The role of cellular signalling pathways and translation initiation factors in Herpesvirus infection

A thesis submitted for the degree of Ph.D

By

Robert Mc Mahon B.Sc. Hons

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

Dr. Derek Walsh

National Institute for Cellular Biotechnology
Dublin City University
Dublin 9, Ireland

2011

Declaration of ownership

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, 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.

Signed: _____ (Candidate) ID No.: _____ Date: _____

This thesis is dedicated to the memory
of my cousin James Corry

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. Derek Walsh for his guidance, knowledge, encouragement and patience during this research thesis, especially during the last stages of the project. Without his constant support and enthusiasm none of this work would have been possible.

I would also like to thank Prof. Martin Clynes and all the staff at the NICB for their time and effort with helping me achieving this thesis, a special mention going out to Mick, Finbar and Niall who would freely give of their time to help me with my experiments. I would also like to thank Paula would kindly agreed to be an examiner for my Viva and help me sort out my corrections. Thanks as well to Yvonne and Carol who were always there to help out with any problem I had.

To my colleagues, a thanks goes out to Iza and Kerstin for there help during my time in the lab, it was a privilege to work with both of you.

To my friends who are all off travelling around the world right now, it was a great few years in Dublin but I'd say the people of Terenure were glad to see the back of us and thank god you went when you did because I'd still be doing this thesis if you didn't!. A big shout out going my friends back home in Ennis, all I can say is on the chown.

I would also like to thank my friends and colleagues at St. Vincent de Paul, especially Rosemarie who was always there with a kind word of wisdom. A special thanks also to Patricia, for just being the way she is.

Finally, I would like to thank my parents, David and Imelda, and my brothers David and Adrian, for always being there for me no matter what. For always encouraging me and believing in me in whatever decisions I made as I went through life. Without their love I would have never gotten very far and this achievement is as much theirs as it is mine. Someday Mam and Dad I hope to repay you for all you've done.

Abbreviations:

4E-BP	-	eIF4E-binding protein
4EGi-1	-	eIF4E/eIF4G interaction inhibitor
ATP	-	adenosine-5'-triphosphate
BSA	-	bovine serum albumin
C-terminus	-	carboxy-terminus
CPE	-	cytopathic effect
DMEM	-	Dulbecco’s modified Eagle’s medium
DNA	-	deoxyribonucleic acid
dsRNA	-	double stranded RNA
E	-	early proteins
eIF	-	eukaryotic initiation factor
FBS	-	foetal bovine serum
FKBP12	-	FK506-Binding Protein, molecular mass of 12 kDa
kDa	-	kilo dalton
Gb	-	glycoprotein B
gC	-	glycoprotein C
gD	-	glycoprotein D
gH	-	glycoprotein H
gL	-	glycoprotein L
GTP	-	guanosine-5'-triphosphate
HVEM	-	Herpes virus entry mediator
ICP	-	infected cell protein
IE	-	immediately early proteins
IFN	-	Interferon

kDa	-	kilo Dalton
kbp (kb)	-	kilo base
L	-	late proteins
LAT	-	latency associated transcript
M	-	mole
Met-tRNAi	-	methionyl-initiator transfer ribonucleic acid
miRNA	-	micro ribonucleic acid
ml	-	millilitre
mM	-	milli mole
Mnk-1	-	MAP kinase interacting kinase 1
mRNA	-	messenger ribonucleic acid
mTOR	-	mammalian target of Rapamycin
MW	-	molecular weight
NHDF	-	normal human diploid fibroblast
N-terminus	-	Amino terminus
ORF	-	open reading frame
PABP	-	poly(A)-binding protein
P-Akt	-	phosphorylated Akt
PBS	-	phosphate-buffered saline
PDK1	-	phosphoinositide-dependent kinase 1
PI3K	-	phosphoinositide 3-kinase
PIKK	-	PI3K-related protein kinase
PIP2	-	phosphatidylinositol-4,5-bisphosphate
PIP3	-	phosphatidylinositol-3,4,5-trisphosphate
PtdIns	-	phosphatidylinositol

RNA	-	ribonucleic acid
RNA Pol II	-	RNA polymerase II
SDS-PAGE	-	sodium dodecyl sulfate-polyacrylamide gel Electrophoresis
PTEN	-	phosphatase and tensin homolog deleted on chromosome 10
Raptor	-	regulatory-associated protein of mTOR
Rheb	-	Ras homolog enriched in brain
Rictor	-	Rapamycin-independent companion of mTOR
RNA	-	ribonucleic acid
RNA Pol II	-	RNA polymerase II
RNAi	-	RNA interference
RT-PCR	-	reverse transcription polymerase chain reaction
SDS-PAGE	-	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS-T	-	tris-buffered saline with Tween 20
tRNA	-	transfer ribonucleic acid
UHP	-	ultra high purity
U_L	-	Unique long region
U_S	-	Unique short region
UTR	-	untranslated region
VacV	-	Vaccinia Virus

Abstract:

As obligate intracellular parasites, viruses rely on host cells to replicate. Hsv-1 is a large double stranded DNA virus which is the cause of common cold sores and corneal blindness in humans. The defining feature of HSV-1 is its ability to exist in two discrete states. During lytic infection, productive virus replication results in cell death. After primary infection HSV-1 enters a second state in non-permissive cells, termed latency in vivo or quiescence in vitro, in which minimal activity of the viral genome allows it to colonize its host. While the lytic state of viral replication is relatively well characterised, understanding of latency has lagged due to a lack of in-vitro models that can facilitate detailed mechanistic study. This thesis investigates aspects of HSV-1 quiescence, specifically the role of host cell signalling and translation initiation during reactivation from a non productive state. To achieve these objectives, a new system was established to study HSV-1 quiescence. The system employs the use of serum starved and temperature elevated primary human cells which allow for efficient suppression of wild type HSV-1 replication, which that results in the formation of HSV-1 quiescent infection. Upon investigation of the kinase pathways required during reactivation from quiescence it was discovered that inhibition of the ERK signalling pathway resulted in a suppression of viral reactivation. Additionally, the activities of the down stream substrate of ERK, Mnk1, along with the mTORC1 substrate 4E-BP, both of which regulate the mRNA translation initiation factor eIF4E were shown to be required for efficient reactivation. To further investigate the role of translation initiation during reactivation, we employed the use 4EGi-1, a recently discovered small molecule inhibitor of eIF4F formation. During our investigations it was discovered that protein synthesis in primary cells was minimally dependent upon eIF4F yet highly sensitive to 4EGi-1 at concentrations that did not alter eIF4F levels but instead, increased the association of inactive eIF2α with initiation complexes. At these relatively low concentrations a potent suppression of mRNA translation was achieved yet tolerable to cells over prolonged periods. Critically, inhibition of translation resulted in suppression of both lytic replication and reactivation from quiescence suggesting that targeting mRNA translation may be a viable therapeutic avenue for treatment of HSV-1 infection.

Section 1.0: Introduction

Section 1: Introduction

Introduction:

1.1. The Herpesviridae

The Herpesviridae are a large family of double stranded DNA viruses. The family is composed of over 200 viruses which are known to infect a range of vertebrate hosts. The viruses derive their name from the Greek word “Herpein” which means to creep and refers to the characteristic latent and recurring infection typical of this group of viruses. There are eight varieties of Herpes virus known to infect humans and they are classified into α (Alpha), β (Beta) and γ (Gamma) subfamilies based on their biological similarities. The *Alphaherpesvirinae* are distinguished by rapidly growing viruses that cause acute diseases, both Herpes simplex virus 1 and 2 (HSV-1 and HSV-2) are included within this subfamily and are known as neurotropic and neuroinvasive viruses. The *Betaherpesvirinae* are characterised as slow growing and highly cell-associated viruses which produce diseases with a prolonged clinical course. Cytomegalovirus and Roseolovirus are members of this subfamily and are leukotropic in nature, establishing latency in these cells until reactivated. The final subfamily within Herpesviridae is the *Gammaherpesvirinae*. The *Gammaherpesvirinae* subfamily contains two viruses known to infect humans. The first of these viruses is the Epstein-Barr virus (EBV), also called human herpesvirus 4 (HHV-4), which is a virus known to infect and replicate in oro-pharyngeal epithelial cells and establish latent infection within B-lymphocyte populations which can result in the causation of Burkitt's lymphoma and Nasopharyngeal carcinoma. The other member of the *Gammaherpesvirinae* known to infect humans is the Kaposi's sarcoma-associated herpesvirus (KSHV) also called human herpesvirus 8 (HHV-8). KSHV is known to target a number of cell types including endothelial cells (and progenitors), B or T-lymphocytes and subsets of monocyte/macrophages present in peripheral blood and in diseased tissues. Similar to Epstein Barr, KSHV can form latency in lymphocytes and is the cause of Kaposi's sarcoma, a common cancer occurring in patients with AIDS. In addition to Kaposi sarcoma, KSHV can cause the lymphoproliferative disorders such as primary effusion lymphoma and some types of multicentric Castleman's disease (Knipe *et al*., 2007).

1.1.1 Herpes simplex virus 1

Herpes Simplex Virus 1 (HSV-1) is a nuclear replicating enveloped double stranded DNA virus and member of the *Alphaherpesvirina* subfamily of the Herpesviridae family. HSV-1 virus is the pathogen responsible for Herpes labialis of the lips, mouth or gums which results in the development of small painful blisters, commonly called cold sores. Although Herpes labialis is a relatively benign condition of mainly aesthetic concern, HSV-1 can cause more severe conditions, notably Herpes Keratitis which can cause blindness and Herpes Encephalitis which destroys brain tissue leaving the patient permanently brain damaged (Barton 2009).

Transmission

Between 60 to 90% of the global adult population harbour neutralizing antibodies against HSV-1 and therefore serve as reservoirs for the virus. The majority of primary infections are attained through direct contact with a lesion or with infected body fluids such as saliva, genital fluids and exudates from active lesions. The transmission of virus is aided by the fact that individuals which harbour latent virus periodically shed infectious HSV-1 in saliva without the formation of symptomatic lesions (Young, Rowe & Buchanan 1976; Oliver *et al.*, 1995; Smith, Robinson 2002). This phenomena of viral shedding is more frequently observed in immunocompromised individuals or in patients undergoing oral surgery (Sacks *et al*., 2004).

1.1.1.1 HSV-1 structure

Genome:

The HSV-1 genome is 152 kb in size with a base composition of 68% G + C. It contains four different regions termed the unique long region (U_L), the unique short region (U_S) , and the two sets of repeats, the repeat long (R_L) and the repeat short (R_S). Each repeat within the genome is present in duplicate copies, differentiated as either the terminal (T) or internal (I). The genome contains three origins of replication situated at the centre of the U_L and flanking the U_S regions. The 9,000 bp long repeat (R_L) encodes both an important immediate early regulatory protein (ICP0) and the promoter of the latency associated transcript (LAT). The U_L which is 108,000 bp in length is known to be the coding region for 56 distinct proteins including the proteins

involved in genome replication and virion formation. The 6,600 bp short repeats (R_S) are the areas that code for immediate early proteins, which act as the transactivators of early genes which then results in viral DNA replication. The 13,000 bp unique short region (U_S) is the coding region for at least 12 ORFs, a number these ORFs are glycoproteins important in viral infectivity and host range in addition to controlling viral responses to host cell defences (Knipe *et al.*, 2007).

Figure 1.1 HSV-1 Genome

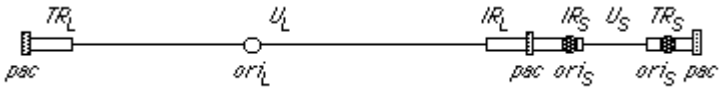

Figure 1.1 illustrating the different regions of the HSV-1 genome, termed the unique long region (U_L), the unique short region (U_S) ,and the two sets of repeats, the repeat long (R_L) and the repeat short (R_S). Each repeat within the genome is present in duplicate copies, differentiated as either the terminal (T) or internal (I). The genome contains three origins of replication (ori) situated at the centre of the U_L and flanking the U_S regions.

