proteins involved in the regulation of cell survival Thus, calpain activity is implicated in a variety of cellular processes, including migration, proliferation and apoptosis (Perrin and Huttenlocker, 2002, Potter et al, 1998)

Treatment of migrating cell with pharmacological inhibitors of calpain activity results in impaired retraction of the rear of the cell, an increase in tail length and suppression of cell movement (Huttenlocher *et al* 1997) Calpains are known to localise at focal adhesion structures and distinct integrin clusters and several focal adhesion components such as FAK, Src, paxillin, talin and β-Integrin subunits are substrates for calpain Calpain-mediated proteolysis has therefore been proposed as a mechanism for promoting disassembly of focal adhesion structures, leading to the turnover of integrin-dependent cell matrix adhesions that is needed for cell movement (Glading *et al*, 2002, Pfaff *et al*, 1999) Spreading and cell motility require calpain degradation of focal adhesions at attachment sites at both the leading and rear edges of cells (Glading *et al*, 2002, Perrin and Huttenlocker, 2002)

Overexpression of calpastatin in NIH-3T3-derived clonal cells impairs the ability of these cells to extent lammelipodia, reduces by 90% the ability of the cells to spread and results in an increase in ezrin content (suggesting that calpastatin overexpression prevents normal degradation of this calpain-sensitive substrate) (Potter *et al*, 1998) Increased expression of calpastatin in 4E-S209-HA 6 cells may therefore cause disruption of focal adhesion disassembly by calpain and therefore affect FAK localisation and cellular morphology and motility

4.4.3 Genes related to neuron guidance, tissue morphogenesis, cellular migration

Recent studies have shown that many proteins that have originally been identified in the nervous system as guidance cues for axons are also involved in regulating tissue morphogenesis, cellular migration and invasion (Hinck, 2004, Tamagnone and Comoglio, 2004) Microarray analysis of 4E-HA 5 and 4E S209-HA 6 cells in comparison to pcDNA 2 control cells identified a number of differentially regulated genes which fall into this category (Table 4 4 3)

	pcDNA 2	4E S209-HA 6	4E-HA 5
ROBO1	1 (181 4)	0 513 (93 17)	0 388 (70 63)
CRMP1	1 (152 9)	0 204 (31 17)	0 259 (39 63)
SLITRK3	1 (6 433)	15 42 (99 2)	20 301 (130 6)
EPHA3	1 (2 267)	189 15 (428 8)	181 78 (412 1)
SEMA3A	1 (33 17)	2 351 (77 97)	3 120 (103 5)
SEMA3C	1 (54 27)	2 04 (110 7)	1 623 (88 1)
DPYSL4 (CRMP3)	1 (61 03)	0 215 (13 1)	0 452 (27 57)

Table 4 4 3 Expression levels of diffentially expressed genes related to neuron guidance, tissue morphogenesis, cellular migration and invasion Normalised and raw (in brackets) expression values are given for these genes

4 4 3 1 Semaphorins, CRMPs

Microarray analysis showed that mRNA levels for the semaphorin family axon guidance genes SEMA3A and SEMA3C were upregulated in 4E-HA 5 and 4E S209-HA 6 cells compared to pcDNA 2 control cells (Section 3 1 2 12 2 6) Messenger RNA levels for the SEMA3A signalling gene CRMP1 and its homolog DPYSL4 (CRMP3) were downregulated in 4E-HA 5 and 4E S209-HA 6 cells compared to pcDNA 2 control cells (Section 3 1 2 12 2 6)

The semaphorins are among the best studied axonal guidance molecules and appear to play key roles in these cellular events To date, more than 20 different semaphorins have been identified They fall into eight groups on the basis of domain organisation

and species of origin but all semaphorins contain a conserved, 500-amino-acid-length "Sema" domain at their amino-terminus

Sema3A, a prototypical class 3 secreted semaphorin, is a potent inhibitor of axonal outgrowth from a specific subset of neurons, including spinal motorneurons and neurons in the embryonic dorsal root ganglion (DRG) and sympathetic ganglion The binding of Sema3A to DRG growth cones rapidly induces actin depolymerisation and growth cone collapse, a cellular response associated with the chemorepulsion of neurites However, semaphorins can provide both repulsive and attractive cues to cells In cortical neurons Sema3A, provides a repulsive signal whereas Sema3C acts as an attractive guidance signal (Goshima *et al*, 2002)

In lung, Sema3A inhibits branching morphogenesis of the fetal mouse lung whereas Sema3C and Sema3F activate branching morphogenesis (Goshima *et al*, 2002, Kagoshima *et al*, 2001) The mhibitory effect of Sema3A on cell growth seen in lung branching morphogenesis may be mediated via CRMP1 CRMP1 has been shown in other cell types to function downstream of SEMA3A in cell growth inhibition/repulsion (Deo *et al*, 2004) Loss of CRMP1 expression is also associated with increased invasiveness of lung cancer cells (Shih *et al*, 2001), this may be due to these cells failing to respond to growth inhibition/repulsion signals from Sema3A as a result of low CRMP1 levels Downregulation of CRMP1 may therefore prevent negative regulation of growth/invasion by semaphorins (Sema3A) and allow positive regulation of growth/invasion by semaphorins (Sema3C) to dominate

The collapsing-response-mediator-protein (CRMP) family consists of five members and is best described in the regulation of axonal-growth cone collapse CRMP1, CRMP2, CRMP3 and CRMP4 family members are approximately 75% identical in protein sequence CRMP5 (also known as CRAM or CRMP3 associated molecule) shares a 50% identity with other CRMPs (Deo *et al*, 2004) CRMPs appear to play a complex role in axon growth as well as microtubule dynamics and axon induction CRMPs localise to the lamellipodia and filopodia of axonal groth cones, suggesting a role in axon guidance (Deo *et al*, 2004)

A cDNA microarray study by Shih *et al* (2001) examining differential gene expression among a panel of lung carcinoma cell lines of varying invasive abilities

identified the gene CRMP-1 as being relatively highly expressed in less invasive lung cancer cell lines. Transfection of CRMP-1 into a highly invasive cell line reduced invasion through a matrigel coated membrane by approximately half (Shih *et al.*, 2001). The CRMP-1 mRNA levels of 80 non-small-cell lung carcinoma tumors were also determined and it was found that patients with high CRMP-1-expressing tumors exhibited statistically significantly longer disease-free and overall survival (Shih *et al.*, 2001). A low expression of CRMP-1 mRNA in lung cancer tissue was significantly associated with advanced disease, lymph node metastasis, early post-operative relapse and shorter survival (Shih *et al.*, 2003).
CRMP3 (DPYSL4) is structurally and functionally homologous to CRMP1 and may therefore also play a similar role in regulating the invasiveness of lung cancer cells (Deo *et al.*, 2004; Shih *et al.*, 2003).

4.4.3.2 ROBO1

Microarray analysis showed that mRNA levels for the axon guidance genes ROBO1 was downregulated in 4E-HA 5 and 4E S209-HA 6 cells compared to pcDNA 2 control cells (Section 3.1.2.12.2.6).
Another family of axonal guidance cue genes which are expressed in the lung are Slit ligands and their Robo receptors (Anselmo *et al.*, 2003; Xian *et al.*, 2001).
The *DUTT1/ROBO1* gene is widely expressed in mammals and codes for a receptor with a domain structure of the NCAM family. Members of the Slit family are likely to be the ligands for mammalian Robo. Slit proteins have been expressed in lung at levels equal to or greater than in adult rat brain (Xian *et al.*, 2001).

In a study by Xian *et al.* (2001) a deleted form of the Robo1 gene, which mimics a naturally occurring, lung tumor-associated human homozygous deletion of exon 2 of DUTT1/ROBO1, was introduced into the mouse germ line. Mice homozygous for this targeted mutation, which eliminates the first Ig domain of Dutt1/Robo1, frequently died at birth of respiratory failure because of delayed lung maturation. Lungs from these mice have reduced air spaces and increased mesenchyme, features that are present some days before birth. Survivors acquire extensive bronchial epithelial abnormalities including hyperplasia. This study shows that ROBO1 plays an important part in regulating lung cell growth and development (Xian *et al.*, 2001).

Analysis of Robo1 gene expression in murine lung development shows its expression correlates with brachial infiltration into the surrounding mesenchyme (Bonner *et al*, 2003, Anselmo *et al*, 2003)

The ROBO1 gene is located on an area of chromosome 3 where allele loss is associated with lung tumor development (Xian *et al*, 2001)

These studies show that the axon guidance molecule ROBO1 plays a role in normal lung development and also lung cancer cell growth The decreased expression of ROBO1 in 4E-HA 5 and 4E S209-HA 6 cells may therefore play a role in regulating the growth and invasiveness of these cells

4 4.3 3 Slitrk3

Microarray analysis showed that mRNA levels for the axon guidance gene SLITRK3 was upregulated in 4E-HA 5 and 4E S209-HA 6 cells compared to pcDNA 2 control cells (Section 3 1 2 12 2 6)

The Slitrk family was identified as neuronal transmembrane proteins that control neurite outgrowth When overexpressed in neuronal cells, induction of a single neurite or inhibition of neurite outgrowth was observed, depending on the Slitrk subtype Structurally, they are characterized by two leucine-rich repeat (LRR) domains located amino-terminally to the transmembrane domain (on exterior of the cell) LRR domains are known to be present in many proteins and mediate protein to protein interaction The LRR domains in the Slitrk family proteins are most similar to those of the Slit family, which are known to control axon guidance and branching Another structural feature is carboxy-terminally located tyrosine residues which are flanked by amino acid sequences similar to the carboxy-terminal domain of trk neurotrophin receptor (Aruga *et al*, 2003)

Increased expression of SLITRK3 in 4E-HA 5 and 4E S209-HA 6 cells may therefore play a role in regulating the growth and invasiveness of these cells

4.4 3 4 EphA3

Microarray analysis showed that mRNA levels for the receptor tyrosine kinase EphA3 was highly upregulated in both 4E-HA 5 (428 8-fold) and 4E S209-HA 6 (412 1-fold) cells compared to pcDNA 2 control cells (Section 3 1 2 12 2 6)

The Eph family of receptor tyrosine kinases regulate the behaviour of various cell types by binding membrane anchored ligands, ephrins, at sites of cell-cell contact (Pasquale, 2005) Signalling through Eph receptor tyrosine kinases is involved in regulating axon guidance, cell adhesion and cell migration during development and disease (Pasquale, 2005) Increased expression of EphA3 in 4E-HA 5 and 4E S209-HA 6 cells may therefore play a role in regulating the growth and invasiveness of these cells

4.4 Invasion related genes

Analysis of the *in vitro* invasiveness of 4E-HA 5, 4E S209-HA 6 and pcDNA 2 cells showed differences in the invasiveness of these cells (Section 3 1 2 10) We therefore decided to identify differentially expressed genes from microarray analysis which were specifically related in cellular invasiveness (Table 4 4)

	pcDNA 2	4E S209-HA 6	4E-HA 5
TACSTD1	1 (18 2)	9 654 (175 7)	1 746 (31 77)
TPBG	1 (88 57)	0 324 (28 67)	1 204 (106 6)
MMP10	1 (681 2)	0 423 (288 4)	0 878 (597 9)
TFPI2*	1 (298 65)	0 006 (1 8665)	0 427 (127 615)

Table 4 4 Expression levels of diffentially expressed invasion related genes Normalised and raw (in brackets) expression values are given for these genes

4 4 4 1 TACSTD1, tumor-associated calcium signal transducer 1

TACSTD1 mRNA expression was upregulated in 4E S209-HA 6 cells compared to 4E-HA 5 and pcDNA 2 cells A recent study by Lader *et al* (2004) analysing the gene expression profile of 22 non-small cell lung cancer cell lines and their invasiveness through Matrigel identified TACSTD1 (Ep-CAM) among the two genes with the highest inverse association with invasion (Lader *et al*, 2004) Increased TACSTD1 expression may therefore inhibit invasiveness in 4E S209-HA 6 cells

4 4 4 2 TPBG, trophoblast glycoprotein

TPBG mRNA expression was decreased in 4E S209-HA 6 cells compared to 4E-HA 5 and pcDNA 2 cells There was also an increase in TPBG mRNA expression in 4E-HA 5 cells compared to pcDNA 2 cells

The human 5T4 oncotrophoblast glycoprotein was discovered by looking for shared surface molecules which would reflect the functional similarities between the growth and invasive properties of trophoblast, the major interfacing cell type between mother and foetus in the placenta and tumour cells It is expressed by many different carcinomas but is detected at only low levels in some normal epithelia

The 5T4 trophoblast glycoprotein is overexpressed in a number of different tumour types, notably ovarian, gastric and colorectal and is associated with poorer clinical outcome This association suggests a role for 5T4 in the progression of malignancy

(Woods et al, 2002, King *et al*, 1999) These studies suggest that decreased TPBG expression in 4E S209-HA 6 cells may cause a decrease the invasiveness of these cells and increased expression 4E-HA 5 cells may increase the invasiveness of those cells

4 4 4.3 MMP10, matrix metalloproteinase 10

MMP10 expression was decreased in 4E S209-HA 6 cells compared to 4E-HA 5 and pcDNA 2 cells

The serine protease MMP10 has a broad range of substrates including the ECM components, laminin, fibronectin and non-fibrillar collagens MMP-10 is also considered to play a role as an activator of other MMPs, converting MMP1, MMP8, MMP7 and MMP9 from their pro-MMP form to their active MMP form (Nakamura *et al*, 1998)

A number of recent studies implicate MMP10 expression in lung cancer growth and metastasis A study of the expression and significance of matrix metalloproteinases in lung carcinomas showed strong overall levels of expression of the stromelysins MMP-3 and MMP-10 in lung adenocarcinomas (Bodey *et al*, 2001)