1.1.1.3 Capsid

The nucleocapsid is the structure which encapsulates the HSV-1 genome. It has a protein shell approximately 15 nm thick and 125 nm in diameter. Its major structural characteristic is its 162 capsomers (150 hexons and 12 pentons) which are constructed from the major capsid protein VP5, and the proteins VP19, VP23, VP26 bound by noncovalent bonding (Knipe *et al*., 2007; Newcomb *et al*., 1994).

1.1.1.2 The tegument

The tegument is an amorphous layer of heterogeneous proteins that occupies the space between the inner capsid and the outer envelope. Over the years the use of cryo-electron tomography and yeast two hybrid screening along with biochemical assays and viral-gene deletions has elucidated much of the structural detail pertaining to the tegument. The tegument is composed of 23 known proteins each of which has a specific function ranging from initiation of viral transcription, control of cellular molecular motors, subversion of cellular antiviral responses and transportation of viral capsids and formation of infectious virions. Many of the genes coding for these

proteins can also be found in both *Betaherpesvirinae* and *Gammaherpesvirinae* (Loret, Guay & Lippe 2008; Varnum *et al.*, 2004).

1.1.1.4 Envelope

The envelope consists of a lipid bilayer studded with 11 glycoproteins. The entry of HSV-1 into the host is facilitated by these glycoproteins embedded in the viral envelope binding to the receptors on the surface of the host cell. During HSV-1 fusion and entry, the envelope glycoprotein C (gC) binds to a proteoglycans on the cell surface called heparan sulphate. Upon binding of gC, glycoprotein D (gD) binds to a receptor called the Herpesvirus entry mediator receptor (HVEM) bringing the membrane surfaces of the cell and virus into close proximity allowing for other glycoproteins embedded in the viral envelope to interact with other cell surface molecules. This binding to HVEM changes gD's configuration allowing it to interact with viral glycoproteins H (gH) and L (gL), which then form a complex. The interaction of these membrane proteins results in the hemifusion state resulting in the interaction between glycoprotein B (gB) and gH/gL which then creates an entry pore for the viral capsid (Subramanian, Geraghty 2007). Once the viral capsid has entered into the cytoplasm, it is transported to the cell nucleus and attaches to the nucleus at a nuclear entry pore. The capsid then ejects its DNA contents via the capsid portal on the nuculeur membrane, which is a structure created using twelve copies of the viral portal protein UL6, which is constructed in a ring configuration (Knipe *et al.*, 2007).

Figure 1.2 HSV-1 Virion

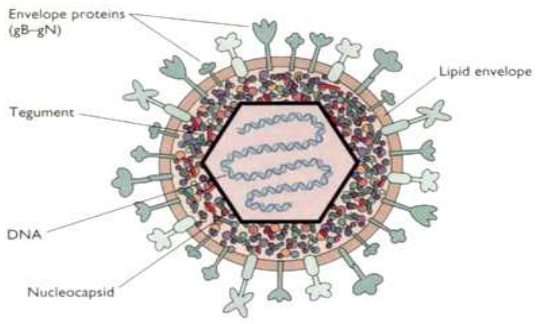

Figure 1.2 An illustration of the basic structural features of the HSV-1 virus. HSV-1 is an enveloped, icosahedral DNA virus. The region between the outer lipid envelope and the nucleocapsid is called the tegument. The DNA of the virus resides in the core. The envelope proteins ("Glycoprotein Spikes") are unique viral proteins, but the envelope itself is derived from the virus host cell. (G. E. Lee *et al.*, 2006).

1.1.1.5 The infectious states of HSV-1

Once the viral DNA has entered the nucleus of a host cell it exist in one of two states, termed lytic or latent.

1.1.1.5.1 Lytic Infection:

Lytic infection is a state defined by the expression of more than 80 HSV-1 proteins in a regulated cascade resulting in the production of infectious progeny. Transcription of viral DNA takes place in the host cell nucleus and the viral proteins are synthesized in the cytoplasm. Transcription of all viral genes is mediated by RNA Pol II and viral proteins can modify RNA Pol II activity and structure (Bastian, Rice 2009)(Wysocka, Herr 2003). The first class of viral proteins synthesized during this process are the viral α or immediate early (IE) genes. The six proteins comprising this class are ICP0, ICP4, ICP22, ICP27, ICP47 and Us 1.5. The transcription of these genes is regulated by the viral protein Vp16 which is present within the tegument surrounding the core of the infecting virion. Around 500 to 1000 Vp16 molecules are delivered to the cell with every incoming virion and these molecules form multicomponent complexes with cellular proteins OCT-1 and HCF which can then recognise and bind TAATGAAAT sequences on the IE gene promoters (Karupiah 2002; Wysocka, Herr 2003).

The immediate early genes have a vast array of functions during lytic infection. The immediate early protein ICP4 is a transactivator which is required for β and γ gene expression, while ICP0 is a ubiquitin ligase that destroys restrictive host factors and is a promiscuous viral gene transactivator (Barklie Clements, Watson & Wilkie 1977; Preston 1979; Watson, Clements 1980; Everett 2000).

The next class of viral genes are the β class or early (E) proteins, with functional viral IE transcription factor ICP4 being essential for β expression. The β genes are largely responsible for replication of viral DNA, nucleotide metabolism and the production of γ or late genes. The β genes are subdivided into two groups: The β1 genes, which are expressed within a short time after the expression of the α proteins and are

exemplified by U_L39 encoding ICP6, the large subunit of ribonucleotide reductase. The second set of β genes, the β2 genes are expressed in a more delayed fashion after α protein expression and are exemplified by U_L23 encoding Thymidine kinase.

The final class of HSV-1 viral genes expressed during lytic infection are the γ or late genes. These genes are expressed after viral DNA synthesis has commenced and mainly encode structural proteins which will compose the viral particle. The γ genes are also subdivided into two groups; the γ1 early/leaky late and the γ2 late or "true late" genes. The chronology of γ gene expression is determined by their dependence on viral DNA synthesis, with γ1 early/late genes such as ICP5 being expressed relatively early in infection but stimulated a few fold upon viral DNA synthesis. In contrast to the γ1 early/late genes, the γ2 late genes such as U_L11 are expressed late in infection only after DNA synthesis has occurred (Knipe *et al*., 2007).

1.1.1.5.2 Virion assembly

After the synthesis of all three classes of viral gene products has occurred the virus is assembled in several stages. Firstly the DNA is packaged into pre-assembled capsids within the nucleus. The filled capsids or "nucleocapsids" then mature into an infectious virion by budding through the inner lamella of the nuclear membrane. After budding, it is currently thought that the virion transits between the outer nuclear membrane to the subcellular space by transit through the Golgi stacks, being de-enveloped and then re-enveloped at the trans Golgi network. Once fully formed, the virus is then released by exocitosis causing the death of the host cell. The virus then spreads to the cells peripheral environment (Knipe *et al*., 2007).

1.1.1.6 Latency

Even though HSV-1 has a wide tropism and can lytically infect a vast array of cell types and animal species, the defining trait of HSV-1 is its ability to establish life long latent infection within host sensory neurons following spread from the primary site of infection.

Latency is established after the virus has entered sensory neurons by fusion at the axonal termini following spread from the epithial site of primary infection. The nucleocapsid then moves by retrograde axonal transport along microtubules to the nucleus of the cell body. Once inside the nucleus the viral genome acquires an endless circular DNA episomal configuration.

In latently infected neurons, the viral genome is modified by nucleosomal chromatin. The Latency Associated Transcript (LAT) promoter and its 5' exon are associated with acetylated H3 histones which are permissive for transcription. In contrast, the lytic gene promoters are associated with heterochromatin forms of histones during latent infection which prevent transcription (Knipe *et al*., 2007).

A key trait with regard to entry into latency is the absence of α gene expression. This lack of expression is most likely to be the primary cause of HSV-1 genome silencing.

One of the possible reasons for lack of α gene expression is probably the lack of nuclear forms of host cofactors required for α transcription. As described previously, Vp16 forms a multicomplex with HCF and OCT-1 which mediate transcription of viral genes in permissive cells. In neuronal cells HCF and OCT-1 are located in the cytoplasm but interestingly migrate to the nucleus during HSV-1 reactivation in explanted ganglia (Kristie, Vogel & Sears 1999; Nogueira *et al*., 2004). During latent infection, lytic gene transcription is suppressed and the only transcript that abundantly accumulates is the "Latency Associated Transcript" (LAT). This transcript yields multiple RNA species upon splicing which are collectively referred to as LATS. First identified by in situ hybridisation in 1984 and three years later by northern blotting, LATs code for no known viral proteins and are found exclusively in the nucleus during latency (Stevens *et al*., 1987). Although the exact functions of the LATs are not completely understood, LATS have been found to regulate the host cell genome and interfere with natural cell death mechanisms such as receptor mediated (Caspase 8) and mitochondrial mediated (Caspase 9) pathways (Henderson *et al*., 2002). In addition, LATS have been found to upregulate heat shock proteins and prevent cold

shock induced apoptosis indicating that LAT expression may prevent the occurrence of a inhospitable cellular environment during the establishment and maintenance of latency (Spivack, Fraser 1987; Perng *et al*., 2000; Ahmed *et al*., 2002; Jin *et al*., 2003; Peng *et al.*, 2004; Atanasiu *et al*, 2006).

During latency in sensory ganglia little or no replicating virus can be detected, but infectious virus is produced in a fraction of neurons periodically in response to environmental cues such as physical and psychological stress. This low level reactivation results in anterograde transport of virus to the peripheral epithelial tissue and can cause a symptomatic or asymptomatic infection depending on the host immune status, as CD8+T cells have been shown to control HSV-1 replication in the nervous systems of mice (Orr *et al*., 2007; Knickelbein *et al*., 2008; Sheridan, Knickelbein & Hendricks 2007).

1.1.1.7 Animal models for the study of HSV-1 latency

As a model which re-creates the exact infection characteristics of human disease remains unattainable and due to the broad array of hosts which HSV-1 can infect, research groups have employed the use of animal models which allow HSV to establish a localised initial infection followed by establishment of a latent infection in neurons, thus allowing for the study of viral pathogenesis, neuroinvasivness, neuropathology and latency. Mice are routinely used for the study of HSV-1 latency due to their reasonable cost relative to more expensive animal models and there are two infection models used in studies. The footpad/dorsal root ganglion model involves the infection of the mouse footpad which is followed by latent infection of the spinal ganglia (Stevens, Cook 1971). This model is thought to mimic aspects of HSV human genital infection and many aspects of latent infection, including identifying the neuron as the site of latency and the expression of LATS during latent infection (Cook, Bastone & Stevens 1974; Cook, Stevens 1976; Stevens *et al.*, 1987).

The second murine model used to study latency is the eye/trigeminal ganglion model, which involves infection of the cornea followed by latency establishment in the trigeminal ganglia. As with the footpad/dorsal model, virus can be recovered from latency by cocultivation of expanted ganglia on feeder cells (Kennedy, Al-Saadi &

Clements 1983). Additionally, reports have shown virus recovery from latency in mouse trigeminal ganglia following Ultraviolet (UV) irradiation and ocular iontophoresis of epinephrine (Willey, Trousdale & Nesburn 1984; Laycock *et al.*, 1991).

The investigation of HSV-1 latency has also been conducted using rabbit models. Like the mouse eye/trigeminal ganglion model latent infection is established following infection of the rabbit cornea. One characteristic of this system is the ability to sporadically recover virus more frequently than in mouse models. Recovery of virus can also be induced by iontophoresis of epinephrine (Nesburn, Elliott & Leibowitz 1967; Tullo *et al.*, 1982; Hill *et al.*, 1986; Shimeld et al. 1990). Additionally, as in the mouse models no infectious virus is detectable in infected ganglia until explantation and culture on feeder cells suggesting viral latency which broadly mimics human infection.

1.1.1.7 Cell Culture models of HSV-1 and latency (Quiescence)

Despite the intrinsic positives of animal models and the progress in empirical understanding made from their use, many questions with regard features of latent phase infection, reactivation and the state of viral genomes can only be best answered using tissue culture models that allow detailed mechanistic studies and genetic biochemical analysis. The major benefits of tissue culture models are the ability to observe the virus at the single cell level and without interference of immunological events that modulate the eventual appearance of virus in the host.

In the past a number of models have been developed to study HSV latency (termed quiescence in vitro) by coercing HSV to a non productive state. Quiescence can be established in sympathetic neurons of rat origin in vitro. These explanted cells are treated with antimitotic agents such as florodeoxyuridine to stop the growth of fibroblasts or Schwann cells that were removed with the neurons during explant. Initial experiments on these cultures reported that quiescence could be established with a loss of 37% of cultures infected with HSV-1 at a multiplicity of infection 0.03, while of the surviving cultures only 12% harbour virus capable of reactivation from quiescence. Further experimentation using these cells showed that treatment of cultures with Anti HSV-1 antibodies two weeks subsequent to infection allowed for the multiplicity of infection to be increased to 1. Of the cultures infected, 63% survived and 53% of which harboured latent virus that could be reactivated upon NGF removal. However when the multiplicity of infection was increased to 2, all cultures were destroyed by productive lytic infection (Wilcox, Johnson 1987).

Additional models from the same group employed the use of Acyclovir. Acyclovir is a guanosine analogue antiviral drug which is a potent inhibitor of viral DNA polymerase. It was shown that pre-treatment with Acyclovir 12 hours prior to infection, and maintenance of Acyclovir in cultures for 7 days, allowed for cultures to be infected at multiplicities of infection of 5 with 100% of the cultures infected surviving. Upon acyclovir removal the cultures could be maintained in a quiescent state for a further 7 days and reactivated by NGF removal (Wilcox, Johnson 1988). However reactivation was often inefficient and did not occur on a population wide level.

The use of other chemical inhibitors such 1-,B3D-arabinofuranosylcytosine have also been applied to primary cultures of explanted ganglia of rat and human origin to suppress viral replication prior to culture maintenance at temperatures above 40.5°C. Although this approach was successful at establishing quiescence the system was limited to infecting with 0.1 plaque-forming units (p.f.u) per cell (Wigdahl, Isom & Rapp 1981). In an attempt to increase the proportion of quiescently infected cells within the culture the same research group pre-treated cells with medium containing (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) and human leukocyte interferon prior to infection at 2.5 plaque-forming unit (pfu) per cell. BVDU is a known inhibitor of viral DNA polymerase (Allaudeen *et al*., 1981), and human interferon is thought to induce a cellular antiviral response and reduce viral replication (Kotwal 1997). In these systems, viral quiescence was sustained after inhibitor removal by increasing the incubation temperature from 37°C to 40.5°C and viral quiescence was maintained for up to 15 days post inhibitor removal while virus reactivation was initiated by decreasing the temperature to 37°C (Wigdahl *et al*., 1982; Wigdahl *et al*., 1983).

As the preparation of dorsal root ganglia is time consuming and inconvenient, other tissue culture models were developed which allow for the study of HSV-1 quiescence. The first of such models is the rat phaeochromocytoma line (PC12), which is differentiated with nerve growth factor. In response to NGF removal the PC12 cells cease to divide and acquire properties characteristic of peripheral nervous tissue. During infection of these differentiated cells, NGF was maintained in the culture media and quiescence could be established and subsequently maintained for several weeks. Upon NGF removal, virus could be recovered indicating reactivation. Alternatively heat shock or forskolin has also been reported to reactivate virus in these systems (Block *et al*., 1994; Danaher *et al*., 1999; Miller, Danaher & Jacob 2006). Further use of these systems showed that the addition of Acyclovir during infection allowed for higher multiplicities of input virus to be used to establish quiescence (Danaher, Jacob & Miller 1999). These models have been used to show the role of Histone Deacytlases (HDACs) and chromatin remodelling during virus reactivation (Danaher *et al*., 2005).

Many of these neuronal models were based in non-human cell types. Due to the ethical and accessibility issues associated with the use of neuronal systems of human origin, primary fibroblasts have been used extensively for the study of HSV-1

quiescence in human cells. The capacity of fibroblasts to harbour quiescent infection is thought to be facilitated by a low metabolic state and expression of factors that resist viral lytic replication which may mimic the state of neurons more accurately than other cell lines (Jamieson *et al*., 1995).

Initial experiments conducted by Crouch and Rapp (Crouch, Rapp 1972) demonstrated HSV-1 replication was strongly suppressed in certain cell types by elevating the temperature of fibroblast cultures to 40.5°C during the initial 6 hours of infection. The fact that this worked in only certain cell types suggested that replication of the virus itself was not sensitive to temperature elevation but that an early stage in the virus life cycle was inhibited in a host cell-specific manner. Consequently, many fibroblast models exploit temperature elevation of cultures to coerce the virus into a non replicative state (Wheeler 1958; Harris, Preston 1991). Although temperature elevation could prevent viral replication, it could only do so at low multiplicities of infection (0.003p.f.u/cell) most likely due to the cytotoxicity of immediate early gene expression by wild type virus in these cells (Johnson, Wang & Friedmann 1994; Harris, Preston 1991). To overcome this problem and allow for infection on a population wide scale, research groups employed the use of viral mutants deficient in various IE genes, in addition to treatment of cultures with chemical inhibitors and or temperature elevation to prevent low-level replication of these mutants (Russell *et al*., 1987; Stow 1989; Harris, Preston 1991; Jamieson *et al*.,1995; Samaniego, Neiderhiser & DeLuca 1998; Preston, Nicholl 1997; Hobbs *et al*., 2001; Everett, Boutell & Orr 2004). However, the use of viral mutants and relatively low efficiency of many models means that our understanding of latency and reactivation remains limited.

1.2 Translation

As obligate intracellular parasites, viruses lack the genes that code for components of the mRNA translational machinery such as ribosomal subunits and translation factors. To ensure viral mRNA translation during both lytic infection and reactivation from latency, the virus must effectively commandeer the host cells translation machinery to compete with the host cells mRNAs for access to limiting amounts of translation initiation factors.

Translation is recognised as an important process for gene regulation as it controls the conversion of mRNA to protein. Its processes are subdivided into three stages, initiation, elongation and termination.

The ribosome complex is a multisubunit structure containing ribosomal RNA (rRNA) and proteins, which is responsible for translation of mRNA to a specific sequence of amino acids. Before mRNA can be converted to protein the ribosome must position itself on the correct area of the mRNA. This process is called translation initiation and the activity of cellular initiation factors that mediate this process can influence both overall global rates of proteins synthesised or influence the relative rates of synthesis of specific subsets of proteins. Whereas the control of global protein synthesis is of potential importance for allowing the cell enter G1 phase of the cell cycle, the control of the translation of specific proteins impacts process such as cell homeostastis, and it is thought that the amounts of a number of proteins which play a key role in cell proliferation and differentiation are controlled at the level of translation (Pain 1996; Gingras *et al*., 2001).

1.2.1 Translation initiation.

Translation initiation is said to be a rate limiting step with regard to protein synthesis as it is at this stage where most steps of physiological control are observed (Figure 1.3). Initiation begins when preexisting 80S ribosomes bound to the 3’ end of mRNA are dissociated by binding of eIF6 to the 60S ribosomal subunit and binding of eIF3 and eIF1A to the 40S ribosomal subunit. Once the 40S subunit is released it can bind eIF2-GTP-Met-tRNA which forms the 43S preinitiation complex. When the 43S

preinitiation complex is formed it is competent at binding mRNA independently, but for the ribosome to assemble on the 5’ end of mRNA it first must avail of ATP hydrolyis and a set of initiation factors termed the eIF4 group. Once the 43S subunit has bound eIF4F through the binding of eIF3 it is thought to traverse and scan the mRNA 5′ untranslated region (5′ UTR) in a linear and processive fashion in a 5′-to-3′ direction until it comes upon an AUG initiation codon in the favourable sequence context. Upon encountering the correct initiation codon the associated initiation factors are released in a process mediated by the GTPase-activating protein (GAP), eIF5, which promotes eIF2 to hydrolyze its bound GTP to GDP. Once the hydrolysis of GTP has occurred, the initiation factors are released from the ribosome and the 60S subunit binds to the complex initiating polypeptide elongation (Gingras, Raught & Sonenberg 1999).

Fig 1.3 Recruitment of Ribsomes to mRNAs

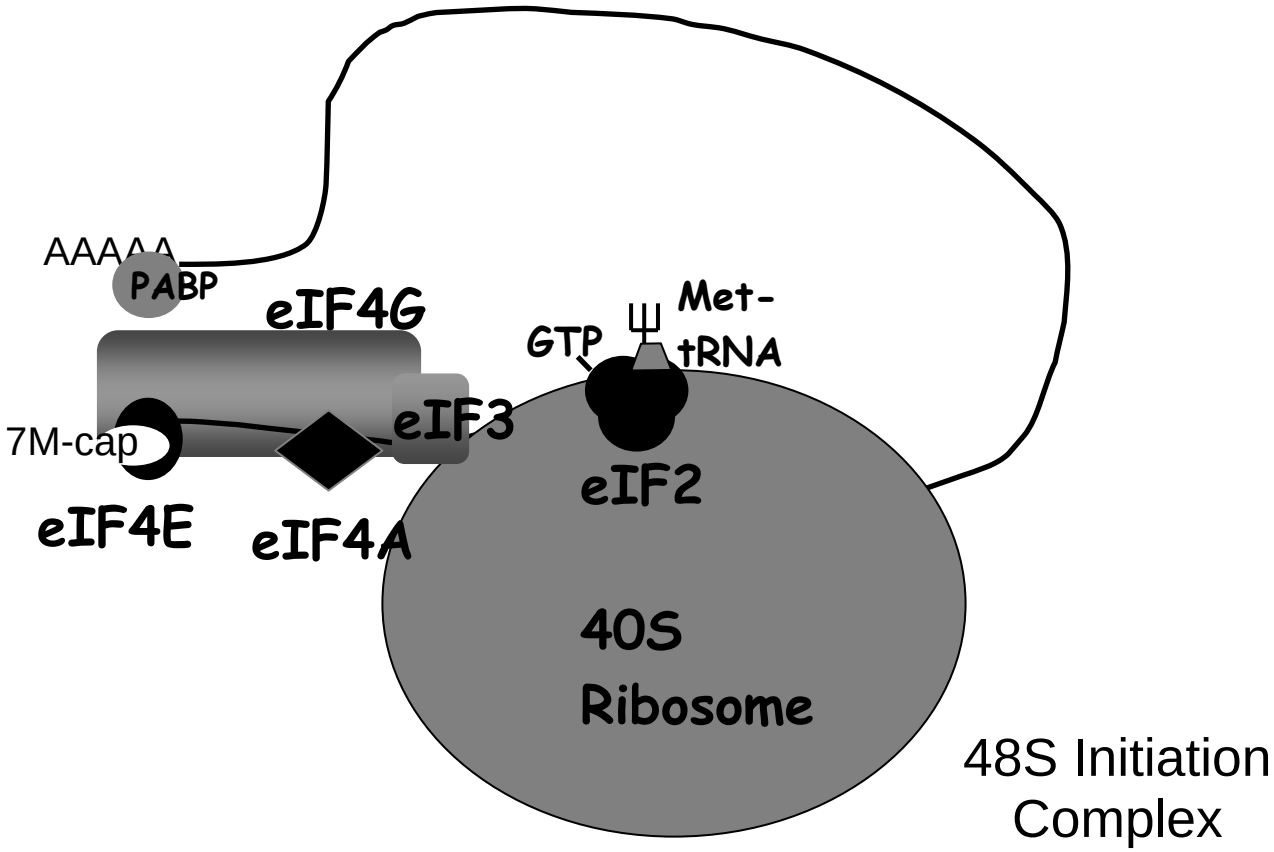

Fig 1.3 The 48S initiation complex is composed of Met-$tRNA_i^{Met}$ which is recruited to the small (40S) ribosomal subunit in a ternary complex (TC) with GTP-bound eIF2, to produce the 43S preinitiation complex (PIC). The 43S PIC interacts with the 5' end of mRNA in a manner stimulated by factors that bind to the m^7G cap of the mRNA. The m^7G cap binding complex termed the eIF4F complex is composed of eIF4E which binds the m^7G cap and the N-terminal region of eIF4G. eIF4G is a protein which has multiple protein binding sites and in addition to eIF4E it also binds the ATP dependent RNA helicase eIF4A, the ribosome binding protein eIF3 and the poly(A) tail binding protein (PABP). The 48S PIC is formed once the mRNA bound eIF4F complex has bound the 40S ribsome. The PIC then scans the mRNA leader until the anticodon Met-$tRNA_i^{Met}$ base-pairs with the "start" AUG codon in the correct site context on the mRNA. When the Met-$tRNA_i^{Met}$ binds this AUG codon the eIF4F complex is then released allowing for the 40s ribsome to bind the 60s ribosome thus forming the 80s ribosome which facilitates polypeptide elongation.