An Indian study on MMP-10 expression in human esophogal squamous cell carcinomas (ESCCs) detected a large proportion (74%) of human ESCCs with MMP-10 overexpression in tumor cell cytoplasm and stromal elements (Mathew *et al*, 2002) MMP-10 overexpression was significantly associated with tumor size, local invasiveness of the tumor and distant organ metastasis, suggestive of its involvement in development and progression of ESCCs (Mathew *et al*, 2002)

Cho *et al* (2004) compared MMP expression in recurring stage I lung cancer to non recurring stage I lung cancer using cDNA arrays, and found MMP10 to be the most frequently upregulated gene in recurring lung cancers This result was validated by quantitative PCR and real time RT-PCR analysis Immunohistochemical analysis of MMP-10 protein expression showed more intense immunoreactivity in recurred stage IB lung cancer than in nonrecurred stage IB lung cancer This study suggests MMP-10 plays an important role in the recurrence of stage IB lung cancer, irrespective of the histological type (Cho *et al*, 2004)

These studies suggest that decreased expression of MMP10 in 4E S209-HA 6 cells may cause a decrease in the mvasiveness of these cells

4 4 4 4 TFPI2

Microarray analysis showed TFPI2 mRNA expression was absent from 4E S209-HA 6 cells and was also down regulated in 4E-HA 5 cells compared to pcDNA 2 control cells (Section 3 1 2 12 2)

TFPI2 plays a role in the invasion of cancer cells through the extracellular matrix (ECM) It is a serine protease inhibitor secreted into the ECM whose expression is often lost in cells derived from tumors of diverse organs TFPI2 inhibits plasmin, trypsin, chymotrypsin, cathepsin, and plasma kallikrein

The role of TFPI2 in cancer progression is not completely elucidated On one hand, TFPI2 has an anti-invasive effect that might be mediated via inhibition of plasmin that activates proteases (e g MMPs), promoting degradation of the extracellular matrix and tumor invasion Several tumor cell lines were less invasive when they were stably transfected with TFPI2 cDNA (Chand *et al*, 2004, Kondun et al, 2001) On the other hand, TFPI-2 has been shown to have a pro-invasive effect in hepatocellular carcinoma cells (Neaud *et al*, 2000) The A549 lung cancer cell line displayed increased invasiveness, as measured by Matrigel invasion assay, when TFPI2 levels were decreased by transfection with antisense mRNA (Lakka *et al*, 2000)

TFPI2 expression is normally associated with inhibition of invasiveness in cancer cells although exceptions are seen in the literature Our array results show pcDNA 2 control transfected cells have the highest level of TFPI2 expression with 4E-HA 5 cells having approximately half the expression level of pcDNA 2 controls and TFPI2 expression absent from 4E S209-HA 6 transfected cells Decreased TFPI2 expression in 4E-HA 5 cells may therefore play a role in the increased invasiveness of these cells Although TFPI2 expression is absent from 4E S209-HA 6 cells they do not display increased invasiveness, this may be due to other factors which regulating invasiveness in these cells

4.4.5 Tumor suppressor genes

Genes known to function as tumor suppressor genes were shown to be highly represented in the group of genes which showed increased expression in 4E S209-HA 6 cells (Cluster 5) compared to 4E-HA 5 and pcDNA 2 control cells (Fig 3 1 2 23) (Section 3 1 2 12 2) The transcriptional upregulation of tumor suppressor genes seen in 4E S209-HA overexpressing cells may indicate that translational changes that occur as a result of decreased eIF4E phosphorylation have a knock on effect on transcriptional regulation of these genes This may have important implications for the role of eIF4E phosphorylation in tumorigenic growth regulation If decreased eIF4E phosphorylation can upregulate tumor suppressor gene expression, inhibitors of the Mnk eIF4E kinases could be used to treat cancers with the aim of increasing the transcription of these genes Further studies of tumor suppressor gene expression in 4E S209-HA overexpressing cells or treatment of cells with eIF4E kinase inhibitors should reveal if this is a general effect or is specific to the cells examined in this study

4.4 6 Transcription factor genes

A number of transcription factors were found to be among genes differentially regulated in 4E-HA 5 and 4E S209-HA 6 cells compared to pcDNA 2 cells (Section 3 1 2 12 2 4) A number of these have previously been shown to play important roles in regulation of lung cell growth and development and are discussed below

4 4 6.1 FOXA1

Microarray analysis showed that mRNA levels for the transcription factor FOXA1 was upregulated in both 4E-HA 5 and 4E S209-HA 6 cells compared to pcDNA 2 control cells (Section 3 1 2 12 2 4)

FOXA1 (HNF3α) belongs to the hepatocyte nuclear factor 3 (HNF3) gene family, which includes HNF3β (FOXA2) and HNF3γ (FOXA3) HNF3α encodes a polypeptide of 473 amino acids in humans The HNF3 genes are members of the forkhead class of DNA-binding proteins, all of which contain a highly conserved 110-amino acid forkhead motif, a variant of the helix-turn-helix motif, first identified in the Drosophila gene fork head (fkh) HNF3α is expressed in embryonic endoderm and adult tissues of endodermal origin including stomach, intestines, liver, and lung The FOXA1/HNF3α gene is amplified and overexpressed in esophageal and lung adenocarcinomas and may have an oncogenic role in these cells (Lin *et al*, 2002) The increased expression of FOXA1 in 4E-HA 5 and 4E S209-HA 6 cells may therefore affect the carcinogenic growth (invasiveness) of these cells

4 4 6 2 NFIB

Microarray analysis showed that the mRNA level for the transcription factor NFIB was decreased in 4E-HA 5 cells compared to 4E S209-HA 6 and pcDNA 2 control cells (Section 3 1 2 12 2 4)

The transcription factor Nuclear factor I-B (NFIB) plays an important role in lung development Lung development is severely impaired in NFIB null mice, causing these mice to die early after birth due to respiratory failure (Grunder *et al*, 2002) As NFIB is known to affect the growth of lung cells during development the decreased

expression of this gene in 4E-HA 5 cells may contribute to the growth characteristics of these cells

4 4 6 3 Etv5

Microarray analysis shows the transcription factor Etv5 (Erm) TO BE downregulated in 4E S209-HA 6 cells and upregulated in 4E-HA 5 cells compared to pcDNA 2 control cells

The transcription factor Etv5 (Erm) is a member of the Pea3 subfamily of Ets transcription factors The Pea3 subfamily includes three members Pea3, Etv5 (Erm), and Er81 (Etv1) All three have a 72-amino-acid N-terminal transcription activation domain, an 85 amino acid winged helix-turn-helix ETS DNA binding domain and a short C-terminal domain They recognise similar DNA sequences flanking the core GGAA/T binding sequence and act as transcriptional activators (Liu *et al*, 2003) The subfamily has been implicated in various cellular processes including proliferation, differentiation and tumorigenesis Members are overexpressed in oncogene-induced mouse mammary tumours and expression of an inhibitor form of Pea 3 reduces the size and the number of tumors (Liu *et al*, 2003)

Expression of a suppressor form of Erm (EngR-Erm) under the control of the promoter of the lung specific gene SpC in mice results in a severe disruption of lung development In addition to defects in branching morphogenesis, the differentiation of distal cell types is inhibited or delayed (Liu *et al*, 2003)

Conserved PEA3 elements that bind members of ETS transcription factors have also been found in all inducible MMP promoters, with the exception of the MMP-12 promoter, they are located adjacent to at least one AP-1 element (Westermarck and Kahari, 1999)

The decreased expression of Etv5 in 4E S209-HA 6 cells corresponds to decreased expression of the matrix metalloproteinase MMP10 in these cells also detected by microarray analysis (Section 3 1 2 12 2 1) The promoter region of the MMP10 gene is known to contain conserved PEA3 elements Etv5 is a member of the PEA3 subfamily of ETS transcription factors and may therefore be responsible for the transcriptional regulation of MMP10 seen here

4.4.7 Possible Connections between transcriptional changes identified in microarray analysis and localised translation.

4 4 7 1 Focal Adhesion Complexes, Src and translational regulation

Microarray analysis has identified a number of focal adhesion and related genes whose expression was differentially regulated in 4E-HA and 4E S209-HA overexpressing cells compared to pcDNA controls These results indicate that signalling via integrins and focal adhesions are likely to be altered within these cells A number of recent studies have indicated that focal adhesion proteins and focal adhesion sites may play a role in the regulation of translational control Src is a well known proto-oncogene which plays a central role in focal adhesion signalling (See section 1 3 1 1) Src mediated phosphorylation of the translational silencing protein hnRNPK is known to reversibly inhibit the binding of hnRNPK to the RNA regulatory element which this protein binds thereby allowing translational activation of mRNAs containing this regulatory element (Ostareck-Lederer *et al*, 2002)
Another recent article by Karni *et al* (2005) revealed that active Src elevates levels of β-catenin by enhancing cap-dependent translation In this study Src was shown to induce phosphorylation of eIF4E and its repressor protein 4E-BP (Karni *et al*, 2005)
Treatment of a number of cell lines with the Src inhibitor PP1 dramatically reduced the phosphorylation of eIF4E on serine 209 (Karni *et al*, 2005)
A study by Hoog *et al* (2004) used specialized mass spectrometry methods to identify proteins interacting with focal adhesion proteins Numerous RNA binding and ribosomal proteins were identified including hnRNP K (Hoog *et al*, 2004)
Subsequent confocal microcopic analysis of spreading cells showed hnRNP K protein localized to focal adhesion associated sites which were termed spreading initiation centers (SICs) (Hoog *et al*, 2004) These results would indicate that Focal Adhesion Complex sites or SICs may be areas where hnRNP K may localize for translational activation of bound mRNAs
These studies therefore show a connection between translational activation and focal adhesion complexes

4.4 7 2 Axon guidance cues and translational regulation

One of the best characterised cellular situations where localised mRNA translation/ protein synthesis is known to occur is neuronal axon growth cone response to growth guidance cues (Piper and Holt, 2004) Studies have shown that axons separated from their cell bodies have the ability to respond to growth guidance cues (Campbell and Holt, 2001) Axons which were separated from their cell bodies and treated with the guidance cue Sema3a showed a rapid increase in protein synthesis Axonal growth cones contained an abundance of ribosomal proteins, capped-RNA and translation initiation factors (eIF4E, 4E-BP and Mnk-1) and treatment with Sema3a and other guidance cues rapidly (5 min) induced the phosphorylation of eIF4E, 4E-BP and Mnk-1 (Campbell and Holt, 2001, 2003) A recent study by Wu *et al* (2005) has also shown that Sema3a induces axonal translation of RhoA mRNA Localisation of RhoA transcripts was found to be mediated by an axonal targeting element located in the mRNA 3'UTR (Wu *et al*, 2005) Localised axonal translation of the EphA2 receptor protein has also been reported and is also regulated by elements within the mRNA 3'UTR (Brittas *et al*, 2002) These results show that localised translational control of protein synthesis may play an important role in regulating axonal growth and migration Many of the factors which regulate axonal growth cone guidance have also been found to play similar roles in regulating cellular growth and migration in organ morphogenesis and cell migration in non-neuronal cells (Hinck, 2004) It may be the case therefore that the translational control mechanisms seen in the neuronal axon are replicated in non-neuronal cells (e g lung)

4.5 F-Actin expression in eIF4E overexpressing DLKP cells

The expression of F-actin was analysed in 4E overexpressing cells using fluorescently labelled phalloidin, which specifically labels F-actin

The pcDNA control transfected cells and 4E-S209-HA mutant overexpressing cells showed diffuse staining distributed throughout the cell (Section 3 1 2 13) In contrast, wild type 4E-HA overexpresssing cells showed intense filamentous F-actin staining and the presence of F-actin structures such as lammelipodia and filopodia, which are associated with cell migration (Section 3 1 2 13) The altered expression of F-actin in wild type 4E overexpressing DLKP may be partially induced by the altered expression of β1 integrin and FAK in these cells The cytoplasmic domain of β1 integrin is known to bind directly to actin binding proteins and can also signal to proteins involved in actin cytoskeletal remodelling such as RhoGTPases via FAK

Microarray analysis identified a large number of genes involved in regulation of the actin cytoskeleton that were differentially regulated in 4E-HA overexpressmg cells (Section 3 1 2 12 2 3)

Microarray analysis also identified a number of genes that are associated with invasiveness in cancers A number of these genes are best characterised for their role in guidance of neuronal axon growth (e g CRMP1, Sema3a) and are also known to play a role in lung morphogenesis These genes are known to induce changes in the actin cytoskeleton thereby altering the growth and migration of target cells (Section 4 4 3)

The altered expression of β1 integrin and FAK proteins (Section 3 1 2 7, 3 1 2 14) and also actin related gene expression seen by microarray analysis (Section 3 1 2 12 2 3) would suggest that altered regulation of actin dynamics is one of the most significant aspects of the changes seen in 4E-HA overexpressmg cells

Proteomic analysis also revealed changes in the levels of proteins involved in actin cytoskeletal regulation and a model for the translational regulation of these genes has beeen proposed (Section 4 3 1 1 10)

These changes are likely to be one of the major factors contributing to the altered morphology, growth and mvasiveness of these cells

4.6 Analysis of BrdU treated DLKP cells for ODC, C/EBPβ, and CBP expression

The work presented in this thesis is derived from the analysis of gene expression in BrdU treated DLKP cells Treatment of DLKP cells with BrdU resulted in the post-transcriptional regulation of a number of growth and differentiation related genes This correlated with increased expression and phosphorylation of the translation initiation factor eIF4E, suggesting translational regulation of gene expression in these cells In this thesis, the translationally regulated proteins ODC and C/EBPβ were examined in eIF4E overexpressing DLKP cells to assess the role of translational regulation in the regulation of growth and differentiation related gene expression These proteins had not previously been examined in BrdU treated cells, so their expression in BrdU treated cells was analysed to determine if their expression was changed