1.2.1.1 Cap structure and poly a tail:

The majority of eukaryotic cellular mRNAs possess an inverted 7- methyguanosine, linked by a 5'-5' triphosphate bridge to the first transcribed residue at the 5'end (Banerjee 1980). This group is referred to as the 'cap' and has many functions including nuclear export, stability, splicing and recognition of mRNA for translation into protein (Lewis, Izaurflde 1997; Cougot *et al*., 2004).

In addition to the cap, most eukaryotic mRNAs possess a 3′ poly(A) tail which can be 50 bases long in yeasts to more than 200 bases long in higher eukaryotes. The poly(A) tail is important for the nuclear export, translation and stability of mRNA (Preiss, Muckenthaler & Hentze 1998; Guhaniyogi, Brewer 2001; Fuke, Ohno 2008).

Figure 1.4 Cap structure

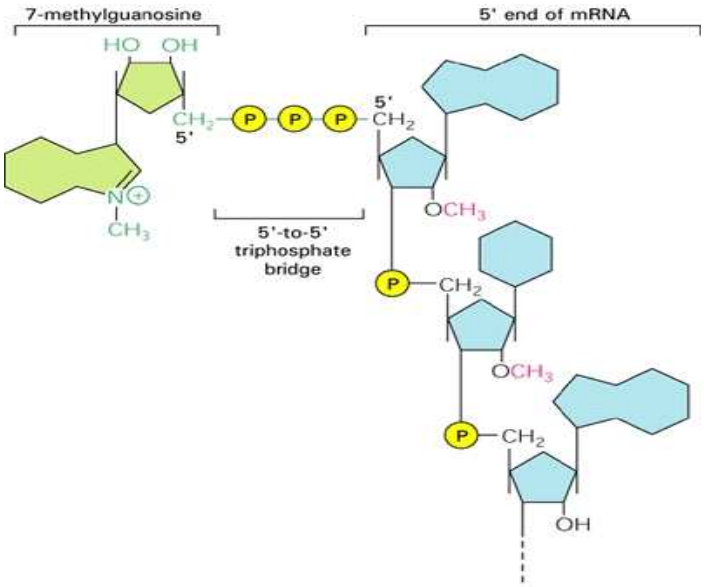

Figure 1.4 The 5' cap structure is found on most eukaryotic messenger RNAs and is required for translation inititaion. The guanosine is methylated on position 7 by a methyl transferase and connected to the 5' end of the mRNA via a 5' to 5' triphosphate linkage (Molecular Biology of the Cell, 4th edition).

1.2.1.1.1 The eIF4F complex:

The eIF4F complex is composed of three subunits, eIF4E, eIF4A, and eIF4G (Gingras, Raught & Sonenberg 1999). eIF4E is responsible for binding the cap complex directly and is itself highly regulated to fine tune cap dependent translation. The eIF4A initiation factor functions within the eIF4F complex as an ATP-dependent RNA helicase. The helicase activity of eIF4A is required to unwind secondary structures within the 5' untranslated region of mRNAs to facilitate ribosome binding (Pain 1996).

eIF4F also binds the poly(A)-binding protein (PABP) which functions to increase translational efficiency. As PABP binds to the poly A tail present on most eukaryotic mRNAs it brings about a circularisation of the mRNA on the eIF4G, where the 5' terminus is bound to eIF4E and the 3' terminus is bound to PABP. This circularisation is thought to function as an integrity check for mRNAs and to stabilise the binding of the mRNA on the complex. Of the three subunits of the translation initiation complex eIF4E is the least abundant and is a major target for regulation. Therefore eIF4E is considered a rate limiting factor in the localisation and binding of mRNA to ribosomes.

1.2.1.1.1.1 eIF4E

The cap facilitates translation of most cellular mRNAs by distinguishing the 5' terminus for interaction with the eIF4E translation factor during the initial stages of translation initiation. It is through this interaction with eIF4E that the 5' end of capped mRNA is recruited to the other components of the translation complex. Consequently eIF4E is essential for cap dependent translation.

The 3D structure of eIF4E is similar in appearance to a cupped hand or baseball glove and is composed of eight antiparallel β strands, three α helices and ten loop structures. The eight antiparallel β strands form a curved β sheet while the α helices provide structural support behind the sheet (Tomoo *et al.*, 2003; Volpon *et al.*, 2006). The C- and N- terminal regions of eIF4E are flexible and the flexibility plays a role in the regulation of eIF4E function. The C- terminal flexible region contains the receiving pocket for the cap and also contains a Ser209 regulatory phosphorylation site. During

cap binding the m^7GDP resides within a narrow slot on the concave surface of eIF4E. Binding of m^7GDP is facilitated by π-π stacking interactions between the base and the eIF4E indole side chains of two tryptophans in the flexible C-terminal region (Trp56 and Trp102) (Tomoo *et al*., 2003; Marcotrigiano *et al*., 1997). Other interactions such as van der Waals contacts between the N-7 methyl group of the guanine ring and Trp166 also organize the π-π stacking between the cap and eIF4E. In addition, positively charged residues in the cap-binding slot (Arg112, Arg157, and Arg162) arrange the negatively charged oxygen atoms of the phosphate moieties in m^7GDP to the correct configuration and hydrogen bonding between amino acids of eIF4E and phosphate groups on the cap also contributes to the stability of binding (Gingras, Raught & Sonenberg 1999).

The N-terminal flexible region of eIF4E contains the binding site for eIF4G. eIF4E binds at position 572–578 in human eIF4G and the consensus eIF4E binding site is YXXXXLΦ, where $\Phi$is usually L, but may also be M or F. This fragment of eIF4G can bind to the dorsal convex surface of eIF4E which is directly behind the cap-binding slot, the binding is facilitated through hydrogen bonds, salt bridges, and van der Waals contacts. The 4E-BPs also possesses the YXXXXLΦ binding motif and therefore 4E-BPs compete with eIF4G for binding with eIF4E (Mader *et al*., 1995; Sonenberg, Gingras 1998; Marcotrigiano *et al*., 1999).

Given the important functions of eIF4E its activity is regulated at multiple levels.
The three mechanisms of eIF4E activity regulation include:

(1) Transcription of eIF4E mRNA
(2) Phosphorylation of the eIF4E
(3) Interaction with the 4E-BP family of translational repressors.

1.2.1.1.1.2 Transcriptional regulation of eIF4E

Previous studies have shown that both eIF4E mRNA and eIF4E protein levels increase in T cells after T-cell receptor (TCR) cross-linking, suggesting that in response to extracellular stimuli eIF4E upregulation may by attributed to transcriptional activation (Mao *et al*., 1992; Rosenwald *et al*., 1993; Boal *et al*., 1993) Indeed, during the cell cycle increased levels of the potent regulator of cell growth MYC are observed along with upregulation of eIF4E mRNA levels, and cells that stably overexpress MYC show the same phenomena (Rosenwald *et al*., 1993). Furthermore the eIF4E promoter contains two functional MYC-binding sites (E boxes) (Jones *et al*., 1996). Therefore, since eIF4E plays a key role in cell growth and proliferation, it seems likely that eIF4E transcription is a downstream result of MYC production and is likely to play a central part of MYC induced cellular transformation and cancers.

1.2.1.1.1.2.1 Regulation of eIF4E through phosphorylation

The second means of regulation of eIF4E is phosphorylation at the C-terminus Ser209 residue. Phosphorylation of this residue is increased following treatment of cells with growth factors, mitogens and hormones, cytokines and stressful conditions (Morley, McKendrick 1997; Pyronnet *et al*., 1999). These increases in Ser 209 phosphorylation appear to be mediated via a series of signalling pathways that respond to a variety of extracellular stimuli and activate specific mitogen-activated protein (MAP) kinase cascades (Davis 1993; Marshall 1994). The first MAP Kinase pathway found to play a role in eIF4E phosphorylation was the extracellular-signal-regulated kinase (ERK) as inhibitors of this pathway reduce eIF4E phosphorylation (Flynn, Proud 1996; Wang *et al*., 1998; Waskiewicz *et al*., 1999).

ERK is activated in response to peptide growth factors, phorbol esters, Ca^{2+} and some G-protein-linked agonists. Typically ERK is stimulated when G-protein coupled receptors, integrins and receptor tyrosine kinases are activated in a process where ligand binding to receptors results in the displacement of GDP for GTP on the G protein Ras. Ras can then activate the family of Raf proteins. Rafs are a group of Serine/Theronine kinases which are maintained in an inactive state in the cytosol by binding with 14-3-3. The 14-3-3 dimer binds the phosphorylated N-Terminal (S259)

and C-terminal (S621) on Raf, keeping Raf in a closed, catalytically inactive configuration. Once Ras recruits Raf it displaces 14-3-3 allowing for Raf to be dephosphorylated by cellular phosphatases. This dephosphorylation causes Raf to change its structural configuration which opens its kinase domain to activating events such as phosphorylation by PKC and Src (Avruch *et al*., 2001; Rodriguez, Viciana *et al.*, 2006). Raf family kinases phosphorylate and activate MAP kinase 1 and 2 (MEK1, MEK2). MEKs are dual specificity kinases that phosphorylate ERK1/2 on the Threonine and Tyrosine residues in the conserved Tyr-Glu-Thr (TEY) motif of the activation loop.

The second Map Kinase pathway found to play a role in eIF4E phosphorylation is referred to as the p38 pathway (Morley, McKendrick 1997; Wang *et al*., 1998). The p38 pathway is composed of several kinases, the most important of which are MEK3 and MEK6 and four known p38 isoforms (α,β,γ and δ). In mammalian cells, the p38 isoforms are activated by environmental stresses and inflammatory cytokines (Cuenda., 2007).

MEK3 and MEK6 are activated by a panoply of kinases in response to various physical and chemical stresses, such as hypoxia, oxidative stress, ischemia, UV irradiation, and cytokines, including tumor necrosis factor alpha and interleukin-1 (IL-1) (Chen *et al*., 2001; Roux, Blenis 2004). MEK6 activate all p38 isoforms, while MEK3 preferentially phosphorylates the p38α and p38ß isoforms. The specificity of p38 activation is due to the the formation of functional complexes between MEK3/6 and different p38 isoforms where MEK3/6 recognises specific sequences in the activation loop of p38. Activation of the p38 isoforms is caused by a MEK3/6-catalyzed phosphorylation of a conserved Thr-Gly-Tyr (TGY) motif in the activation loop of p38 at Thr180 and Tyr182.

Both ERK1/2 and p38 kinases phosphorylate proteins containing Serine/Threonine residues that are followed by a Proline (S/T-P). ERK and p38 can phosphorylate substrates at various locations within the nucleus and the cytoplasm (Roux, Blenis 2004). The use of two-hybrid screens in addition to novel phosphorylation screens looking for ERK substrates discovered a kinase termed the MAPK signal integrating kinase, or Mnk, with a unique ability to interact with both p38 and ERK2

(Waskiewicz *et al*., 1997; Fukunaga, Hunter 1997). Mnk1 and 2 share 88% similarity in their catalytic domains and their N-and C- termini share 77% and 65% similarity, respectively. Both Mnk isoforms interact with eIF4F *in vivo* (Waskiewicz *et al*., 1999; Scheper *et al*., 2001). The Mnks directly bind with the C terminal domain of eIF4GI and eIF4GII in mammalian cells and therefore eIF4G acts to bring the Mnk kinase in close proximity to phosphorylate the eIF4E substrate. As such 4E is phosphorylated when it is part of the 4F complex. Consequently, when Mnk's are absent from the eIF4F complex, eIF4E has been shown to be less phosphorylated than when Mnk's are bound to eIF4G (Waskiewicz *et al*., 1999).

Although both Mnks are responsive to ERK and p38 stimulation in vitro their basal activities differ *in vivo* (Fukunaga, Hunter 1997; Waskiewicz *et al*., 1997). *In vivo* Mnk1 has a low level of activity, which is increased when either the ERK or the p38 MAP kinase α/β pathways are stimulated (Wang *et al*,. 1998; Waskiewicz *et al*., 1997; Fukunaga, Hunter 1997). Conversely, Mnk2 has a high basal activity which is not enhanced upon ERK or p38 MAP kinase pathway stimulation (Scheper *et al*., 2001). Additionally, the finding that Mnk2 activity can be reduced upon inhibition of both the ERK and p38 pathways has led to suggestions that low basal activities of these pathways in unstimulated cells is sufficient to allow for high basal Mnk2 activity (Scheper *et al*., 2001). As Mnk1 and Mnk2 differ in their basal activities and regulation, the ratio of Mnk1 to Mnk2 may have a large bearing on eIF4E phosphorylation. In cells that primarily contain Mnk1, the level of eIF4E phosphorylation will depend on the stimulation of ERK and p38 along with the level of functioning eIF4F complexes which mediate the Mnk1-eIF4E interaction, whereas in cells containing mainly Mnk2, the amount of eIF4F will be the limiting factor.

To date conflicting reports have surfaced with regard to the exact mechanistic function eIF4E phosphorylation performs during the process of translation initiation. Initial experiments suggested that eIF4E increases in binding affinity to the cap structure upon phosphorylation (Bu, Haas & Hagedorn 1993; Minich *et al*., 1994). One hypothesis based on analysis of the co-crystal structure of eIF4E bound to the cap proposed that the Ser209 located behind the cap binding slot could form a retractable salt bridge over the mRNA by binding to a Lys159 residue which lies opposite from Ser209 and in the putative mRNA path. This retractable clamp would thus stabilize the mRNA in the cap-binding slot and consequently phosphorylation would enhance

the binding of eIF4E to capped mRNAs and long cap analogs (Marcotrigiano *et al*., 1997). This hypothesis was subsequently dispelled by more recent crystallographic studies using larger ligands (m^7GpppG m^7GpppA) showing that a salt bridge formation was not possible as the distance between the Lys 159 and Ser209 was too vast (Tomoo *et al*., 2002; Niedzwiecka *et al*., 2002; Tomoo *et al*., 2003). The initial studies which led to the hypothesis that eIF4E increases in binding affinity to the cap structure upon phosphorylation were performed before the Mnks had been identified, and used chromatography on RNA-cellulose to separate phosphorylated from unphosphorylated eIF4E. The fractions of eIF4E that were unbound to the resin was found in the phosphorylated form, while the bound material was unphosphorylated. Using fluorescence methods, it was found that the fraction containing the phosphorylated eIF4E had three to four times greater affinity for m^7GTP and for capped (globin) RNA, but questions over this approach arose on the basis that the resolution of these forms on RNA-Sepharose was unclear. Additionally, it was possible that fractions used in these assays were contaminated with other proteins that affect the affinity of eIF4E for capped RNA. For example, it was plausible that the fractions contained 4E-BPs or eIF4G, which increase the binding of eIF4E to the cap. This caveat to the investigation was not known at the time and could have biased the results obtained.

More recent studies into the role of eIF4E phosphorylation employed Mnks to produce stoichiometrically phosphorylated eIF4E in vitro. The binding of eIF4E to cap analogue (m^7GTP) was examined by fluorescence quenching and it was found that phosphorylated eIF4E bound with a lower affinity (2.5-fold difference) than the unphosphorylated protein. The same group also conducted surface plasmon resonance to examine binding of eIF4E to a capped oligonucleotide. The capped mRNA ligand was immobilized on a biotin group at its 3'-end, which allows very tight binding to the streptavidin chip surface and is thought to more accurately resemble the physiological ligand of eIF4E. In these experiments, phosphorylation of eIF4E reduced its ligand affinity by five fold and acidic mutations at Ser209 which mimic the charge of phosphate, also decreased the affinity of eIF4E, although to a lesser degree than phosphorylation (Scheper *et al*., 2002).

Thus, the reduction in affinity for the cap could reduce translation rates, or by releasing eIF4F from the cap promote scanning and translation initiation. Indeed

many studies have shown that eIF4E phosphorylation is either positively of negitively modulated in response to viral infection. For example upon infection with either Herpes Simplex Virus-1 (HSV-1) or Human Cytomegalovirus (HCMV), phosphorylated eIF4E accumulates rapidly through the exclusive activation of the p38 kinase pathway (Walsh, Mohr 2004; Walsh *et al*., 2005). Whereas following infection of cells with adenovirus, unphosphorylated eIF4E accumulates and this correlates with the inhibition of host protein synthesis (Huang, Schneider 1991). In contrast, studies analysing eIF4E phosphorylation in various systems have shown that phosphorylation of eIF4E may not be a requirement for efficient translation (McKendrick *et al*., 2001; Morley, Naegele 2002) and may even have a negative effect on translation (Knauf, Tschopp & Gram 2001).

1.2.1.1.1.3 4E binding proteins

Another mechanism that regulates eIF4E activity is its interaction with a family of repressor proteins termed the eIF4E-binding proteins (4E-BPs) which function as a metabolic brake to control translation in response to nutrient availability, stress and growth signals (Teleman, Chen & Cohen 2005). These proteins were initially identified by the far western technique which isolated two small 12kDa proteins that bind to eIF4E. These proteins, termed 4E-BP1 and 4E-BP2 share 56% amino acid homology and were found to inhibit cap-dependent translation both *in vivo* and in an *in vitro* cell free translation assay (Pause *et al*., 1994).

Binding of the 4E-BPs to eIF4E does not alter the affinity of eIF4E for the cap, as experiments using a matrix-bound cap have found that eIF4E/4E-BP1 binding does not alter eIF4E cap binding (Pause *et al*., 1994). The mechanism of translation inhibition arises from 4E-BPs ability to prevent eIF4E association with eIF4G, therefore preventing the formation of the eIF4F complex (Mader *et al*., 1995; Poulin *et al*., 1998).

1.2.1.1.1.3.1 Regulation of 4E-BP affinity through phosphorylation

The phosphorylation of specific Serine/Threonine residues on 4E-BP regulates its affinity for eIF4E. Hypophosphorylated 4E-BPs bind strongly to eIF4E, but upon phosphorylation a decrease in affinity for eIF4E and a relief in translational repression is observed. 4E-BP phosphorylation occurs on sites Thr37, Thr46, Ser65, Thr70, Ser 83, Ser101 Ser112 and phosphorylation occurs in a hierarchical manner. Firstly, phosphorylation on Thr37 and Thr46 is required as a priming event for the subsequent phosphorylation of Thr70, which is then followed by phosphorylation of Ser65 and the release of eIF4E (Gingras, Raught & Sonenberg 2001; Mothe-Satney *et al*., 2000). Phosphorylation of Ser101 seems to be constitutive and has been reported to be required for efficient phosphorylation of Ser65 *in vivo* (Wang *et al*., 2003). The roles of phosphorylated Ser83 and Ser112 are less clear; although priming events have been postulated, subsequent studies discovered no effects on hierarchical phosphorylation events when these serine residues were mutated to alanines (Ferguson, Mothe-Satney & Lawrence 2003).

Growth factors, cytokines, mitogens, G protein coupled receptors, hormones (insulin, angiotensin) and adenovirus infection have been reported to induce phosphorylation of 4E-BP (Feigenblum, Schneider 1996; Gingras, Sonenberg 1997; Rao *et al*., 1994; Wang *et al*., 1998; Graves *et al*., 1995), whereas heat shock and poliovirus infection have been reported to decrease phosphorylation (in specific cell types) (Gingras *et al*., 1996; Scheper *et al*., 1997), which led to the suggestion that 4E-BP was activated through MAPK kinase pathways. This hypothesis was subsequently disproved by studies which reported inhibition of 4E-BP activity in cells treated with the inhibitors Wortmannin and Rapamycin which have no inhibitory effect on MAPK pathways (von Manteuffel *et al*., 1996; Gingras *et al*., 1999).

A link between AKT activity and the levels of 4E-BP1 phosphorylation was first proposed when HEK 293 cells expressing a dominant negative mutant of AKT blocked insulin mediated phosphorylation of 4E-BP1. The study also highlighted that upon AKT activation 4E-BP becomes phosphorylated on the same residues as occurs upon serum stimulation and that a hyper activated form of AKT was insensitive to Wortmannin but was sensitive to the inhibitor Rapamycin (Gingras *et al*., 1998). These results pointed to a kinase downstream of AKT being responsible for 4E-BP phosphorylation.

With 4E-BP1 established as a target for regulation by PI3K–AKT signalling the Frap/mTOR complex was elucidated to be responsible for 4E-BP phosphorylation when a Rapamycin resistant Frap/mTOR1 conferred Rapamycin resistance to 4E-BP1 phosphorylation (Brunn *et al*., 1997) and studies have shown that mTOR plays an essential role in the phosphorylation of 4E-BP1 in response to insulin treatment and nutrient levels in cells (Raught, Gingras 1999; Proud 2002).

Currently, the mechanism by which mTOR activates 4E-BPs remains ambiguous. Whether mTOR directly or indirectly phosphorylates 4E-BP remains unclear but reports have emerged suggesting that Raptor may play a role as a scaffold protein that bridges mTOR to its substrates (Hara *et al*., 2002). Additionally, the C-terminus of 4E-BP contains a TOR signalling (TOS) motif which was found to be required for phosphorylation at mTOR regulated sites (Schalm *et al*., 2003). This motif is also required for binding to Raptor suggesting a role for the regulation of 4E-BP (Schalm

et al., 2003; Choi, McMahon & Lawrence 2003; Nojima *et al*., 2003). Another four amino acid sequence named the RAIP has been shown to play a role in the association with raptor and mTOR-mediated phosphorylation of 4E-BP1; however its function is mainly accessory whereas TOS motif has an essential function to this process in response to amino acid stimulation (Eguchi *et al.*, 2006).

1.2.1.1.1.3.2 PI3K-AKT-mTOR Signalling.

Wortmannin inhibits the phosphoinositide 3-kinase (PI3K), which in response to a variety of growth factors are a family of lipid kinases which phosphorylate the hydroxyl group at position three of the inositol ring of phosphatidylinositols. PI3Ks are involved in cellular functions such as growth, proliferation, differentiation, motility, survival and intracellular trafficking (Vanhaesebroeck *et al*., 1997; Courtney, Corcoran & Engelman 2010).

Activation of PI3K upon ligand binding to receptor tyrosine kinases results in the production of phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3) or phosphatidylinositol (3,4) diphosphate (PtdIns(3,4)P2), which in turn bind to the pleckstrin homology domain of AKT. AKT is a serine–threonine kinase that has three family members: AKT1, AKT2 and AKT3, which are encoded by three different genes (Datta, Brunet & Greenberg 1999). Since PtdIns(3,4,5)P3 and PtdIns(3,4)P2 are restricted to localise to the plasma membrane, this results in AKT localisation to the plasma membrane. Likewise, the phosphoinositide-dependent protein kinase 1 (PDK1) also contains a pleckstrin homology domain that allows it to bind to PtdIns(3,4,5)P3 and PtdIns(3,4)P2, causing PDK1 to also translocate to the plasma membrane upon activation of PI3K (Chan, Rittenhouse & Tsichlis 1999). PDK1 colocalization with AKT causes PDK1 to phosphorylate AKT on residue threonine 308 causing partial activation of AKT. AKT is fully activated upon phosphorylation of serine 473 by the TORC2 complex of the mTOR protein kinase (Sarbassov *et al*., 2005). Once phosphorylation occurs at both sites, AKT detaches from the plasma membrane and can then phosphorylate its substrates within the cell. AKT/PKB has been implicated in a variety of cellular processes, including proliferation and cell growth, apoptosis, regulation of gene expression and translational control (Chan, Rittenhouse & Tsichlis 1999).