4 6 1 ODC expression in BrdU treated DLKP cells

Polyamines are ubiquitous cellular components that are involved in normal and neoplastic growth Polyamine biosynthesis is tightly regulated in mammalian cells by the activities of ornithme-decarboxylase (ODC) and S-adenosyl methionone decarboxylase ODC is a rate limiting enzyme for polyamine biosynthesis and is recognised as a proto-oncogene (Shantz and Pegg,1999) Overexpression of ODC causes transformation of NIH3T3 cells ODC mRNA contains a lengthy GC rich 5'UTR, rendering it poorly translated Overexpression of eIF4E in NIH 3T3 cells has been shown to increase ODC protein levels and depletion of eIF4E using anti-sense eIF4E suppressed ODC mRNA translation in eIF4E overexpressing cells (Mamame *et al*, 2004, Shantz and Pegg,1999)

Immunocytochemical analysis of expression of ODC in BrdU treated cells showed increased expression in these cells (Fig 3 2 1) Previous studies in this laboratory have shown that treatment of DLKP cells with BrdU induced increased expression of eIF4E As increased eIF4E levels lead to increased expression of ODC, the increased expression of eIF4E may account for the increase observed Increased production of

polyamines as a result of increased ODC levels may cause further changes in gene expression and regulation of growth

4.6.2 C/EBPα and C/EBPβ expression in BrdU treated DLKP cells

In this thesis we have analysed the expression of the transcription factors C/EBPα and C/EBPβ These transcription factors are known to be translationally regulated (Calkhoven *et al*, 2000) and also play a role in regulating the expression of differentiation related genes in the lung (Cassel and Nord, 2003) Immunocytochemical and Western blot analysis of C/EBPα expression shows that this protein is not present in either untreated or BrdU treated cells (Fig 3 3 1, 3 3 2) Low expression of C/EBPα may be significant as previous studies suggest C/EBPα is down regulated in a large proportion of lung cancers and that it has growth inhibitory properties in airway epithelial cells (Halmos *et al*, 2002) C/EBPα-deficient mice show hyperproliferation of type II pneumocytes and disturbed alveleolar architecture indicating its role in normal lung development (Flodby *et al*, 1996)

Immunocytochemical analysis of C/EBPβ expression in untreated DLKP cells shows strong cytoplasmic staining for C/EBPβ Treatment of DLKP cells with BrdU caused a shift in staining from predominantly cytoplasmic staining in untreated cells to nuclear staining in BrdU treated DLKP cells (Fig 3 3 3) C/EBPβ has previously been shown to be subject to regulation by nucleo-cytoplasmic transport (Ramji and Foka, 2002) Western blot analysis of C/EBPβ expression showed an overall decrease in C/EBPβ protein levels in BrdU treated cells (Fig 3 3 4)

4.6.3 CBP expression in BrdU treated DLKP cells

CREB-binding protein (CBP) proteins are known to play a role in the expression of a number of lung differentiation related genes and are known to interact with a wide range of transcription factors CBP and p300 are transcriptional co-activator proteins that play a central role in co-ordinating and integrating multiple signal dependent events with the transcriptional apparatus, allowing the appropriate level of gene activity to occur in response to different physiological cues that influence, for example, proliferation, differentiation and apoptosis(Goodman and Smolik, 2001)

The transcription regulating properties of p300 and CBP appear to be exerted through multiple mechanisms They act as protein bridges, thereby connecting different sequence-specific transcription factors to the transcription apparatus Providing a protein scaffold upon which to build a multicomponent transcriptional regulatory complex is likely to be an important feature of p300/CBP control Another key property is the presence of histone acetyltransferase (HAT) activity, which endows p300/CBP with the capacity to influence chromatin activity by influencing nucleosomal histones Other proteins, including YY1 and c-Myc are also subject to regulation through acetylation by p300 and CBP (Chan and La Thangue, 2001, Yao *et al*, 2001, Vervoorts *et al*, 2003) p300 has also been reported to interact with the transcription factor C/EBPβ which results in activation of C/EBPβ and also triggers phosphorylation of p300 (Mink *et al*, 1997, Schwartz *et al*, 2003)

CBP has been found to be involved in the transcriptional regulation of the lung differentiation specific genes Surfactant Protein A (SP-A) and Surfactant Protein (SP-B) (Naltner *et al*, 2000, Yi *et al*, 2002) CBP was found to stimulate the SP-B promoter synergistically with the transcription factors TTF-1 and RAR in the H441 pulmonary adenocarcinoma cells (Naltner *et al*, 2000) CBP, TTF-1 and SRC-1 synergistically activated SP-A promoter activity in A549 lung adenocarcinoma cells (Yi *et al*, 2002)

Immunocytochemical analysis of CBP and p300 in developing mouse lung showed nuclear staining for both CBP and p300 in almost all cell types at various stages of

lung development indicating a role for CBP and p300 as general transcriptional co-activators in this organ (Naltner *et al*, 2000)

Immunocytochemical analysis of CBP and p300 expression in untreated DLKP cells showed strong expression of CBP and low expression of p300 (Fig 3 4 1) Western blot analysis showed a decrease in the level of CBP protein expression in BrdU treated cells (Fig 3 4 2) As CBP and P300 are known to have a wide ranging effect on transcriptional regulation the decreased expression of CBP in BrdU treated DLKP cells may affect the transcriptional regulation of a large number of genes

4.7 Analysis of Gene expression in DLKP cells grown in Serum Free Media treated with Hormone Supplemented Media

As an initial part of our study into regulation of gene expression in growth and differentiation of DLKP cells we decided to analyse the expression of growth and differentiation related genes after treatment of DLKP cells with a cocktail of hormones and growth factors known to be involved in regulating growth and development in normal lung Cells were grown in serum free media in order to prevent interference from growth factors and hormones present in serum supplemented media and allow increased reproducibility

Unfortunately, no major changes were observed in the expression of genes analysed upon treatment of DLKP cells grown in serum free media with hormone supplemented media and these studies were discontinued

5.0 Conclusions

The main focus of this thesis was analysing the effects of overexpression of the translation initiation factor eIF4E on regulation of gene expression, growth and functional characteristics in the poorly differentiated lung carcinoma cell line DLKP Previous studies in this lab had shown increased expression and phosphorylation of eIF4E to be associated with post-transcriptional regulation of gene expression in DLKP cells treated with the differentiation modulating agent BrdU
The following conclusions were made as a result of our research

- Stably transfected DLKP cells overexpressing high levels of 4E-HA (4E-HA 5) showed increased expression of the epithelial marker Keratin 8 Keratin 8 expression wasn't detected in 4E S209-HA overexpressing cells and 4E-HA overexpressing DLKP cells (4E-HA 10) Immunocytochemical analysis of Keratin 8 expression in transiently transfected DLKP cells show hetereogenous staining for Keratin 8 This matches heterogenous staining for eIF4E protein seen in these cells indicating that Keratin 8 may be upregulated in cells expressing high levels of 4E protein These results show that eIF4E overexpression may cause increased Keratin 8 expression if expressed at a sufficiently high level in DLKP cells The level of Keratin staining seen in 4E-HA overexpressing cells is not at the level seen in BrdU treated cells This indicates other factors may cause increased Keratin 8 expression in BrdU treated DLKP cells though increased 4E expression may be a contributing factor Immunocytochemical analysis of Keratin 18 and 19 expression in stably transfected 4E-HA and 4E S209-HA cells showed no increase in expression of these cells indicating 4E alone does not control expression of these proteins

- Immunocytochemical analysis showed increased β1 mtegrin expression in DLKP cells overexpressing high levels of 4E-HA (4E-HA 5) Moderately 4E-HA overexpressing cells (4E-HA 10) and 4E S209-HA overexpressing cells showed no increase in β1 integrin expression These results suggest that high levels of eIF4E expression can result in increased β1 integrin expression in these cells Immunocytochemical analysis of α subunit integrin expression showed increased expression of α3 expression both 4E-HA overexpressing

clones No increases in other α integrin proteins were detected in 4E S209-HA overexpressing cells

- The polyamine metabolism regulating protein Ornithine Decarboxylase (ODC) was upregulated in 4E-HA and 4E-S209 HA overexpressing clones This protein is known to be translationally regulated and previous studies have shown upregulation of ODC in 4E overexpressing cell lines The increased expression of ODC in 4E S209-HA overexpressing cells indicates that phosphorylation of 4E may not be necessary for increased translational regulation of ODC expression Microarray analysis detected no increase in ODC mRNA expression suggesting that post-transcriptional regulation is responsible for this increase

- The highly 4E-HA overexpressing DLKP clone 4E-HA 5 displayed increased invasiveness through reconstituted basement membrane compared to pcDNA controls and 4E-S209-HA cells 4E-HA clone 10 which expresses a lower level of transfected 4E-HA did not show increased invasiveness 4E S209-HA overexpressing cells also did not display increased invasiveness compared to pcDNA control transfected cells This would suggest that increased 4E expression can result in increased invasiveness in DLKP cells although 4E expression needs to be at a sufficiently high level for this to occur The effect of the mutation of the 4E S209 phosphorylation site in 4E S209-HA overexpressing cells on invasiveness cannot be deduced from this experiment as the level of transfected protein was not at the same level as the highly expressing, invasive 4E-HA transfected cell line We therefore cannot tell whether the lack of increased invasiveness is due to the mutation of the S209 phosphorylation site or the lower level of transfected 4E S209-HA protein We can deduce that the mutation of the 4E phosphorylation site did not cause a reduction in invasiveness below the level seen in pcDNA control cells and therefore it would appear that the introduction of non-phosphorylatable 4E does not have an inhibitory effect on invasiveness
- Proteomics analysis using the 2D-DIGE system revealed a number of proteins of various functional categories which were differentially expressed in 4E-HA

and 4E S209-HA overexpressing DLKP cells compared to pcDNA control cells. Of particular interest were a number of proteins involved in regulating cytoskeletal dynamics whose expression was down regulated in 4E S209-HA overexpressing cells. A common regulatory element in the mRNA of these proteins was identified which led to the development of a hypothesis for localised translation of these proteins. The possible involvement of eIF4E in regulation of localised translation of these proteins represents a novel aspect of translational regulation by eIF4E which may contribute to its role in oncogenesis.

- 4E S209-HA cells also showed upregulation of proteins involved in proteasomal degradation pathways which suggests possible disruption of protein translation and processing in these cells.
- A smaller number of proteins than expected were found to be upregulated in wild type 4E-HA overexpressing cells. Of the proteins upregulated in 4E overexpressing cells, the main protein of interest identified is the RasGAP-associated endoribonuclease G3BP, which showed a 1.86 fold increase in expression compared to pcDNA control cells. Recent studies have shown overexpression of this protein induces the formation of cellular bodies known as stress granules. Stress granules are described as cytoplasmic foci at which untranslated mRNAs accumulate in cells subjected to environmental stress. Stress granules have been shown to contain poly-A mRNA complexed with eIF4E and other translation initiation factors. Upregulation of G3BP in 4E-HA overexpressing cells may represent a mechanism whereby translation initiation is kept under control by sequestering eIF4E-bound mRNAs in stress granules and also by possible activation of mRNA degradation.
- 2D DIGE proteomic analysis was also used to detect differentially-expressed proteins between the two 4E-HA-overexpressing clones used in this experiment. As 4E-HA expression and invasiveness in 4E-HA 5 cells was at a higher level than 4E-HA 10 cells we hoped to identify proteins that are involved in regulating invasiveness and also may be affected by increased 4E-HA expression. A number of differentially-expressed proteins were identified which correlated with the invasive profiles of these cells and also may have been subject to translational regulation by increased 4E-HA expression.

- Oligonucleotide microarray analysis of the mRNA expression levels of genes in eIF4E overexpressing cells was conducted to determine the effects of eIF4E overexpression on transcriptional regulation downstream of its effects on translation regulation Microarray analysis showed changes in the expression of a large number of genes with diverse cellular functions This indicates changes in transcriptional regulation occur as a result of eIF4E overexpression The highly overexpressing 4E-HA clone 4E-HA 5 and 4E S209-HA overexpressing clone 6 were analysed in comparison to pcDNA 2 control cells A large number of differentially expressed genes were identified which function in pathways regulating cellular morphology and migration including cytoskeletal genes, focal adhesion/integrin signalling genes, invasion regulation genes and neuron guidance cue genes

- Microarray analysis showed a large number of genes were differentially expressed at the mRNA level in the 4E-HA and 4E S209-HA overexpressing cells examined One of the main areas of interest in our studies was the expression of integrins in our cell lines and the possible effects of integrin expression in regulating growth and differentiation The Focal Adhesion Kinase (FAK) gene which plays a central role in integrin signalling, was found to be down-regulated in the 4E-HA and 4E S209-HA overexpressing clones analysed Western blot analysis confirmed that protein expression of this gene was down-regulated Immunoflourescent confocal microscope analysis also showed decreased expression and altered localisation of FAK in these cells FAK is considered to play a central role in integrin signalling and is an important regulator of cellular morphology, growth, motility and invasion This result also provides verification of results from microarray analysis

- Both proteomic and oligonucleotide microarray analysis of gene expression identified changes in the expression a large number of genes involved in regulating actin cytoskeletal dynamics in 4E-HA and 4E S209-HA overexpressing cells It was therefore decided to analyse the actin cytoskeleton

in these cells using flourescently labelled phalloidin which binds specifically to filamentous (F) actin structures 4E-HA 5 cells which express high levels of transfected 4E-HA showed a dramatic change in F-actin compared to 4E S209-HA and pcDNA control cells 4E-HA 5 cells showed intense staining for actin structures such as stress fibres, lammelopodia and filopodia Regulation of F-actin structures is intimately linked to cell morphology, motility and invasiveness 4E-HA 5 cells exhibit a high level of invasiveness and the high intensity staining seen for F-actin structures associated with cellular motility would indicate F-actin regulation may be playing a significant role in mediating the invasive properties of these cells These results also contribute to the hypothesis for localised translation of actin cytoskeletal genes mRNAs developed from our proteomic analysis

- Western blot analysis of C/EBPβ expression in BrdU treated DLKP cells showed decreased expression of C/EBPβ in BrdU treated cells Immunocytochemical analysis of C/EBPβ expression showed increased nuclear localisation of C/EBPβ in BrdU treated DLKP cells
- Expression of the transcriptional co-activator CBP is reduced in BrdU treated DLKP cells

6.0 Future work

1 Future work comparing the effects of wild type eIF4E overexpression vs S209 phosphorylation site mutant eIF4E overexpression should involve samples more closely matched in the expression level of transfected eIF4E protein The wild type and S209 mutant eIF4E overexpressing DLKP clones selected for examination in a large part of this thesis did not all express the same level of transfected eIF4E protein Further work involving transient transfections, or analysis of more evenly matched clones, should reinforce previous observations or determine if differing levels of eIF4E overexpression affect previous observations

2 Further examination of the role of eIF4E expression and eIF4E phosphorylation in regulating the invasiveness of DLKP cells could be carried out using transiently transfected cells and other eIF4E overexpressing clones eIF4E overexpression in other non-invasive or low level-invasive lung carcinoma cell lines could be examined to examine if eIF4E affects the invasiveness of lung carcinoma cells in a similar manner to DLKP

3 Integrin expression is known to regulate the ability of cells to adhere to extracellular matrix components and also affects the migratory and invasive capabilities of cells The effect of increased integrin expression in eIF4E overexpressing cells could be examined further by using blocking antibodies to assess if blockage of integrin binding reduces adhesion or invasion in these cells

4 Overexpression of wild type eIF4E caused changes in the organisation of the actin cytoskeleton in DLKP cells The effect of eIF4E overexpression on the actin cytoskeleton of other cell lines could be examined to determine if this is a general effect of eIF4E overexpression The actin cytoskeleton is known to be important for generating motile force in migrating cells The abilty of eIF4E overexpressing cells to migrate could also be assessed by in vitro wound assays

5 The expression and localisation of the integrin-signalling protein FAK was altered in eIF4E overexpressing cells FAK expression could be analysed further with phosphospecific antibodies which bind to the activated form of this protein It would also be interesting to analyse if FAK is co-localised in cells with β1 integrin Microarray analysis also identified other focal adhesion proteins which were differentially expressed in 4E-HA and 4E-S209-HA overexpressing cells and in our hypothesis for localised translation the focal adhesion protein Src is involved in translational activation It would therefore be of interest to further analyse the expression of focal adhesion proteins in these cells

6 Microarray analysis has shown changes in the mRNA levels of a large number of genes in eIF4E overexpressing cells The regulation of the expression of these genes by eIF4E overexpression could be examined further and the effects of these genes on the phenotype of these cells could be examined by altering the expression levels of the corresponding protein using RNAi

7 Actively translating mRNAs are usually associated with multiple ribosomes and form large structures called poyribosomes or polysomes mRNA associated with polysomes can be separated from translationally inactive mRNAs by sucrose gradient centrifugation These two pools of mRNA can then be analysed by microarrays to determine what mRNAs are being actively translated This analytical method could be employed to analyse the translational status of mRNAs in eIF4E overexpressing cells

8 We have proposed a hypothesis where cytoskeletal regulatory mRNAs are localised and translated in areas of cellular outgrowth Analysis of proteins and mRNAs present in areas of cellular outgrowth (pseudopodia) can be achieved through a procedure for purification of pseudopodia which separates pseudopodial projections which extend through a porous polycarbonate membrane (similar to invasion assay) from the cell body The proteins and mRNA profile of harvested pseudopodia can then be analysed by a variety of methods

7.0 Bibliography

Adhikary, S and Eilers, M (2005) Transcriptional regulation and transformation by Myc proteins *Nat Rev Mol Cell Biol*, 6, (8), 635-645

Anselmo, M A , Dalvin, S , Prodhan, P , Komatsuzaki, K , Aidlen, J T , Schnitzer, J J , Wu, J Y and Kinane, T B (2003) Slit and robo expression patterns in lung development *Gene Expr Patterns*, 3, (1), 13-19

Aranda-Abreu, G E , Hernandez, M E , Soto, A , Manzo, J (2005) Possible Cis-acting signal that could be involved in the localization of different mRNAs in neuronal axons *Theor Biol Med Model*, 2, 33

Aruga, J , Yokota, N and Mikoshiba, K (2003) Human SLITRK family genes genomic organisation and expression profiling in normal brain and brain tumor tissue *Gene*, 315, 87-94

Asamoto, M and Cohen, S M (1994) Prohibitin gene is overexpressed but not mutated in rat bladder carcinomas and cell lines *Cancer Lett*, 83, 201–207

Barazi, H O , Zhou, L , Templeton, N S , Krutzsch, H C , Roberts, D D (2002) Identification of heat shock protein 60 as a molecular mediator of alpha 3 beta 1 integrin activation *Cancer Res*, 62, (5), 1541-1548

Baron, A , Migita, T , Tang , D and Loda, M (2004) Fatty acid synthase a metabolic oncogene in prostate cancer? *J Cell Biochem*, 91, (1), 47-53

Bauer, C , Diesinger, I , Brass, N , Steinhart, H , Iro, H and Meese, E U (2001) Translation initiation factor eIF-4G is immunogenic, overexpressed, and amplified in patients with squamous cell lung carcinoma *Cancer*, 92, (4), 822-829

Bauer, C , Brass, N , Diesinger, I , Kayser, K , Grasser, F A and Meese, E U (2002) Overexpression of the eukaryotic translation initiation factor 4G (eIF4G-1) in squamous cell lung carcinoma *Int J Cancer*, 98, (2), 181-185

Bennett, V and Baines, A J (2001) Spectrin and ankyrin-based pathways metazoan inventions for integrating cells into tissues *Physiol Rev* , 81, (3), 1353-1392

Beugnet, A , Wang, X and Proud, C G (2003) The TOR-signaling and RAIP motifs play distinct roles in the mTOR-dependent phosphorylation of initiation factor 4E-binding protein 1 *J Biol Chem* , 278, (42), 40717-40722

Bishop, A E (2004) Pulmonary epithelial stem cells *Cell Prolif*, 37, 89-96

Bodey, B , Bodey, B Jr , Groger, A M , Siegel, S E and Kaiser, H E (2001) Invasion and metastasis the expression and significance of matrix metalloproteinases in carcinomas of the lung *In Vivo*, 15, (2), 175-180

Bomsztyk, K , Denisenko, O and Ostrowski, J (2004) hnRNP K one protein multiple processes *Bioessays*, 26, (6), 629-638

Bonner, A E , Lemon, W J and You, M (2003) Gene expression signatures identify novel regulatory pathways during murine lung development implications for lung tumorigenesis *J Med Genet* , 40, 408-417

Brakebusch, C and Fassler, R (2003) The integrin-actin connection, an eternal love affair *EMBO J* , 22, (10), 2324-33

Bretscher, A , Chambers, D , Nguyen R and Reczek, D (2000) ERM-Merlin and EBP50 protein families in plasma membrane organization and function *Annu Rev Cell Dev Biol* , 16, 113-143

Brittis, P A , Lu, Q and Flanagan, J G (2002) Axonal protein synthesis provides a mechanism for localized regulation at an intermediate target *Cell*, 110, (2), 223-35

Brunn, G J , Hudson, C C , Sekulic, A , Williams, J M , Hosoi, H , Houghton, P J , Lawrence, J C and Abraham, R T (1997) Phosphorylation of the translation repressor PHAS-I by the mammalian target of rapamycin *Science*, 277, 99-101

Bu, X , Haas, D W and Hagedorn, C H (1993) Novel phosphorylation sites of eukaryotic initiation factor-4F and evidence that phosphorylation stabilizes interactions of the p25 and p220 subunits *J Biol Chem* , 268, (7), 4975-4978

Buttery, R C , Rintoul, R C and Sethi, T (2004) Small cell lung cancer the importance of the extracellular matrix *Int J Biochem Cell Biol* , 36, 1154-1160

Bzowska, A , Kulikowska, E and Shugar, D (2000) Purine Nucleoside Phosphorylases properties functions and clinical aspects *Pharm Ther* , 88, 349-425

Calkhoven, C F , Muller, C and Leutz, A (2000) Translational control of C/EBPα and C/EBPβ isoform expression *Genes Dev* , 14, 1920-1932

Campbell, D S and Holt, C E (2001) Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation *Neuron*, 32, (6), 1013-1026

Campbell, D S and Holt, C E (2003) Apoptotic pathway and MAPKs differentially regulate chemotropic responses of retinal growth cones *Neuron*, 37, (6), 939-952

Cassel, T N , Moller, L N , Andersson, O , Gustafsson, J A and Nord, M (2000) C/EBPα and C/EBPδ Activate the Clara Cell Secretory Protein Gene through Interaction with Two Adjacent C/EBP-Binding Sites *Am J Respir Cell Mol Biol* , 22, 469-480

Cassel, T N , Nord, M (2003) C/EBP transcription factors in the lung epithelium *Am J Physiol Lung Cell Mol Physiol* , 285, 773-781

Castro, C M , Yang, Y , Zhang, Z , Linnoila, R I (2000) Attenuation of pulmonary neuroendocrine differentiation in mice lacking Clara cell secretory protein *Lab Invest* , 80, (10), 1533-1540

Chakrabarti, A and Maitra, U (1992) Release and recycling of eukaryotic initiation factor 2 in the formation of an 80 S ribosomal polypeptide chain initiation complex *J Biol Chem* , **267**, (18), 12964-72

Chan, H M and La Thangue, N B (2001) p300/CBP proteins HATs for transcriptional bridges and scaffolds *J Cell Sci* , 114, 2363-2373

Chand, H S , Du, X , Ma, D , Inzunza, H D , Kamei, S , Foster, D , Brodie, S and Kisiel, W (2004) The effect of human tissue factor pathway inhibitor-2 on the growth and metastasis of fibrosarcoma tumors in athymic mice *Blood*, 103, (3), 1069-77

Chen, G , Gharib, T G , Huang, C C , Taylor J M G , Misek, D E , Kardi, S , Giordano, T J , Iannettoni, M D , Orringer, M B , Hanash, S M and Beer, D G (2002) Discordant Protein and mRNA Expression in Lung Adenocarcinomas *Mol Cell Proteom* , 1, (4), 304-313

Chen, G , Gharib, T G , Thomas, D G , Huang, C C , Misek, D E , Kuick, R D , Giordano, T J , Iannettoni, M D , Orringer, M B , Hanash, S M and Beer, D G (2003) Proteomic Analysis of eIF-5A in Lung Adenocarcinomas *Proteomics*, 3, 496-504

Cho, N H , Hong, K P , Hong, S H , Kang, S , Chung, K Y AND Cho, S H (2004) MMP expression profiling in recurred stage IB lung cancer *Oncogene*, 23, (3), 845-851

Choi, K M , McMahon, L P and Lawrence, J C Jr (2003) Two motifs in the translational repressor PHAS-I are required for efficient phosphorylation by mTOR and for recognition by raptor *J Biol Chem* , 278, (22), 19667-19673

Choma, D P , Pumiglia, K and DiPersio, C M (2004) Integrin alpha3beta1 directs the stabilization of a polarized lamellipodium in epithelial cells through activation of Rac1 *J Cell Sci* , 117, (17), 3947-3959

Clement, A , Campus, J , Farmer, S R and Brody, J S (1990) Constitutive expression of growth related mRNAs in proliferating and nonproliferating lung epithelial cells in primary culture Evidence for growth-dependent translational control *Proc Natl Acad Sci USA*, 87, 318-322

Clemens M J and Bommer, U A (1999) Translational control the cancer connection *Int J Biochem Cell Biol* , 31, 1-23

Coraux, C , Delplanque, A , Hinnrasky, J , Peault, B , Puchelle, E and Gallard, D (1998) Distribution of Integrins During Human Fetal Lung Development *J Histochem Cytochem* , 46, (7), 803-810

Condeelis, J , Singer, R H (2005) How and why does beta-actin mRNA target? *Biol Cell*, 97, (1), 97-110

Coraux, C , Zahm, J -M , Puchelle, E and Gaillard, D (2000) β1-Integrins are involved in migration of human fetal tracheal epithelial cells and tubular morphogenesis *Am J Physiol Lung Cell Mol Physiol* , 279, 224-234

Crowe, D L and Ohannesian, A (2004) Recruitment of focal adhesion kinase and paxillin to β1 integrin promotes cancer cell migration via mitogen activated protein kinase activation *BMC Cancer*, 4, 18

Cuesta, R , Xi, Q and Schneider, R J (2000) Adenovirus specific translation by selective disassembly of cap-initiation complex *EMBO J* , 19, 3465-3474

Culjkovic B, Topisirovic I, Skrabanek L, Ruiz-Gutierrez M, Borden KL (2005). eIF4E promotes nuclear export of cyclin D1 mRNAs via an element in the 3'UTR. *J. Cell. Biol.*, 169 (2), 245-256.