Frap/mTOR is the mammalian homologue of yeast TOR (Target of Rapamycin) (Helliwell *et al*., 1994). Mammalian TOR (mTOR) is a large 298 kDa Serine/Threonine protein kinase that belongs to the family of PI3K-related protein kinases (PIKKs)(Keith, Schreiber 1995). mTOR exists as part of two functional complexes, mTORC1 and mTORC2. mTORC1 is composed of mTOR, FRAP and its regulatory protein Raptor, along with PRAS40, DEPTOR and mammalian LST8/G-protein β-subunit like protein (mLST8/GβL) which stimulates the in vitro kinase activity of mTOR. mTOR can be inhibited by Rapamycin, a macrocyclic antibiotic and immunosupressant which binds the cystolic protein FKBP12. The subsequent Rapamycin/ FKBP12 complex then binds to FRAP causing a potent inhibition of the mTORC1 complex (Chan 2004; Kim *et al*., 2003; Kim et al. 2002; Hara *et al*., 2002; Loewith et al. 2002; Sancak *et al.*, 2007; Peterson *et al.*, 2009; Haar *et al*., 2007).

Alternatively, the Rapamycin insensitive mTOR Complex 2 (mTORC2) is composed of mTOR, FRAP, Rictor and GβL along with the mammalian stress-activated protein kinase interacting protein 1 (mSIN) (Frias *et al*., 2006; Jacinto *et al*., 2006; Jacinto *et al*., 2004; Dos D. Sarbassov *et al*., 2004; Yang *et al*., 2006). In addition to their differential sensitivity to Rapamycin, mTORC1 and 2 are stimulated in alternate ways and have distinct substrate specificities. mTORC1 responds to viral infection, amino acids, growth factors, energy and oxygen levels (Walsh, Mohr 2004; Nobukuni *et al*., 2005; Xu *et al.*, 2010; Schneider, Younis & Gutkind 2008), whereas mTORC2 activation is less understood, but seems to be activated only by growth factors although recent reports suggest Vaccinia virus may also activate this protein (Zaborowska, Walsh 2009).

mTOR is also directly regulated by factors such the tuberous sclerosis complex (TSC). The TSC complex is formed by TSC1 and TSC2 and causes the inhibition of the mTORC1 activator GTPase Ras-homolog enriched in brain (Rheb) by causing a hydrolyzion of its GTP to GDP (Inoki et al., 2003). TSC2 is itself activated by phosphorylation by the AMP-activated protein kinase (AMPK) (Inoki, Zhu & Guan 2003) and AMPK activation is the result of a high AMP to ATP ratio in the cell. AMPK also causes the phosphorylation and inactivation of Raptor, which is required for efficient phosphorylation of mTOR, thus AMPK inhibits mTORC1 activity by

TSC-dependent and TSC-independent mechanisms (Gwinn et al., 2008). AMPK activity is itself regulated through phosphorylation by the tumor suppressor LKB1 (Kyriakis 2003; Marignani 2005).

AKT inactivates TSC1/2 by phosphorylating TSC2 (Manning *et al*., 2002). Additionally, AKT inhibits PRAS40, a regulator of mTORC1 that negates Rheb function (Haar *et al*., 2007; Sancak *et al*., 2007). The activation of mTORC2 is not well understood, but as mentioned previously this complex directly activates AKT (and AKT-related kinases) by phosphorylation and seems to be part of a possible mTOR –AKT regulatory loop.

Counteracting AKT function is the tumor suppressor phosphatase and tensin homolog deleted on chromosome ten (PTEN) which is a lipid phosphatase that converts phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3) to phosphatidylinositol (3,4) diphosphate (PtdIns(3,4)P2) therefore quelling the signalling from PI3K (Ramaswamy *et al*., 1999).

Figure 1.5 Intracellular signalling pathways regulating the phosphorylation of the translation initiation factor eIF4E and the 4E-BPs and the kinase inhibitors used in this study.

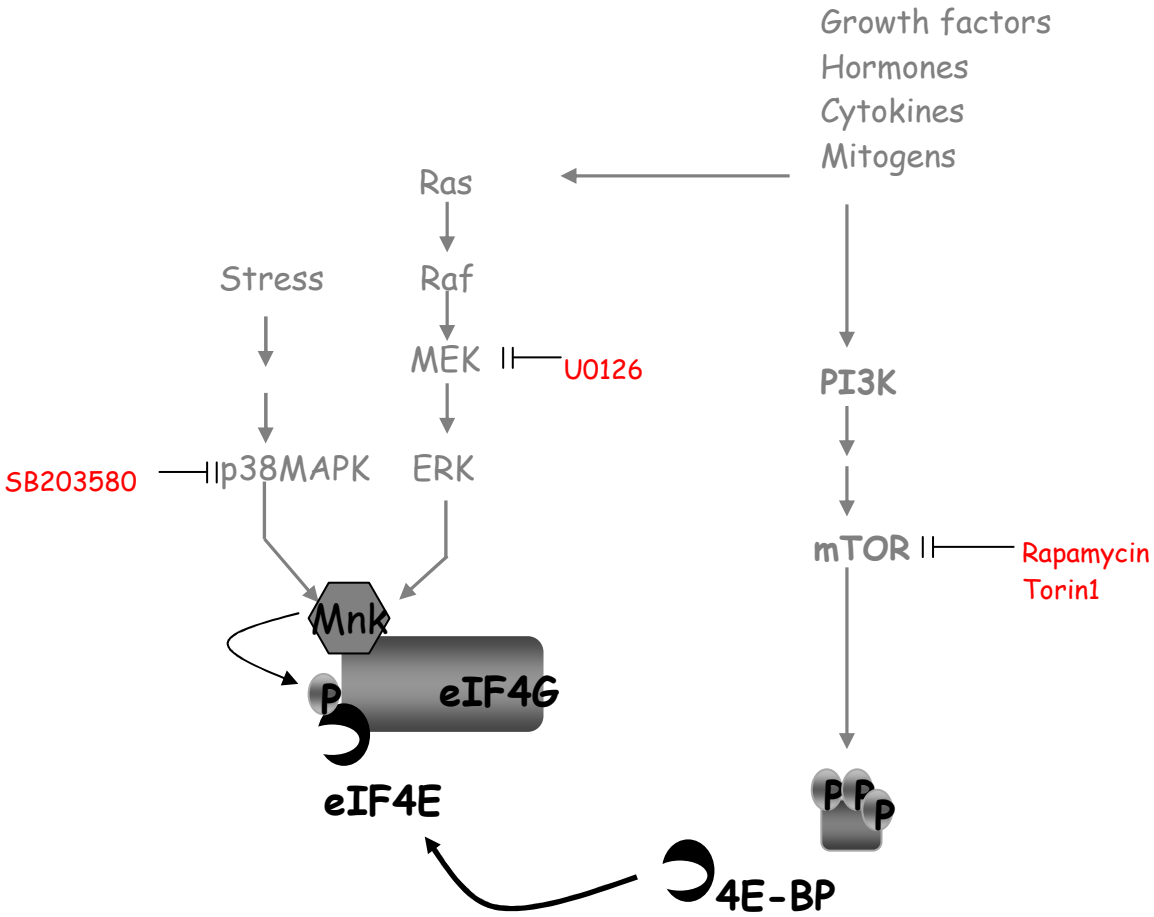

Figure 1.5 The stimulation of signalling pathways can regulate both the availably and activity eIF4E. Hypophosphorylated 4E-BPs inhibits translation through sequestering eIF4E, which can either be free or bound to the cap complex. Upon activation of the PI3K pathway, mTOR can hyperphosphorylate 4E-BP which results in the release eIF4E allowing it to bind to eIF4G, so permitting translation to proceed. Additionally, stimulation of either the stress activated p38 pathway or the mitogen activated ERK pathway can result in the phosphorylation of Mnk. Mnk can bind to the C-terminus of eIF4G and phosphorylate eIF4E on its Ser209 residue.

1.2.1.2 Eukaryotic initiation factor eIF2

Another rate limiting step in the process of mRNA translation initiation is the regulation of eIF2 activity. The function of eIF2 is to mediate the binding of the initiator Met-tRNA to the ribosome in a GTP-dependent manner during the initiation of translation of all cytoplasmic mRNAs in eukaryotic cells.

When in a GTP-bound state the eIF2 complex binds with Met-tRNA$_i$. The eIF2·GTP·Met-tRNA$_i$ 'ternary' complex is then able to bind to the 40S ribosomal subunit. The Met-tRNA$_i$ recognises and binds to the start codon on the mRNA. Upon binding at the AUG start codon, hydrolysis of eIF2-bound GTP occurs in a process mediated by eIF5 (Das, Ghosh & Maitra 2001). Subsequent to hydrolysis of GTP, the newly formed GDP-bound eIF2 is released from the ribosome and mRNA translation begins. To return to its active GTP-bound state, eIF2 must undergo nucleotide exchange, and this function is performed by eIF2B. eIF2 is composed of three subunits termed α, β, and γ. The α subunit contains a phosphate acceptor at Ser 51. The β-subunit contains multiple phosphorylation sites (residues 2, 13, 67, 218) and three lysine clusters in the N-terminal domain (NTD) which mediate the interaction with guanine nucleotide exchange factors (GEFs). The γ-subunit comprises three guanine nucleotide binding sites and is known to be the main docking site for GTP/GDP (Kimball 1999).

eIF2B facilitates the release of GDP from eIF2 as it is a GDP-dissociation stimulator protein (GDS) (Williams *et al.*, 2001). The best characterized mechanism for regulating eIF2B activity involves phosphorylation of the eIF2α subunit at Ser51. Upon eIF2α phosphorylation, eIF2B binds to eIF2 with a much greater affinity but cannot be released after GTP transfer, thus prohibiting the binding of new Met-tRNA. As eIF2B is present at low levels relative to eIF2, eIF2α phosphorylation sequesters eIF2B and is the major regulator of global translation initiation.
eIF2B is comprised of five subunits termed α, β, ,γ, δ and ε. eIF2Bα/β/δ form a regulatory subcomplex that sensitises eIF2B to inhibition by eIF2α phosphorylation (Pavitt, Yang & Hinnebusch 1997; Krishnamoorthy *et al.*, 2001). The ε subunit contains the proteins catalytic domain (Gomez, Mohammad & Pavitt 2002; Boesen *et al.*, 2004) and forms a binary 'catalytic' subcomplex with eIF2Bγ, with which it has

partial sequence homology (Pavitt, Yang & Hinnebusch 1997). In addition, the extreme C-terminus of the ε subunit is involved in interacting with eIF2 in a process that requires phosphorylation on sites within this region (Asano *et al*., 1999).

eIF2α phosphorylation is regulated in mammalian cells by four different eIF2α kinases. This common substrate has led to these four kinases being classified as part of the integrated stress response (ISR) (Harding, Ron 2002). Through work facilitating transgenic mice which have targeted knock-outs of each kinase or where eIF2α phosphorylation was eliminated by a targeted Ser51 to alanine knock in to remove the eIF2α phosphorylation site, the characteristic nature and importance of these kinases with regard the integrated stress response has been elucidated. Of importance was that experiments using these mice showed that loss of integrated stress response kinase activity or eIF2α phosphorylation resulted in as loss of mRNA translation inhibition in response to ER stress thus confering a reduced survival capacity (Scheuner *et al*., 2001).

1.2.1.2.1 eIF2α kinases

Heme-regulated inhibitor (HRI).

The Heme-regulated inhibitor was initially identified during studies on reticulocytes. Reticulocytes are the precursors of red blood cells which produce large amounts of globins. When reticulocytes are heme depleated mRNA translation shuts off, an event which is the result of eIF2α phosphorylation (Chen, London 1995). Studies have elucidated HRI as the kinase which regulates eIF2α phosphorylation upon heme depleation by providing evidence that that HRI activity was incresingly quelled by increasing heme levels in reticulocytes. Furtheremore studies employing targeted knock-out mice have shown that HRI is essential for the shuting off protein synthesis in reticulocytes deprived of heme (Han *et al.*, 2001).

PKR

Protein kinase activated by dsRNA (PKR) or Eukaryotic translation initiation factor 2-alpha kinase 2, is a kinase activated by double-stranded (ds) RNA. This function is facilitated by its N-terminal RNA-binding domains (RBDs). Upon RNA binding, PKR dimerises, resulting in the activation of the enzyme by autophosphorylation. PKR is expressed at low levels in many cell types but is induced by interferons (IFNs) (Hovanessian 2007). PKR plays a primary role in protecting the cell from invading viral infection. During viral replication dsRNA is produced which then activates PKR and leads to the phosphorylation of eIF2α resulting in translational inhibition, thus causing a repression of viral replication. The importance of PKR for controlling viral replication is highlighted by the fact that viruses have evolved many strategies to inhibit PKR which include mechanisms to counteract the induction or effects of interferons (Goodbourn, Didcock & Randall 2000) in addition to preventing the activation of dsRNA-dependent pathways and avoiding the repressive effects of the PKR and 2′-5′ oligoadenylate synthetase/RNase L system (Goodbourn, Didcock & Randall 2000).

PERK (PKR like endoplasmic reticulum kinase)

PERK (PKR like endoplasmic reticulum kinase or EIF2AK3) is a kinase which is activated in response to misfolded protein in the endoplasmic reticulum. PERK has a kinase domain with homology to other eIF2α kinases and its N-terminal domain has homology to the IRE1, which is a protein involved in the unfolded protein response (UPR) (Harding, Ron 2002). Under normal cell conditions the IRE1-domain of PERK binds to the chaperone BiP/GRP78. Upon ER stress, BiP dissociates from the IRE1-domain of PERK and consequently binds to proteins in the ER lumen. Once BiP is no longer bound to PERK, PERK molecules can then dimerise. The dimerisation of PERK results in its activation and leads to the phosphorylation of eIF2α which then causes an inhibition of mRNA translation. As many proteins are destined for the endoplasmic reticulum this inhibition thus allows the the organelle time to deal with the existing load of proteins it has to process (Kapoor, Sanyal 2009).

GCN2

GCN2 is a kinase found in yeast and mammals that functions to inhibit translation during amino acid starvation. It has a kinase domain that is homologous PKR, PERK and HRI and has a C-terminal domain similar to histidinyl-tRNA synthetases (HisRSs). During amino acid depleation, uncharged tRNAs bind to the C-terminal domain of Gcn2p. The resulting interaction activates Gcn2 and causes the induction of eIF2α phosphorylation. Gcn2 has also been shown to phosphorylate eIF2α in response to ultraviolet radiation (Zhang *et al*., 2002; Deng *et al*., 2002).

1.2.2 Mechanisms of Translational Control

As previously described, mRNA translation is of paramount importance in regulating gene expression. Regulation of translation initiation facilitates the cells ability to respond rapidly to stimuli. In response to such stimuli global rates of translation initiation can increase in many cell types and mRNAs which normally remain translationally repressed become translated. Many of these mRNAs produce growth factors and proto oncogenes involved in proliferation and differentiation.

In addition to the activity of translation initiation factors, the efficiency of mRNA translation depends on the degree of innate complexity with in the mRNA structure. Most cellular mRNAs in vertebrates contain 5′ UTR which are from 10 to 200 bases long, whereas 60% of mRNAs which encode growth factors contain 5′ UTRs with more than 200 bases (Willis 1999).

Within these UTRs there can exist various cis-acting elements which can regulate translational efficiency. Under normal conditions (e.g cells in a resting state) these elements prohibit efficient translation whereas under conditions favouring growth, repressive cis acting elements within UTRs can no longer prevent translation initiation. The following are repressive elements found within 5'UTRs.

(i) The sequence flanking the initiation codon

The scanning model currently posits that the 40S ribosomal subunit which is bound to Met-tRNA and various initiation factors binds initially at the 5'-end of mRNA and then traverses the mRNA in a linear proccessive fashion, upon reaching the first AUG codon in a favourable context, translation initiation begins. The model thus suggests that both the AUG start codon position relative to the 5'end and context of the neighbouring codons within the sequence influence the selection of the initiation site (Kozak 1987).

(ii) Long UTRs with complex secondary structures

The majority of cellular mRNAs have 5'UTRs of up to 100 nucleotides in length.

However, many mRNAs which code for oncoproteins, growth factors, transcription factors and signal transduction components have long 5'UTRs which can have a high

GC content (70 to 90%). This degree of length and GC content suggests a high degree of mRNA secondary structures which have the ability to form stem loop structures. These secondary structures have an inhibitory effect on the ribosomes capacity to scan from the cap to the AUG start codon and need high eIF4F activity for efficient translation (Baim *et al*., 1985; Pelletier, Sonenberg 1985; Kozak 1987; Cigan, Pabich & Donahue 1988).

(iii) Upstream open reading frames

A subset of mRNAs contain long leader sequence which contain one or more upstream open reading frames (uORFs) in the 5'UTR. The presence of uORFs can repress the translation of downstream cistrons, and in some cases these uORFs are also involved in the selective translation of specific mRNAs (Geballe, Morris 1994).

(iv) Binding sites for specific regulatory proteins

Many mRNAs contain binding regions for inhibitory proteins in their 5' UTR. These proteins can compete with the 43S complex for binding to mRNA thus preventing translation initiation (Hentze, Kühn 1996).

(v) Oligopyrimidine tracts at the extreme 5′ terminus (5′ TOP mRNAs)

One common feature found in sequenced mammalian ribosomal protein mRNAs is the oligopyrimidine tract at the 5' terminus. This element, referred to as the 5' terminal oligopyrimidine tract (5'TOP), is usually composed of a cytidine residue at the cap site followed by an uninterrupted sequence of 7-13 pyrimidine nucleotides. Considerable evidence has been presented showing that 5′ TOP is a cell-growth-dependent translational *cis*-regulatory element in mammalian cells (Kaspar *et al*., 1992; Biberman, Meyuhas 1997).

(vi) Internal ribsome entry sites

Another mechanism by which ribosomes may be recruited to eukaryotic mRNAs is by direct binding of the 40S subunit to internal structures within the mRNA. This internal binding negates the need for the 5′ end containing the cap, consequently certain initiation factors are not required for mRNA translation initiation. Internal binding of the ribosome to sites termed internal ribosome entry sites (IRES) was first identified

for two picornaviruses, poliovirus and encephalomyocarditis virus (Pelletier, Sonenberg 1988; Jang *et al*., 1988).

Picornavirus mRNAs translate by a cap-independent mechanism as they do not contain a 5’ cap on their mRNA. Additionally, they contain long 5′ UTRs (Belsham, Sonenberg 1996), have significant secondary structure and contain numerous redundant AUGs (Belsham, Sonenberg 1996). In addition to viral RNA, IRESes also occur in capped RNAs of cellular origin such mRNAs for BiP, fibroblast growth factor-2 (FGF-2) (Nanbru et al. 1997), MYC proto-oncogene product, and vascular endothelial growth factor (VEGF) (Macejak, Sarnow 1991; Vagner *et al*. 1995; Subkhankulova, Mitchell, Willis 2001; Huez *et al*. 1998). It is hypothesised that the extensive secondary and tertiary structure of the IRES region can be recognized by the translation machinery in various ways and is responsible for ribosome binding. This is likely mediated by RNA structure due to the fact that IRESs vary in length and lack identifiable consensus sequences. Supporting this theory was computer analysis conducted by Le and Maizel which presented a common structural motif for the IRESes of several cellular genes (BiP, platelet-derived growth factor 2, VEGF, and FGF-2) as well as IRES of HCV and picornaviruses. This structural motif is composed of a Y-shaped pseudoknot stem-loop which is followed by a smaller simple stem-loop (Le, Maizel 1997).

1.3 Viral manipulation of translation initiation factor functions

As obligate intracellular parasites viruses have evolved many techniques to manipulate host mRNA translation to favour viral replication. To this end many, viruses also target eIF4F.

1.3.1 RNA viruses and eIF4F

Given the role of eIF4F in cap dependent translation many viruses target its activity. RNA viruses do not require intact eIF4F to efficiently translate their mRNA and consequently have evolved mechanisms to inhibit host cap dependent translation while promoting cap independent translation of their own mRNA. RNA viruses such as poliovirus and encephalomyocarditis virus code positive-stranded viral mRNA that is translated by an internal ribosome entry mechanism, which facilitates internal ribosome entry and mRNA translation independent of the m^7cap structure. Typically, they facilitate ribsome binding, either directly or through binding to the C-terminal region of eIF4G, obviating the need for cap binding initiation factors. During virus infection, virus-encoded proteases cleave the eIF4G component of eIF4F at the N-terminal domain. The N-terminal cleavage product contains the binding site for eIF4E and PABP, whereas the C-terminal product contains binding sites for eIF4A and eIF3, which has ribosome binding activity.

Once eIF4G is cleaved host cell cap dependent translated is curtailed allowing viral IRES dependent translation to proceed unimpeded by competition for ribosomal engagement (Schneider, Mohr 2003).

1.3.1.2 DNA Viruses

HSV-1:

HSV-1 encodes mRNAs which are capped. As a result it endeavours to sequester host cells initiation factors while suppressing host cell translation during productive infection. HSV-1 encodes the mRNA U_L 41 and its protein product is termed the Virion host shutoff protein or Vhs. Vhs is a 58 kDa polypeptide, which is packaged in the tegument of HSV-1 virions. Its primary function is to shut off host cell translation

and it achieves this by degrading host cell mRNA by associating with the translation factors eIF4A and eIF4H, thus allowing viral mRNA to compete efficiently for host cell initiation factors (Feng, Everly & Read 2001).

During lytic infection HSV-1 uses multiple strategies to stimulate the translational machinery to ensure that its mRNAs are translated efficiently. Firstly, during the initial stages of infection the stress activated kinase p38 is activated, a process which is thought to be mediated by the viral protein ICP27 (Gillis, Okagaki & Rice 2009). The activation of this pathway results in increased Mnk1 activity which increases the phosphorylation of eIF4E bound to eIF4G (Walsh, Mohr 2004; Walsh, Mohr 2006). Indeed, prevention of eIF4E phosphorylation by inhibiting p38 or Mnk-1 phosphorylation significantly reduced HSV-1 mRNA translation and viral replication in quiescent primary human cells (Walsh, Mohr 2004). In addition to stimulating the eIF4E kinase Mnk-1, HSV-1 promotes phosphorylation of the translational repressor 4E-BP1 and its degradation by the cellular proteasome allowing eIF4E to bind to eIF4G and form active eIF4F. Although 4E-BP1 is phosphorylated through mTOR, the exact viral protein responsible for mTOR activation is unknown (Walsh, Mohr 2004).
In addition to stimulating signalling pathways during lytic infection, HSV-1 encodes ICP6 whose product is a 140kDa protein which acts as a chaperone to promote the assembly of eIF4F complexes in quiescent cells which have been lytically infected (Walsh, Mohr 2006).

Human Cytomegalovirus (HCMV):

HCMV like HSV-1 produces mRNAs which are capped on their 5’ ends. While HCMV cannot totally suppress the synthesis of host polypeptides during infection, HCMV infection results in the phosphorylation of the cap binding protein eIF4E and the translational repressor 4E-BP1 early in infection. Additionally during infection, HCMV increases the overall abundance of eIF4F components, eIF4G, eIF4E and PABP which promotes assembly of eIF4F complexes resulting in a reduction of competition between viral and host cellular mRNA (Mohr, Walsh 2005)

KSHV:

While little is known about the requirements for the mRNA translation apparatus during KSHV latent or lytic infection, a recent report detailing reactivation of KSHV from latency in Primary effusion lymphoma derived B cells showed both phosphorylation of 4E-BP and eIF4E as well as eIF4F assembly were initiated upon viral reactivation (Arias *et al*., 2009). Critically the report also showed that Mnk1 inhibition caused a reduction of the accumulation of the viral gene transactivator RTA and therefore suggests that cap dependent translation is required by KSHV during reactivation from latency (Arias *et al*., 2009).

Adenovirus:

During late stages of adenovirus infection, cap-dependent translation is inhibited. Adenovirus inhibition of cellular protein synthesis correlates with a strong decrease in eIF4E phosphorylation (Huang, Schneider 1991; Zhang, Feigenblum & Schneider 1994) but this inhibition does not involve eIF4E sequestration by the 4E-binding proteins. Instead the Adeno virus late L4 100-kilodalton (L4 100K) binds to eIF4G displacing Mnk1 and preventing eIF4E phosphorylation thus inhibiting cap dependent protein synthesis (Cuesta *et al*., (Cuesta *et al*., 2000).