Daly N., Meleady, P., Walsh, D. and Clynes, M. (1998). Regulation of keratin and integrin gene expression in cancer and drug resistance. *Cytotechnology*, 27, 321-344.

De Benedetti, A. and Rhoads, R.E. (1990). Overexpression of eukaryotic protein synthesis initiation factor 4E in HeLa cells results in aberrant growth and morphology. *Proc. Natl. Acad. Sci. USA*, 87, 8212–8216.

De Benedetti, A., Joshi-Barve, S., Rinker-Schaeffer, C. and Rhoads, R.E. (1991). Expression of antisense RNA against initiation factor eIF-4E mRNA in HeLa cells results in lengthened cell division times, diminished translation rates, and reduced levels of both eIF-4E and the p220 component of eIF-4F. *Mol. Cell. Biol.*, 11, 5435–5445.

De Benedetti, A., Joshi, B., Graff, J.R. and Zimmer, S.G. (1994). CHO cells transformed by the translation factor eIF4E display increased c-myc expresion but require overexpression of Max for tumorigenicity. *Mol.Cell. Diff.*, 2, 347-371.

De Benedetti, A. and Harris, A.L. (1999). eIF4E expression in tumors: its possible role in progression of malignancies. *Int. J. Biochem. Cell Biol.*, 31, 59-72.

De Benedetti, A. and Graff, J.R. (2004). eIF-4E expression and its role in malignancies and metastases. *Oncogene*, 23, (18), 3189-3199.

Deo, R.C., Schmidt, E.F., Elhabazi, Togashi, H., Burley, S.K. and Strittmatter (2004). Structural bases for CRMP function in plexin-dependent semaphorin3A signalling. *EMBO J.*, 23, 9-22.

DesMarais, V., Ichetovkin, I., Condeelis, J. and Hitchcock-DeGregori, S. E. (2002). Spatial regulation of actin dynamics: a tropomyosin-free, actin-rich compartment at the leading edge. *J. Cell Sci.* 115, 4649-4660

DesMarais, V , Ghosh, M , Eddy, R and Condeelis, J (2005) Cofilin takes the lead *J Cell Sci* ,118, (1), 19-26

Disatnik, M H , Boutet, S C , Pacio, W , Chan, A Y , Ross, L B , Lee, C H and Rando, T A (2004) The bi-directional translocation of MARCKS between membrane and cytosol regulates integrin-mediated muscle cell spreading *J Cell Sci* , 117, (19), 4469-4479

Downward, J (1998) Lipid regulated kinases some common themes at last *Science*, 279, 673

Du, Z , Yu, J , Chen, Y , Andino, R , James, T L (2004) Specific recognition of the C-rich strand of human telomeric DNA and the RNA template of human telomerase by the first KH domain of human poly(C)-binding protein-2 *J Biol Chem* , 279, (46), 48126-48134

Fahraeus, R (2005) Do peptides control their own birth and death? *Nat Rev Mol Cell Biol* , 6, (3), 263-267

Filatov, V L , Katrukha, T V , Bulargina, T V and Gusev, N B (1999) Troponin Structure, Properties and Mechanism of Functioning *Biochem (Moscow)*, 64, (9), 969-985

Fine, A (2004) Marrow cells as progenitors of lung tissue *Blood Cells Mol Dis* , 32, 95-96

Flier, A and Sonnenberg, A (2001) Function and interactions of integrins *Cell Tissue Res* , 305, (3), 285-298

Flynn, A and Proud, C G (1996) Insulin stimulation of the phosphorylation of initiation factor 4E is mediated by the MAP kinase pathway *FEBS Lett* , 389, 162-166

Flodby, P , Barlow, C , Kylefjord, H , Richter, L A and Xanthopoulus, K G (1996) Increased Hepatic Cell Proliferation and Lung Abnormalities in Mice Deficient in CCAAT/Enhancer Binding Protein α *J Biol Chem* , 271, (40), 24753-24760

Francis, P and Kuhajda, M D (2000) Fatty-acid synthase and human cancer new perspectives on its role in tumor biology *Nutrition*, 16, (3), 202-208

Fuchs, E (1998) Keratins as biochemical markers of epithelial differentiation *Trends Genet* , 4, 277-281

Fukunaga, R and Hunter, T (1997) Mnk1, a new MAP kinase activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates *EMBO J* , 16, 1921-1933

Gamberi, C , Izaurralde, E , Beisel, C and Mattaj, I W (1997) Interaction between the Human Nuclear Cap-Binding Protein Complex and hnRNP F *Mol Cell Biol* , 17, (5), 2587-2597

Garneau, D , Revil, T , Fisette, J F and Chabot, B (2005) Heterogeneous Nuclear Ribonucleoprotein F/H Proteins Modulate the Alternative Splicing of the Apoptotic Mediator Bcl-x *J Biol Chem* , 280, (24), 22641-22650

Ghosh, M , Song, X , Mouneimne, G , Sidani, M , Lawrence, D S and Condeelis J S (2004) Cofilin promotes actin polymerization and defines the direction of cell motility *Science*, 304, (5671), 743-746

Giancotti, F G and Ruoslahti, E (1999) Integrin Signaling *Science*, 285, 1028-1032

Giangreco, A , Reynolds, S D , Stripp, B R (2002) Terminal bronchioles harbour a unique airway stem cell population that localises to the bronchioalveolar duct junction *Am J Pathol* , 161, 173-182

Gilchrist, A J , Meuser, R , Turchinsky, J , Shaw, A R E , Pasdar, M and Dixon, W T (2002) Cell Adhesion-Mediated Transformation of a Human SCLC Cell Line Is Associated with the Development of a Normal Phenotype *Exp Cell Res* , 276, 63-78

Gilles, C , Polette, M , Zahm, J M , Tournier, J M , Volders, L , Foidart, J M , Birembaut, P (1999) Vimentin contributes to human mammary epithelial cell migration *J Cell Sci* , 112, (24), 4615-4625

Gingras, A -C , Kennedy, S G , O'Leary, M A , Sonenberg, N and Hay, N (1998) 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway *Genes Dev* , 12, 502-513

Gingras, A C , Raught, B and Sonenberg, N (2001) Regulation of translation initiation by FRAP/ mTOR *Genes Dev* , 15, 807-826

Glading, A , Lauffenburger, D A and Wells, A (2002) Cutting to the chase calpain proteases in cell motility *Trends Cell Biol* , 12, 46-54

Goodman, R H and Smolik, S (2000) CBP/p300 in cell growth, transformation and development *Genes Dev* , 14, 1553-1577

Goshima, Y , Ito, T , Sasaki, Y and Nakamura, F (2002) Semaphorins as signals for cell repulsion and invasion *J Clin Invest* , 109, 993-998

Graffe, J R , Boghaert, E R , De Benedetti, A , Tudor, D M and Zimmer, S G (1995) Reduction of translation initiation factor 4E reduces tumor growth, invasion and metastasis of ras-transformed cloned rat embryo fibroblast *Int J Cancer*, 60, 255-263

Graffe, J R , De Benedetti, A , Olson, J W , Tamez, P , Casero, R A and Zimmer, S G (1997) Translation of ODC mRNA and polyamine transport are suppressed in ras-transformed CREF cells by depleting translation initiation factor 4E *Biochem Biophys Res Comm* , 240, 15-20

Gross, J D , Moerke, N J , von der Haar, T , Lugovskoy, A A , Sachs, A B , McCarthy, J E G and Wagner, G (2003) Ribosomal Loading onto the mRNA Cap Is Driven by Conformational Coupling between eIF4G and eIF4E *Cell*, 115, 739-750

Grunder, A , Ebel, T T , Mallo, M , Schwarzkopf, G , Shimizu, T , Sippel, A E , Schrewe, H (2002) Nuclera factor I-B (*Nfib*) deficient mice have severe lung hypoplasia *Mech Dev* , 112, (1-2), 69-77

Guan, J L , Trevithick, J E and Hynes, R O (1991) Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein *Cell Regul* , 2, (11), 951-964

Halmos, B , Huettner, C S , Kocher, O , Ferenczi, K , Karp, D D and Tenen, D G (2002) Down-Regulation and Antiproliferative role of C/EBPα in Lung Cancer *Cancer Res* , 62, 528-534

Han, J -Y , Kim, H S , Lee, S H , Park, W S , Lee, J Y and Yoo, N J (2003) Immunohistochemical expression of integrins and extracellular matrix proteins in non-small cell lung cancer correlation with lymph node metastasis *Lung Cancer*, 41, 65-70

Hanks, S K , Calalb, M B , Harper, M C and Patel, S K (1992) Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin *Proc Natl Acad Sci USA*, 89, (18), 8487-8491

Hara, K , Maruki, Y , Long, X , Yoshino, K , Oshiro, N , Hidayat, S , Tokunaga, C , Avruch, J and Yonezawa, K (2002) Raptor a binding partner of target of rapamycin (TOR), mediates TOR action *Cell*, 110, 177-189

Hauck, C R , Sieg, D J , Hsia, D A , Loftus, J C , Gaarde, W A , Monia, B P and Schlaepfer, D D (2001) Inhibition of Focal Adhesion Kinase Expression or Activity Disrupts Epidermal Growth Factor-stimulated Signling Promoting the Migration of Invasive Human Carcinoma Cells *Cancer Res* , 61, 7079-7090

Hauck, C R , Hsia, D A , Ilic, D and Schlaepfer, D D (2002) vSrc SH3-enhanced Interaction with Focal Adhesion Kinase at β1 Integrin-containing Invadopodia Promotes Cell Invasion *J Biol Chem* , 277, 15, 12487-12490

Hayes, M J , Rescher, U , Gerke, V , Moss, S E (2004) Annexin-actin interactions *Traffic*, 5, (8), 571-576

He, Y C and Crouch, E (2002) Surfactant protein D gene regulation-Interactions among the conserved CCAAT/enhancer-binding protein elements *J Biol Chem* , 277, (22), 19530-19537

Hendrix, M J , Seftor, E A , Seftor, R E , Trevor, K T (1997) Experimental co-expression of vimentin and keratin intermediate filaments in human breast cancer cells results in phenotypic interconversion and increased invasive behavior *Am J Pathol* , 150, (2), 483-495

Hinck, L (2004) The versatile roles of "axon guidance" cues in tissue morphogenesis *Dev Cell* , 7, (6), 783-793

Hong, K U , Reynolds, S , Giangreco, A , Hurley, C M and Stripp B R (2001) Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion *Am J Respir Cell Mol Biol* , 24, 671-681

Hong, K U , Reynolds, S D , Watkins, S , Fuchs, E and Stripp, B R (2004) In vivo differentiation potential of tracheal basal cells evidence for multipotent and unipotent subpopulations *Am J Physiol Lung Cell Mol Physiol* , 286, 643-649

Hood, J D and Cheresh, D A (2002) Role of integrins in cell invasion and migration *Nat Rev Cancer*, 2, 91-100

Hoog, C.L., Foster, L.J., Mann, M. (2004). RNA and RNA binding proteins participate in early stages of cell spreading through spreading initiation centers. *Cell*, 117, (5), 649-662.

Hosack, D.A., Dennis, G. Jr,, Sherman. B.T., Lane, H.C., Lempicki, R.A. (2003). Identifying biological themes within lists of genes with EASE. *Genome Biol.*, 4, (10), R70.

Hu, L., Lau, S.H., Tzang, C.H., Wen, J.M., Wang, W., Xie, D., Huang, M., Wang, Y., Wu, M.C., Huang, J.F., Zeng, W.F., Sham, J.S., Yang, M. and Guan, X.Y. (2004). Association of Vimentin overexpression and hepatocellular carcinoma metastasis. *Oncogene*, 23, (1), 298-302.

Huang, Y.S., Richter, J.D. (2004) Regulation of local mRNA translation. *Curr. Opin. Cell Biol.*, 16, (3), 308-313.

Huttenlocher, A., et al. (1997). Regulation of cell migration by the calcium dependent protease calpain. *J. Biol. Chem.*, 272, 32719-32722.

Imai, J., Maruya, M., Yashiroda, H., Yahara, I., Tanaka, K. (2003). The molecular chaperone Hsp90 plays a role in the assembly and maintenance of the 26S proteasome. *EMBO J.*, 22, (14), 3557-3367.

Jagus, R., Joshi, B. and Barber, G.N. (1999). PKR, apoptosis and cancer. *Int. J. Biochem. Cell. Biol.*, **31**, 123-138)

Jolly, C. and Morimoto, R.I. (2000). Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *J. Natl. Cancer Inst.*, 92, (19), 1564-1572.

Jones, R.M., Branda, J., Johnston, K.A., Polymenis, M., Gadd, M., Rustgi, A., Callanan, L. and Schmidt, E.V. (1996). An essential E box in the promoter of the gene encoding the mRNA cap binding protein (eukaryotic initiation factor 4E) is a target for activation by c-myc. *Mol. Cell. Biol.*, 16, 4754-4764.

Joshi, B , Cai, A L , Keiper, B D , Minich, W B , Mendez, R , Beach, C M , Stepinski, J , Stolarski, R , Darzynkiewicz, E and Rhoads, R E (1995) Phosphorylation of eukaryotic protein synthesis initiation factor 4E at ser-209 *J Biol Chem* , 270, 14597–14603

Kagoshima, M , Ito, T , Kitamura, H and Goshima, Y (2001) Diverse gene expression and function of semaphorins in developing lung positive and negative regulatory roles of semaphorins in lung branching morphogenesis *Genes Cells*, 6, 559-571

Kanner, S B , Reynolds, A B and Parsons J T (1989) Immunoaffinity purification of tyrosine-phosphorylated cellular proteins *J Immunol Meth* , 120, (1), 115-124

Karni, R , Gus, Y , Dor, Y , Meyuhas, O , Levitzki, A (2005) Active Src elevates the expression of beta-catenin by enhancement of cap-dependent translation *Mol Cell Biol* , 25, (12), 5031-5039

Kedersha, N , Stoecklin, G , Ayodele, M , Yacono, P , Lykke-Andersen, J , Fitzler, M J , Scheuner, D , Kaufman, R J , Golan, D E , Anderson, P (2005) Stress granules and processing bodies are dynamically linked sites of mRNP remodeling *J Cell Biol* , 169, (6), 871-884

Keith, C T and Schreiber, S L (1995) PIK-related kinases DNA repair, recombination, and cell cycle checkpoints *Science*, 270, 50–51

Kim, D H , Sarbassov, D D , Ali, S M , King, J E , Latek, R R , Erdjument-Bromage, H , Tempst, P and Sabatini, D M (2002) mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery *Cell*, 110, 163-175

King, K W , Sheppard, F C , Westwater, C , Stern, P L and Myers, K A (1999) Organisation of the mouse and human 5T4 oncofoetal leucine-rich glycoprotein genes and expression in foetal and adult murine tissues *Biochim Biophys Acta*, 1445, 257-270

Kleijn, M , Scheper, G C , Voorma, H O and Thomas, A M (1998) Regulation of translation initiation factors by signal transduction *Eur J Biochem* , **253**, 531-544

Klimek-Tomczak, K , Wyrwicz, L S , Jain, S , Bomsztyk, K and Ostrowski, J (2004) Characterization of hnRNP K protein-RNA interactions *J Mol Biol* , 342, (4), 1131-1141

Knaupf, U , Tschopp, C and Gram, T (2001) Negative Regulation of Protein Translation by Mitogen Activated Protein Kinase-Interacting Kinases 1 and 2 *Mol Cell Biol* , 21, (16), 5500-5511

Konduri, S D , Rao, C N , Chandrasekar, N , Tasiou, A , Mohanam, S , Km, Y , Lakka, S S , Dinh, D , Olivero, W C , Gujrati, M , Foster, D C , Kisiel, W and Rao, J S (2001) A novel function of tissue factor pathway inhibitor-2 (TFPI-2) in human glioma invasion *Oncogene*, 20, (47), 6938-6945

Kornberg, L J , Earp, H S , Turner, C E , Prockop, C and Juliano, R L (1991) Signal transduction by integrins increased protein tyrosine phosphorylation caused by clustering of beta 1 integrins *Proc Natl Acad Sci USA*, 88, (19), 8392-8396

Kostourou, V , Troy, H , Murray, J F , Cullis, E R , Whitley, G S , Griffiths, J R and Robinson, S P (2004) Overexpression of dimethylarginine dimethylaminohydrolase enhances tumor hypoxia an insight into the relationship of hypoxia and angiogenesis in vivo *Neoplasia*, 6, (4), 401-411

Kozak, M (1987) An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs *Nucleic Acids Res* , 15, (20), 8125-8148

Kozak, M (1991) An analysis of vertebrate mRNA sequences intimations of translational control *J Cell Biol* , 115, (4), 887-903

Kreidberg, J A , Donovan, M J , Goldstein, S L , Rennke, H , Shepherd, K , Jones, R C and Jaenisch, R (1996) Alpha 3 beta1 Integrin has a crucial role in kidney and lung organogenesis *Development*, 122, 3537-3547

Lachance, P E , Miron, M , Raught, B , Sonenberg, N and Lasko, P (2002) Phosphorylation of eukaryotic translation initiation factor 4E is critical for growth *Mol Cell Biol* , 22, (6),1656-1663

Lader, A S , Ramoni, M F , Zetter, B R , Kohane, I S and Kwiatowski, D J (2004) Identification of a Transcriptional Profile Associated with In Vitro Invasion in Non-Small Cell Lung Cancer Cell Lines *Cancer Biol Ther* , 3, (7)

Lakka, S S , Konduri, S D , Mohanam, S , Nicolson, G L and Rao, J S (2000) In vitro modulation of human lung cancer cell line invasiveness by antisense cDNA of tissue factor pathway inhibitor-2 *Clin Exp Metastasis*, 18, (3), 239-244

Lambrechts, A , Van Troys, M and Ampe, C (2004) The actin cytoskeleton in normal and pathological cell motility *Int J Biochem Cell Biol* , 36, 1890-1909

Lang, S H , Hyde, C , Reid, I N , Hitchcock, I S , Hart, C A , Bryden, A A , Villette, J M , Stower, M J and Maitland, N J (2002) Enhanced expression of vimentin in motile prostate cell lines and in poorly differentiated and metastatic prostate carcinoma *Prostate*, 52, (4), 253-263

Law, E , Gilvarry, U , Lynch, V , Gregory, B , Grant, G and Clynes, M (1992) Cytogenetic comparison of two poorly differentiated human lung squamous cell carcinoma lines *Cancer Genet Cytogenet* , 59, (2), 111-118

Lawrence, J B and Singer, R H (1986) Intracellular localization of messenger RNAs for cytoskeletal proteins *Cell* 45, 407-415

Lazaris-Karatzas, A , Montine, K S and Sonenberg, N (1990) Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap *Nature*, 345, 544–547

Lee, J H , Chang, S H , Shim, J H , Lee, J Y , Yoshida, M and Kwon, H (2003) Cytoplasmic localization and nucleo-cytoplasmic shuttling of BAF53, a component of chromatin-modifying complexes *Mol Cells*, 16, (1), 78-83

Lewis, J D and Izaurralde, E (1997) The role of the cap structure in RNA processing and nuclear export *Eur J Biochem* , **247**, (2), 461-469

Lewis, S A , Tian, G , and Cowan, N J (1997) The α- and β- tubulin folding pathways *Trends Cell Biol* , 7, 479-483

Leyton, J , Garcia-Marin, L , Jensen, R T and Moody T W (2002) Neurotensin causes phosphorylation of focal adhesion kinase in lung cancer cells *Eur J Pharmacol* , 442, 179-186

Lin, L , Miller, C T , Contreras, J I , Prescott, M S , Dagenais, S L , Wu, R , Yee, J , Orringer, M B , Misek, D E , Hanash, S M , Glover, T W , Beer, D G (2002) The hepatocyte nuclear factor 3 alpha gene, HNF3alpha (FOXA1), on chromosome band 14q13 is amplified and overexpressed in esophageal and lung adenocarcinomas *Cancer Res* , 62, (18), 5273-5279

Lipfert, L , Haimovich, B , Schaller, M D , Cobb, B S , Parsons, J T and Brugge, J S Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125FAK in platelets *J Cell Biol* , 119, (4), 905-9112

Liu, Y , Fallon, L , Lashuel, H A , Liu, Z and Lansbury, P T Jr (2002) The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility *Cell*, 111, (2), 209-218

Liu, Y , Jiang, H , Crawford, H C , Hogan, B L (2003) Role for ETS domain transcription factors Pea3/Erm in mouse lung development *Dev Biol* , 261 (1), 10-24

Lobo, C , Ruiz-Bellido, M A , Aledo, J C , Marquez, J , Nunez De Castro, I and Alonso F J (2000) Inhibition of glutaminase expression by antisense mRNA decreases growth and tumourigenicity of tumour cells *Biochem J* , 348, (2), 257-261

Lubman, R L , Zhang, X -L , Zheng, J , Ocampo, L , Lopez, M L , Veeraraghavan, S , Zabski, S M , Danto, S I and Borok, Z (2000) Integrin α3-subunit expression modulates alveolar epithelial cell monolayer formation *Am J Lung Cell Mol Physiol* , 279, 183-193

Lynch, M , Chen, L , Ravitz, M J , Mehtani, S , Korenblat, K , Pazin, M J and Schmidt, E V (2004) hnRNP K binds a core polypyrimidine element in the eukaryotic translation initiation factor 4E (eIF4E) promoter, and its regulation of eIF4E contributes to neoplastic transformation *Mol Cell Biol* , 25, (15), 6436-6453

Mader, S , Lee, H , Pause, A and Sonenberg, N (1995) The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4γ and the translational repressors 4E-binding proteins *Mol Cell Biol* , 15, 4990-4997

Mamane, Y , Petroulakis, E , Rong, L , Yoshida, K , Ler, L W and Sonenberg, N (2004) eIF4E--from translation to transformation *Oncogene*, 23, (18), 3172-3179

Marcotrigiano, J , Gingras, A C , Sonenberg, N and Burley, S K (1997) Co-Crystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP *Cell*, 89, 951-961

Marte, B M and Downward, J (1997) PKB/AKT – Connecting phosphoinositide 3-kinase to cell survival and beyond *Trends Biochem Sci* , 22, 355-358

Mathew, R , Khanna, R , Kumar, R , Mathur, M , Shukla, N K and Ralhan, R (2002) Stromelysin-2 overexpression in human esophegal squamous cell carcinoma potential clinical implications *Cancer Detect Prev* , 26, (3), 222-228

Matsumori , M , Itoh, H , Toyoshima, I , Komatsuda, A , Sawada, K , Fukuda, J , Tanaka, T , Okubo, A , Kinouchi, H , Mizoi, K , Hama, T , Suzuki, A , Hamada, F , Otaka, M , Shoji, Y and Takada, G (2002) Characterisation of the 105 kDa molecular chaperone Identification, molecular properties and localization *Eur J Biochem* , 269, (22), 5632-5641

Matsuoka, Y , Li, X and Bennett, V (2000) Adducin structure, function and regulation *Cell Mol Life Sci* , 57, (6), 884-895

McBride, S , Meleady, P , Baird, A , Dinsdale, D and Clynes, M (1998) Human Lung Carcinoma Cell Line DLKP Contains 3 Distinct Subpopulations with Different Growth and Attachment Properties *Tumor Biol* , 19, 88-103

McBride, S , Walsh, D , Meleady, P , Daly, N , Clynes, M (1999) Bromodeoxyuridine induces keratin protein synthesis at a posttranscriptional level in human lung tumour cell lines *Differentiation*, 64, (3), 185-193

McKendrick, L , Morley, S J , Pain, V M , Jagus, R and Joshi, B (2001) Phosphorylation of eukaryotic initiation factor 4E (eIF4E) at Ser209 is not required for protein synthesis *in vitro* and *in vivo* *Eur J Biochem* , 268, 5375-5385

McKendrick, L , Pain, V M and Morley, S J (1999) Translation initiation factor 4E *Int J Biochem Cell Biol* , **31**, 31-35

Medina, M A (2001) Glutamine and cancer *J Nutr* , 131, 2539S-2542S

Meleady, P and Clynes, M (2001) Bromodeoxyuridine Induces Integrin Expression at Transcriptional (α_2 Subunit) and Post-Transcriptional (β1 Subunit) Levels, and Alters the Adhesive properties of Two Human Lung Tumour Cell Lines *Cell Adhes Comm* , 8, (1), 45-59

Meleady, P and Clynes, M (2001) Bromodeoxyuridine increases keratin 19 protein expression at a posttranscriptional level in two human lung tumor cell lines *In Vitro Cell Dev Biol Anim*, 37, (8), 536-542

Melker, A A de and Sonnenberg, A (1999) Integrins alternative splicing as a mechanism to regulate ligand binding and integrin signaling events Bioessays, 21, (6), 499-509

Mignone, F , Grillo, G , Licciulli, F , Iacono, M , Liuni, S , Kersey, P J , Duarte, J , Saccone, C and Pesole, G (2005) UTRdb and UTRsite a collection of sequences and regulatory motifs of the untranslated regions of eukaryotic mRNAs *Nucleic Acids Res*, 33, D141-6

Mingle, L A , Okuhama, N N , Shi, J , Singer, R H , Condeelis, J and Liu, G (2005) Localization of all seven messenger RNAs for the actin-polymerization nucleator Arp2/3 complex in the protrusions of fibroblasts *J Cell Sci*, 118 (11), 2425-2433

Minich, W B , Balasta, M L , Goss, D J and Rhoads, R E (1994) Chromatographic resolution of *in vivo* phosphorylated and nonphosphorylated eukaryotic translation initiation factor eIF-4E Increased cap affinity of the phosphorylated form *Proc Natl Acad Sci USA*, 91, 7668-7672

Mink, S , Haenig, B and Klempnauer, K -H (1997) Interaction and functional collaboration of p300 and C/EBPβ *Mol Cell Biol*, 17, (11), 6609-6617

Miranti, C K and Brugge, J S (2002) Sensing the environment a historical perspective on integrin signal transduction *Nat Cell Biol*, 4, (4), E83-90

Mishra, V S , Henske, E P , Kwiatkowski, D J and Southwick, F S (1994) The human actin-regulatory protein Cap G gene structure and chromosome location. *Genomics*, 23, (3), 560-565

Mizejewski, G J (1999) Role of Integrms in Cancer Survey of Expression Patterns *Proc Soc Exp Biol Med*, 222, (2), 124-138

Moll, R , Franke, W W and Schiller, D L (1982) The Catalog of Human Cytokeratins Patterns pf Expression in Normal Epithelia, Tumors and Cultured Cells *Cell*, 31, 11-24

Moreb, J S , Gabr, A , Vartikar, G R , Gowda, S , Zucali, J R and Mohuczy, D (2005) Retinoic acid down-regulates aldehyde dehydrogenase and increases cytotoxicity of 4-hydroperoxycyclophosphamide and acetaldehyde *J Pharmacol Exp Ther* , 312, (1), 339-345

Morley, S J and McKendrick, L (1997) Involvement of stress activated protein kinase and p38/RK mitogen-activated protein kinase signaling pathways in the enhanced phosphorylation of initiation factor eIF4E in NIH 3T3 cells *J Biol Chem* , 272, 17887-17893

Morley, S J and Naegele, S (2002) Phosphorylation of Eukaryotic Initiation Factor (eIF) 4E Is Not Required for *de Novo* Protein Synthesis following Recovery from Hypertonic Stress in Human Kidney Cells *J Biol Chem* , 277, (36), 32855-32859

Moro, L , Perlino, E , Marra, E , Languino, L R and Greco, M (2004) Regulation of β1a and β1c Integrin expression in Prostate Carcinoma Cells *J Biol Chem* , 279 (3), 1692-1702

Murai, K K and Pasquale, E B (2003) 'Eph'ective signalling forward reverse and crosstalk *J Cell Sci* , 116, 2823-2832

Nakamura, H , Fujii, Y , Ohuchi, E , Yamamoto, E and Okada, Y (1998) Activation of the precursor of human stromelysin 2 and its interactions with other matrix metalloproteinases *Eur J Biochem* , 253, 67-75

Naltner, A , Ghaffari, M , Whitsett, J A and Yan, C (2000a) Retinoic acid stimulation of the human surfactant protein B promoter is thyroid transcription factor 1 site-dependent *J Biol Chem* , 275, (1), 56-62

Naltner, A , Wert, S , Whitsett, J A and Yan, C (2000b) Temporal/spatial expression of nuclear receptor coactivators in the mouse lung *Am J Physiol Lung Cell Mol Physiol* , 279, 1066-1074

Nave, B T , Ouwens, M , Withers, D J , Alessi, D R and Shepherd, P R (1999) Mammalian target of rapamycin is a direct target for protein kinase B identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation *Biochem J* , 344, 427-431

Neaud, V , Hisaka, T , Monvoisin, A , Bedin, C , Balabaud, C , Foster, D C , Desmouliere, A , Kisiel, W and Rosenbaum, J (2000) Paradoxical Pro-invasive Effect of the Serine Proteinase Inhibitor Tissue Factor Pathway Inhibitor-2 on Human Hepatocellular Carcinoma Cells *J Biol Chem* , 275, 35565-35569

Nguyen, B P , Ren, X D , Schwartz, M A and Carter, W G (2001) Ligation of integrin alpha 3beta 1 by laminin 5 at the wound edge activates Rho-dependent adhesion of leading keratinocytes on collagen *J Biol Chem* , 276, (47), 43860-43870

Niedzwiecka, A , Marcotrigiano J , Stepinski, J , Jankowska-Anyszka, M , Wyslouch-Cieszynska, A , Dadlez, M , Gingras, A C , Mak, P , Darzynkiewicz, E , Sonenberg, N , Burley, S K and Stolarski, R (2002) Biophysical Studies of eIF4E Cap-binding Protein Recognition of mRNA 5' Cap Structure and Synthetic Fragments of eIF4G and 4E-BP1 Proteins *J Mol Biol* , 319, 615-635

Nishiya, N , Tachibana, K , Shibanuma, M , Mashimo, J I and Nose, K (2001) Hic-5-Reduced cell spreading on Fibronectin Competitive Effects between Paxillin and Hic-5 through interaction with Focal Adhesion Kinase *Mol Cell Biol* , 21, (16), 5332-5345

Nolasco, S , Bellido, J , Goncalves, J , Zabala, J C , Soares, H (2005) Tubulin cofactor A gene silencing in mammalian cells induces changes in microtubule cytoskeleton, cell cycle arrest and cell death *FEBS Lett* , 579, (17), 3515-3524

O'Neill, G M , Fashena, S J and Golemis, E A (2000) Integrin signalling a new Cas(t) of characters enters the stage *Trends Cell Biol* , 10, 111-119

Osaka, H , Wang, Y L , Takada, K , Takizawa, S , Setsuie, R , Li, H , Sato, Y , Nishikawa, K , Sun, Y J , Sakurai, M , Harada, T , Hara, Y , Kimura, I , Chiba, S , Namikawa, K , Kiyama, H , Noda, M , Aoki, S , Wada, K (2003) Ubiquitin carboxy-terminal hydrolase L1 binds to and stabilizes monoubiquitin in neuron *Hum Mol Genet* , 12, 1945-1958

Ostareck, D H , Ostareck-Lederer, A , Shatsky, I N , Hentze, M W (2001) Lipoxygenase mRNA silencing in erythroid differentiation The 3'UTR regulatory complex controls 60S ribosomal subunit joining *Cell*, 104, (2), 281-90

Ostareck-Lederer, A , Ostareck, D H , Cans, C , Neubauer, G , Bomsztyk, K , Superti-Furga, G , Hentze, M W (2002) c-Src-mediated phosphorylation of hnRNP K drives translational activation of specifically silenced mRNAs *Mo l Cell Biol* , 22, (13), 4535-4543

O'Sullivan, F (1999) In vitro models of human lung epithelial cell differentiation PhD Thesis Dublin City University

Pain, V M (1996) Initiation of protein synthesis in eukaryotic cells *Eur J Biochem* , 236, 747-771

Panaretou, B , Siligardi, G , Meyer, P , Maloney, A , Sullivan, J K , Singh, S , Millson, S H , Clarke, P A , Naaby-Hansen, S , Stem, R , Cramer, R , Mollapour, M , Workman, P , Pipe, P W , Pearl, L H , Prodromou, C (2002) Activation of the ATPase Activity of Hsp90 by the Stress-Regulated Cochaperone Aha1 *Mol Cell* , 10, (6), 1307-1318

Park, K S , Cho, S Y , Kim, H and Paik, Y K (2002) Proteomic alterations of the variants of human aldehyde dehydrogenase isozymes correlate with hepatocellular carcinoma *Int J Cancer*, 97, (2), 261-265

Parsons, J T (2003) Focal adhesion kinase the first ten years *J Cell Sci* , 116, 1409-1416

Pasquale, E B (2005) Eph receptor signalling casts a wide net on cell behaviour *Nat Rev Mol Cell Biol* , 6, (6), 462-475

Patel, J , McLeod, L E , Vries, R G , Flynn, A , Wang, X and Proud, C G (2002) Cellular stresses profoundly inhibit protein synthesis and modulate the states of phosphorylation of multiple translation factors *Eur J Biochem* , 269, 3076-3085

Pause, A , Belsham, G J , Gingras, A -C , Donze, O , Lin, T A , Lawrence Jr , J and Sonenberg, N (1994) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function *Nature*, 371, 762-767

Perrin, B J and Huttenlocker, A (2002) Molecules in focus Calpain *Int J Biochem Cell Biol* , 34, 722-725

Pfaff, M , Du, X and Ginsberg, M H (1999) Calpain cleavage of Integrin β cytoplasmic domains *FEBS Lett* , 460, 17-22

Potter, D A , Tirnauer, J S , Jansenn, R , Croall, D E , Hughes, C N , Fiacco, K A , Mier, J W , Maki, M and Herman, I M (1998) Calpain regulates actin remodelling during cell spreading *J Cell Biol* , 141, 647-662

Pincheira, R , Chen, Q and Zhang, J T (2001) Identification of a 170-kDa protein over-expressed in lung cancers *British J Cancer*, 84, (11), 1520-1527

Piper, M and Holt, C (2004) RNA translation in axons *Annu Rev Cell Dev Biol* , 20, 505-523

Proud, C G (2002) Regulation of mammalian translation factors by nutrients *Eur J Biochem* , 269, 5338-5349

Ptushkina, M , von der Haar, T , Karim, M M , Hughes, J M X and McCarthy, J E G (1999) Repressor binding to a dorsal regulatory site traps human eIF4E in a high cap-affinity state *EMBO J* , 18, 4068-4075

Pyronet, S , Imataka, H , Gingras, A C , Fukunaga, R , Hunter, T and Sonenberg, N (1999) Human eukaryotic translation initiation factor 4G (eIF4G) recruits Mnk1 to phosphorylate eIF4E *EMBO J* , 1, 270-279

Rajalingam, K , Wunder, C , Brinkmann, V , Churin, Y , Hekman, M , Sievers, C , Rapp, U R and Rudel, T (2005) Prohibitin is required for Ras-induced Raf-MEK-ERK activation and epithelial cell migration *Nat Cell Biol* , 7, (8), 837-843

Ramji, D P , Foka, P (2002) CCAAT/enhancer-binding proteins structure, function and regulation *Biochem J* , 365, (3), 561-575

Rao, R V and Bredesen, D E (2004) Misfolded proteins, endoplasmic reticulum stress and neurodegeneration *Curr Opin Cell Biol* , 16, (6), 653-662

Raught, B and Gingras, A C (1999) eIF4E activity is regulated at multiple levels *Int J Biochem Cell Biol* , 31, 43-57

Rechsteiner, M and Hill, C P (2005) Mobilising the proteolytic machine cell biological roles of proteasome activators and inhibitors *Trends Cell Biol* , 15, (1), 27-33

Reimann, I , Huth, A , Thiele, H, Thiele, B J (2002) Suppression of 15-lipoxygenase synthesis by hnRNP E1 is dependent on repetitive nature of LOX mRNA 3'-UTR control element DICE *J Mol Biol* , 315, (5), 965-974

Rescher, U and Gerke, V (2004) Annexins–unique membrane binding proteins with diverse functions *J Cell Sci* , 117, (13), 2631-2639

Reynolds, S D , Giangreco, A , Power, J H T , Stripp, B R (2000) Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration *Am J Pathol* , 156, 269-278

Revenu, C , Athman, R , Robine, S and Louvard, D (2004) The co-workers of actin filaments from cell structures to signals *Nat Rev Cell Mol Biol* , 5, (8), 635-646

Rinker-Schaeffer, C W , Graff, J R , De Benedetti, A , Zimmer, S G and Rhoads, R E (1993) Decreasing the level of translation initiation factor 4E with antisense RNA causes reversal of ras – mediated transformation and tumorigenesis of cloned rat embryo fibroblasts *Int J Cancer*, 55, 841–847

Rintoul, R C and Sethi, T (2002) Extracellular matrix regulation of drug resistance in small cell lung cancer *Clin Sci* , 102, 417-424

Roberts, E L , Newton, R P, and Axford A T (2004) Plasma purine nucleoside phosphorylase m cancer patients *Clin Chim Acta* , 344 (1-2), 109-114

Rosenwald, I B , Rhoads, D B , Callanan, L D , Isselbacher, K J and Schmidt, E V (1993) Increased expression of eukaryotic translation initiation factors eIF-4E and eIF-2 alpha in response to growth induction by c-myc *Proc Natl Acad Sci USA*, 90 6175-6178

Rosenwald, I B , Hutzler, M J , Wang, S , Savas, L and Fraire, A E (2001) Expression of eukaryotic translation initiation factors 4E and 2α is increased frequently in bronchioalveolar but not in squamous cell carcinoma of the lung *Cancer (Phila)*, 92, 2164-2171

Rosenwald, I B (2004) The role of translation in neoplastic transformation from a pathologist's point of view *Oncogene*, 23, (18), 3230-3247

Roberts, E L , Newton, R P, and Axford A T (2004) Plasma purine nucleoside phosphorylase in cancer patients *Clin Chim Acta* , 344 (1-2), 109-114

Ross, A C (2004) On the sources of retinoic acid in the lung understanding the local conversion of retinol to retinoic acid *Am J Physiol Lung Cell Mol Physiol* , 286, (2), 247-248

Sakamoto, H , Mashima, T , Sato, S , Hashimoto, Y , Yamori, T , Tsuruo, T (2001) Selective activation of apoptosis program by S-p-bromobenzylglutathione cyclopentyl diester in glyoxalase I-overexpressing human lung cancer cells *Clin Cancer Res* , 7, (8), 2513-2518

Schaller, M D , Borgman, C A , Cobb, B S , Vines, R R , Reynolds, A B and Parsons, J T (1992) pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions *Proc Natl Acad Sci USA*, 89, (11), 5192–5196

Schalm, S S , Fingar, D C , Sabatini, D M and Blenis, J (2003) TOS Motif-Mediated Raptor Binding Regulates 4E-BP1 Multisite Phosphorylation and Function *Curr Biol* , 13, 797-806

Scheper, G C , Morrice, N A and Proud, C G (2001) The MAP kinase signal integrating kinase Mnk2 is an eIF4E kinase with high basal activity in mammalian cells *Mol Cell Biol* , 21, 743-754

Scheper, G C , van Kollenburg, B , Hu, J , Luo, Y , Goss, D J and Proud, C G (2002) Phosphorylation of Eukaryotic Initiation Factor 4E Markedly Reduces Its Affinity for Capped mRNA *J Biol Chem* , 277, (5), 3303-3309

Scheper, G C and Proud, C G (2002) Does phosphorylation of the cap binding protein eIF4E play a role in translation initiation? *Eur J Biochem* , 269, 5350-5359

Schlaepfer, D D , Mitra, S K and Ilic, D (2004) Control of motile and invasive cell phenotypes by focal adhesion kinase *Biochim Biophys Acta*, 1692, 77-102

Schmid, R S , Shelton, S , Stanco, A , Yokota, Y , Kreidberg, J A and Anton, E S (2004) alpha3beta1 integrin modulates neuronal migration and placement during early stages of cerebral cortical development *Development*, 131, (24), 6023-6031

Schoch, K G , Lori, A , Burns, K A , Eldred, T , Olsen, J C and Randell, S H (2004) A subset of mouse tracheal epithelial basal cells generates large colonies in vitro *Am J Physiol Lung Cell Mol Physiol* , 286, 631-642

Schubert, U *et al* (2000) Rapid degradation of a large fraction of newly synthesized proteins by proteasomes *Nature*, 404, 770–774

Schwartz, C , Beck, K , Mink, S , Schmolke, M , Budde, B , Wenning, D and Klempnauer, K H (2003) Recruitment of p300 by C/EBPbeta triggers phosphorylation of p300 and modulates coactivator activity *EMBO J* , 22, (4), 882-892

Seki, N , Takasu, T , Mandai, K , Nakata, M , Saeki, H , Heike, Y , Takata, I , Yoshihiko, S , Hanafusa, T and Eguchi, K (2002) Expression of Eukaryotic Initiation Factor 4E in Atypical Adenomatous Hyperplasia and Adenocarcinoma of the Peripheral Lung *Clin Cancer Res* , 8, 3046-3053

Sethi, T and Rozengurt, E (1991) Multiple neuropeptides stimulate clonal growth of small cell lung cancer effects of bradykinin, vasopressin, cholecystokinin, galanin and neurotensin *Cancer Res* , 51, 13, 3621-3623

Shantz, L M and Pegg, A E (1999) Translational regulation of ornithine decarboxylase and other enzymes of the polyamine pathway *Int J Biochem Cell Biol* , 31, 43-57

Shav-Tal, Y and Singer, R H (2005) RNA localization *J Cell Sci* , 118, (18), 4077-4081

Sheppard, D (2003) Functions of Pulmonary Integrins From Development to Disease *Physiol Rev*, 83, 673-686

Shen, C and Nathan, C (2002) Nonredundant antioxidant defense by multiple two-cysteine peroxiredoxins in human prostate cancer cells *Mol Med.*, 8, (2), 95-102

Shih, J Y , et al (2001) Collapsin response mediator protein-1 a lung cancer invasion suppressor gene with nerve *J Natl Cancer Inst*, 93, 1392-1400

Shih, J Y , Lee, Y C , Yang S C , Hong, T M , Huang, C Y and Yang, P C (2003) Collapsin response mediator protein-1 a novel invasion-suppressor gene *Clin Exp Metastasis*, 20, (1), 69-76

Singh, S , Sadacharan, S , Su, S , Belldegrun, A , Persad, S , Singh, G (2003) Overexpression of vimentin role in the invasive phenotype in an androgen-independent model of prostate cancer *Cancer Res*, 63, (9), 2306-2311

Sonenberg, N and Denver, T E (2003) Eukaryotic translation initiation factors and regulators *Curr Opin Struct Biol*, 13, 56-63

Stumpo, D J , Graff, J M , Albert, K A , Greengard, P and Blackshear, P J (1989) Molecular cloning, characterization, and expression of a cDNA encoding the "80- to 87-kDa" myristoylated alanine-rich C kinase substrate a major cellular substrate for protein kinase C *P N A S*, 86 (11) 4012–4016

Strudwick, S and Borden, K L B (2002) The emerging roles of translation initiation factor eIF4E in the nucleus *Differentiation*, 70, 10-22

Sugahara, K , Sadohara, T , Sugita, M , Iyama, K -I and Takiguchi, M (1999) Differential expression of CCAAT enhancer binding protein family in rat alveolar epithelial cell proliferation and in acute lung injury *Cell Tissue Res*, 297, 261-270

Sun, C X , Robb, V A and Gutmann, D H (2002) Protein 4 1 tumor suppressors getting a FERM grip on growth regulation *J Cell Sci*, 115, 3991-4000

Tamagnone, L and Comoglio, P M (2004) To move or not to move? *EMBO Rep* , 5, (4), 356-361

Tanahashi, N *et al* (2000) Hybrid proteasomes Induction by interferon-γ and contribution to ATP-dependent proteolysis, *J Biol Chem* , 275, 14336–14345

Thornalley, P J (2003a) Glyoxalase 1 - structure, function and a critical role in the enzymatic defence against glycation Biochem Soc Trans , 31, (6), 1343-1348
Thornalley, P J (2003b) Protecting the genome defence against nucleotide glycation and emerging role of glyoxylase 1 overexpression in multidrug resistance in cancer therapy *Biochem Soc Trans* , 31, (6), 1372-1377

Tomoo, K , Shen, X , Okabe, K , Nozoe, Y , Fukuhara, S , Morino, S , Ishida, T , Taniguchi, T , Hasegawa, H , Terashima, A , Sasaki, M , Katsuya, Y , Kitamura, K , Miyoshi, H , Ishikawa, M and Miura, K (2002) Crystal structures of 7-methylguanosine 5'-triphosphate (m^7GTP)- and P^1-7-methylguanosme-P^3-adenosine-5', 5'-triphosphate (m^7GpppA)-bound human full-length eukaryotic initiation factor 4E biological importance of the C-terminal flexible region *Biochem J* , 362, 539-544

Tomoo, K , Shen, X , Okabe, K , Nozoe, Y , Fukuhara, S , Morino, S , Sasaki, M , Taniguchi, T , Miyagawa, H , Kitamura, K , Miura, K and Ishida, T (2003) Structural Features of Human Initiation Factor 4E, Studied by X-ray Crystal Analyses and Molecular Dynamics Simulations *J Mol Biol* , 328, (2), 365-383

Topisirovic, I , Ruiz-Gutierrez, M and Borden, K L (2004) Phosphorylation of the eukaryotic translation initiation factor eIF4E contributes to its transformation and mRNA transport activities *Cancer Res* , 64, (23), 8639-8642

Tourriere, H , Gallouzi, I E , Chebli, K , Capony, J P , Mouaikel, J , van der Geer, P and Tazi, J (2001) RasGAP-associated endoribonuclease G3BP Selective RNA

degradation and phosphorylation-dependent localization *Mol Cell Biol*, 21, (22), 7747-7760

Tourriere, H, Chebli, K, Zekri, L, Courselaud, B, Blanchard, J M, Bertrand, E, Tazi, J (2003) The RasGAP-associated endoribonuclease G3BP assembles stress granules *J Cell Biol*, 160, (6), 823-831

Tourriol, C, Bornes, S, Bonnal, S, Audigier, S, Prats, H, Prats, A C and Vagner, S (2003) Generation of protein isoform diversity by alternative initiation of translation at non-AUG codons *Biol Cell*, 95, 169-178

Ueda, T, Watanabe-Fukunaga, R, Fukuyama, H, Nagata, S and Fukunaga, R (2004) Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development *Mol Cell Biol*, 24, 15, 6539-6549

Vanhaesebrook B, Leevers S J, Panayotou, G and Waterfield, M D (1997) Phosphoinositide 3-kinases a conserved family of signal transducers *Trends Biochem Sci*, 22, 267-272

Van der Velden, A W and Thomas, A A M (1999) The role of the 5' untranslated region of an mRNA in translational regulation during development *Int J Biochem Cell Biol*, 31, 87-106

Vervoorts, J, Luscher-Firzlaff, J M, Rottmann, S, Lilischkis, R, Walsemann, G, Dohmann, K, Austen, M and Luscher, B (2003) Stimulation of c-MYC transcriptional activity and acetylation by recruitment of the cofactor CBP *EMBO Rep*, 4, (5), 484-490

Von der Haar, T, Ball, P D and McCarthy, J E G (2000) Stabilisation of Eukaryotic Initiation Factor 4E Binding to the mRNA 5'Cap by Domains of eIF4G *J Biol Chem*, 275, (39), 30551-30555

Waggoner, S A and Liebhaber, S A (2003) Identification of mRNAs Associated with αCP2-Containing RNP Complexes *Mol Cell Biol*, 23, (19), 7055-7067

Wallace, H M and Fraser, A V (2004) Inhibitors of polyamine metabolism review article *Amino Acids*, 26, (4), 353-365

Walsh, D , Meleady, P , Power, B , Morley, S J and Clynes, M (2003) Increased levels of the translation initiation factor eIF4E in differentiating epithelial lung tumor cell lines *Differentiation*, 71, (2), 126-134

Wang, X , Flynn, A , Waskiewicz, A J , Webb, B L J , Vries, R G , Baines, I A , Cooper, J and Proud, C G (1998) The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cells stresses and cytokines is mediated by distinct Map kinase pathways *J Biol Chem* , 273, 9373-9377

Wang, K J , Wang, R T and Zhang, J Z (2004) Identification of tumor markers using two-dimensional electrophoresis in gastric carcinoma *World J Gastroenterol* , 10, 2179–2183

Warburton, D , Schwarz, M , Tefft, D , Flores-Delgado, G , Anderson, K D and Cardoso, W V (2002) The molecular basis of lung morphogenesis *Mech Dev* , 92, (1), 55-81

Waskiewicz, A J , Flynn, A , Proud, C G and Cooper, J A (1997) Mitogen activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2 *EMBO J*, 16, 1909-1920

Waskiewicz, A J , Johnson, J C , Penn, B , Mahalingam, M , Kimball, S R and Cooper, J A (1999) Phosphorylation of the Cap-Binding Protein Eukaryotic Translation Initiation Factor 4E by Protein Kinase Mnk1 In Vivo *Mol Cell Biol*, 19, (3),1871-1880

Watkins, S J and Norbury, C J (2002) Translation initiation and its deregulation during tumorigenesis *British J Cancer*, 86, 1023-1027

Webb, B L J and Proud, C G (1997) Eukaryotic initiation Factor 2B (eIF2B) *Int J Biochem Cell Biol*, 29, (10), 1127-1137

Westermarck, J and Kahari, V -M (1999) Regulation of Matrix Metalloproteinases in Tumor Invasion *Faseb J*, 13, 781-792

Willis, A E (1999) Translational control of growth factor and proto-oncogene expression *Int J Biochem Cell Biol*, 31, 59-72

Willis, D, Li, K W, Zheng, J Q, Chang, J H, Smit, A, Kelly, T, Merianda, T T, Sylvester, J, van Minnen, J and Twiss, J L (2005) Differential transport and local translation of cytoskeletal, injury-response, and neurodegeneration protein mRNAs in axons *J Neurosci*, 25, (4), 778-791

Witke, W, Li, W, Kwiatowski, D J and Southwick, F S (2001) Comparison of CapG and Gelsolin null macrophages demonstration a unique role for CapG in receptor-mediated ruffling, phagocytosis and vesicle rocketing *J Cell Biol*, 154, (4), 775-784

Woods, A M, Wang, W W, Shaw, D M, Ward, C M, Carroll, M W, Rees, B R, Stern, P L (2002) Characterization of the murine 5T4 oncofoetal antigen a target for immunotherapy in cancer *Biochem J*, 366, (1), 353-65

Wu, K Y, Hengst, U, Cox, L J, Macosko, E Z, Jeromin, A, Urquhart, E R and Jaffrey, S R (2005) Local translation of RhoA regulates growth cone collapse *Nature*, 436, (7053), 1020-1024

Xian, J, Clark K J, Fordham, R, Pannell, R, Rabbitts, T H and Rabbits, P H (2001) Inadequate lung development and bronchial hyperplasia in mice with a targeted deletion in the *Dutt1/Robo1* gene *Proc Natl Acad Sci USA*, 98, 15062-15066

Yamada, S , Wirtz, D and Columbe, P A (2002) Pairwise Assembly Determines the Intrinsic Potential for Self-Organisation and Mechanical Properties of Keratin Filaments *Mol Biol Cell*, **13**, 382-391

Yamano, T , *et al* (2002) Two distinct pathways mediated by PA28 and hsp90 in major histocompatibility complex class I antigen processing *J Exp Med*, 196, 185–196

Yao, Y -L , Yang, W -M and Seto, E (2001) Regulation of Transcription Factor YY1 by Acetylation and Deacetylation *Mol Cell Biol*, 21, (17), 5979-5991

Yap, C T , Simpson, T I , Pratt, T , Price, D J and Maciver, S K (2005) The motility of glioblastoma tumour cells is modulated by intracellular cofilin expression in a concentration-dependent manner *Cell Motil Cytoskeleton*, 60, (3), 153-165

Yewdell, J W , Anton, L C and Bennink, J R (1996) Defective ribosomal products (DRiPs) a major source of antigenic peptides for MHC class I molecules? *J Immunol*, 157, 1823–1826

Yi, M , Tong, G -X , Murry, B and Mendelson, C R (2002) Role of CBP/p300 and SRC-1 in Transcriptional Regulation of the Pulmonary *Surfactant Protein-A* (*SP-A*) Gene by Thyroid Transcription Factor-1 (TTF-1) *J Biol Chem* , 277, (4), 2997-3005

Yoshida et al , (1998) Human Aldehyde Dehydrogenase Gene Family *Eur J Biochem* , 251, 549-557

Yoshimasu, T , Sakurai, T , Oura, S , Hirai, I , Tanino, H , Kokawa, Y , Naito Y , Okamura, Y , Ota, I , Tani, N and Matsuura, N (2004) Increased expression of integrin $\alpha 3\beta 1$ in highly brain metastatic subclone of a human non-small cell lung cancer cell line *Cancer Sci* , 95, 142-148

Yuminamochi et al , (2003) Expression of the LIM Proteins Paxillin and Hic-5 in Human Tissues *J Histochem Cytochem* , 51, (4), 513-521

Zhenge, M and McKeown-Longo, T J (2002) Regulation of HEF1 Expression and Phosphorylation by TGF-β1 and Cell Adhesion *J Biol Chem* , 277, (42), 39599-39608

Zuberek, J , Wyslouch-Cieszynska, A , Niedzwiecka, A , Dadlez, M , Stepinski, J , Augustyniak, A , Gingras, A C , Zhang, Z , Burley, S K , Sonenberg, N , Stolarski, R and Darzynkiewicz, E (2003) Phosphorylation of eIF4E attenuates its interaction with mRNA 5' cap analogs by electrostatic repulsion Intein-mediated protein ligation strategy to obtain phosphorylated protein *RNA*, 9, 52-61