Adenovirus late mRNAs are translated despite the inhibition of host cell protein synthesis as late adenovirus mRNAs contain a 200-nucleotide 5′ noncoding region, known as the tripartite leader. The tripartite leader mediates translation by a novel initiation mechanism termed ribosome shunting. During ribosome shunting the 40S ribosomal subunit associates to the 5’ cap structure with eIF4G but is directed by its tripartite leader to translocate to the downstream initiating AUG in a nonlinear fashion, this causes the ribosome to shunt over and bypass the intervening RNA regions. Ribosome shunting in late Adenovirus infected cells has been found to be enhanced with dephosphorylation of eIF4E and inhibition of host cell protein synthesis with 100K fulfilling this function (Yueh, Schneider 1996; Yueh, Schneider 2000).

1.3.2 Viral strategies for inhibition of PKR and eIF2 phosphorylation.

HSV-1 has two mechanisms for preventing the activation of PKR. The first is the expression of the γ34.5 protein which inhibits the downstream effects of PKR by recruiting the cellular protein phosphatase 1 (PP1) thus forming a complex causing the dephosphorylation eIF-2α (He, Gross & Roizman 1998). HSV-1 also encodes a protein called Us11, which functions to bind double stranded viral RNA and mask it from PKR thus preventing eIF2α phosphorylation. Another function of Us11 is its ability to bind to the N-terminal domain of PKR directly preventing PKR activation by PACT, which is itelf activated by stress conditions in the absence of foreign dsRNA (Cassady, Gross 2002; Peters *et al*., 2002).

Kaposi sarcoma-associated herpesvirus (KSHV) produces two proteins that obstruct PKR's action: vIRF2 and LANA2. vIRF2 binds to PKR and prevents its autophosphorylation (Burysek, Pitha 2001). LANA2 is homologous to cellular IRF-4 (Rivas *et al*., 2001) and inhibits apoptosis and PKR-mediated translational inhibition (Esteban *et al*., 2003).

Epstein–Barr virus (EBV) produces the RNA species EBER-1. EBER-1 mRNA is not translated but is key to the Epstein–Barr viruses endeavour to transform cells (Clemens 2006). In-vitro EBER-1 has been found to inhibit PKR by binding to its dsRNA domain, thus preventing dsRNA activators present in the cell from binding to PKR (Sharp *et al*.. 1993). EBV also encodes for the SM protein which is a post transcriptional regulator of gene expression that also fuctions to bind dsRNA preventing PKR activation (Poppers *et al*., 2003).

Vaccinia virus expresses two proteins, E3L and K3L, which can inhibit intracellular IFN-induced pathways. E3L prevents activation of PKR and OAS, (2′-5′ oligoadenylate synthetase) by binding to and cloaking dsRNA molecules (Shors *et al*., 1997; Rivas *et al*., 1998). E3L can also directly bind to PKR which leads to heterodimer formation and repression of PKR function (Romano *et al*., 1998; Sharp *et al*., 1998). The K3L protein inhibits the dimerisation and autophosphorylation of PKR, thus preventing an inhibition of protein synthesis (Carroll *et al*., 1993; Davies *et al*., 1992).

The Influenza virus codes for a protein called NS1 which is similar to E3L and has a dsRNA-binding domain that inhibits IFN-α/β synthesis in addition to PKR activation (LU *et al*., 1995; Hatada, Saito & Fukuda 1999; Bergmann *et al*., 2000). Like HSV-1 Us11, NS1 can also prevent PKR activation by inhibiting its cellular activator PACT (Li *et al*., 2006).

Human immunodeficiency virus type 1 (HIV-1) encodes a protein labelled Tat which is a transcriptional transactivator that can also act as a substrate homologue of eIF2α, preventing PKR activation (Brand, Kobayashi & Mathews 1997).

Aims of thesis.

While our understanding of the processes involved in HSV-1 lytic replication has become more refined with time, the characterisation of HSV-1 latency has been obfuscated by a lack of infection models which reflect the situation of HSV-1 latency in-vivo. To date, in-vivo studies employ the use of animal models but questions over their relevancy to human infection has led to uncertainty over the results garnered from such studies. Consequently, without relevant in vivo models researchers must rely on tissue culture models to illuminate the mechanistics of HSV-1 non-productive infection but current models are relatively inefficient for conducting such studies. Considering these problems, the main aims of this thesis were as follows:

- To understand the mechanistics of non productive infection (quiescence) by developing a tissue culture model using human cells that facilitates high multiplicities of infection with wild type HSV-1.
- Investigate the roles of signalling pathways during HSV-1 quiescence and reactivation from quiescence.
- Investigate the role of translation initiation factors during HSV-1 reactivation from quiescence and lytic replication

Section 2: Materials and Methods

2.0 Materials and Methods

2.1.1 Reagents

Acrylamide
Acetic acid (Merck)
APS (Ammonium persulfate) (Sigma)
β-Mercaptoethanol (Merck)
Bis-acrylamide
Bromophenol Blue
Buffer solutions pH 10, 7 and 4 (Merck)
Crystal violet (Merck)
DMSO (dimethyl sulfoxide) (catalogue no. D2650, Sigma)
EDTA (ethylene diamine tetraacetic acid) (Sigma)
Ethanol (Merck)
Glycerol
Glycine 98 % (Sigma)
Glutamic acid
Glycerophosphate
HCl (Hydrochloric Acid, min. 37 %) (Riedel-de Haen)
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma)
Histidine
Hydrogen peroxide (Sigma)
iso-Butanol (Sigma)
L-cystein (Sigma)
L-methionine (Sigma)
Methanol (Merck)
$MgCl_2$ (Sigma)
NaOH (Sigma)
NaCl (Sigma)
Na_3VO_4 (sodium orthovanadate, catalouge no. 567540, Calbiochem)
NP-40 alternative (nonylphenyl polyethylene glycol) (Calbiochem)
Nuclease-free water, 0.2 μm filtered (Ambion)
PBS (phosphate buffered saline, Dulbecco A) (BR0014G, Oxoid)
Phalloidin (Sigma)
Protease inhibitor cocktail tablets (CompleteR Mini-tablets, Roche)
RNase (catalogue no. R4642, Sigma)
7-methyl GTP Sepharose (Ge Healthcare)
Sepharose 4B beads (catalogue no. 4B200, Sigma)
SDS (sodium dodecylsulfate polyacrylamide) (Sigma)
TEMED (N,N,N',N'-tertramthyl ethylendiamine) (Merck)
Tris (tris(hydroxymethyl)aminomethane) (Sigma)
Tween 20 (Merck)
Water UHP (Ultra High Purity) (Maxima, ELGA)
U0126 (Calbiochem)
Urea
Sb 203580 (Calbiochem)
Sodium dodecyl sulfate (Sigma)
Rapamycin (Calbiochem)
Cgp57380 (Calbiochem)

4EGi-1 (Calbiochem)
4EGi-1 (Santa cruz)
MG132 (Calbiochem)
Tetramethylethylenediamine (TEMED) (Merck)
Torin1 (gift of Nathanael Gray)
Trypan blue (Sigma-Aldrich).
[^{35}S] Methionine/Cysteine (Perkin Elmer)

2.1.2 Equipment

Container with printed label, 30 ml (greiner bio-one)
Hotplate stirrer (SB162, Stuart)
Laminar flow cabinet (Lamin Air, Model 1.2, Holten)
Leica DFC 500 microscope
Microcentrifuge (catalogue no. 37001-296, Galaxy 14D, VWR)
Microprocessor pH meter (pH210, HANNA instruments)
Mini protean 3 cell, iso electric focusing Rig (Bio rad Cat no165,3301)
Mini see-saw rocker (SSM4, Stuart)
LS 6500 Liquid Scintillation Counter (Beckmann)
Micro pipettes 1-10 µl, 10-100 µl, 20-200 µl, 100-1000 µl (VWR)
Pipettes 1 ml, 2 ml, 5 ml (costar, USA)
Pipettes 10 ml, 25 ml (greiner bio-one)
Power Supply (PowerPac™ HV, catalogue no. 164-5056, BIO-RAD)
Speed Gel SG 200 gel dryer (Savant, Farmingdale, NY)
Steri-cycle CO2 Incubator (Thermo Electron Corporation)
Tips, natural bevelled (TipOne, Starlab)
Vortex Mixer (PV-1, Grant Bio)
Vacuum Millipore

2.1.3 Cells

The utilized cell lines were primary normal human diploid fibroblasts (NHDFs) (Clonetics, Walkersville, Maryland, United States), Vero and HeLa cells (kindly provided by Dr. I. Mohr, New York University). The sub-culturing of Adherent cells was conducted on a routine basis under strict aseptic technique. To do this the waste medium was removed by aspiration from the 10cm² plates and cells were washed with 10mls of pre-warmed (37°C) PBS to sequester naturally occurring trypsin inhibitor which is present in residual serum. Subsequent to the wash step the dishes were incubated at 37°C with 1ml trypsin/EDTA (TV) solution (0.25% trysin (Gibco; 043-05090), 0.01% EDTA (Sigma; EDs solution in PBS A (Oxoid;BR14a). After 5 mins the cells were checked under the microscope to confirm detachment. Once cells were

detached, 9 mls of growth medium was added to the plate. The plate was then split according to specifications into fresh 10cm² plates

2.1.4 Medium

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM Sigma) supplemented with 2 mM L-glutamine (invitrogen, UK), 1 mM sodium pyruvate (invitrogen, UK), 50 units of penicillin, and 50 μg per ml of streptomycin (both invitrogen, UK). Medium with 5 % foetal bovine serum (FBS) (catalogue no. F7524, Sigma) was used for culturing cells, whereas 0.2 % FBS supplementation was added to the medium for starving NHDFs. Human serum used to prevent viral spread was derived from platelet-poor human plasma, sterile-filtered, (mycoplasma and virus tested) (Sigma).

2.1.5 Trypan blue cell viability assay

For viability assays 10cm² dishes containing NHDF cells were trypsinized with 1ml trypsin for five minutes at 37°C. 9mls of 5% DMEM was added to the plate once cells were trypsinized. 40μl of the cell suspension was placed in an eppendorff along with 40μl of 0.4% trypan blue. The sample was mixed and allowed stand for 5 minutes at room temperature. 10μl of the stained cells were placed in a Hemocytometer. The percentage of viable cells was calculated by dividing the amount of dye excluding cells by the total number of cells and multiplying by 100.

2.1.6 Growing and Titering virus

For HSV-1 stocks, Vero cells were seeded at half confluence in 10cm² dishes containing 10mls 5% FBS DMEM for 24 hours before addition of virus. One hour before the addition of virus the medium was changed to 1% FBS DMEM. Cells were subsequently infected at m.o.i 0.001. The culture was then returned to the 37°C incubator for 72 hours. Cells were then scraped and medium containing cells from 10cm² dishes was pooled into a suitable container (50ml Falcon tube). The Falcon tubes were then stored in a –80°C freezer.

HSV-1 titration was conducted on permissive Vero cells. Vero cells were seeded at 200,000 cells per well in 5% FBS DMEM for 24 hours before addition of virus. One hour before the addition of virus the medium was changed to 1.5mls of 1% FBS DMEM in each well. The cell lysates taken from virally infected cells were serially diluted in 1% FBS DMEM to the appropriate dilution and 500µl of the chosen dilutions were added to the appropriate wells and cultures were returned to the 37° incubator for 72 hours. On day three of incubation the cells were removed and fixed with 2ml 10% TCA for 10 minutes. The cells were then stained with 2mls crystal violet for 15mins (plate was rocked at room temp).

After staining, the plates were washed with water and allowed air dry.

2.1.7 Preparation of working sample buffer and cell lysis:

Table 1: preperation of Sample buffers

1X sample buffer	**2X sample buffer**
50 % 2 X Laemmli	95 % 2 X Laemmli
45% UHP water	----------
5% β-Mercaptoethanol	5% β-Mercaptoethanol
4 µl 8% bromophenol blue/ml buffer	4 µl 8% Bromophenol blue/ml buffer

Table 2: preperation of 2X Laemmli

2 X Laemmli
12.5 % 1 M Tris, pH 6.8
20 % SDS (20 %)
40 % Glycerol (50 %)
27.5 % UHP water

The working sample buffer was prepared according to the above table 1. The buffer contains the negatively charged detergent sodium dodecylsulfate and the reducing agent β-Mercaptoethanol. The purpose of these reagents is to confer a negative charge and disrupt the tertiary structure and quaternary structure of proteins. SDS binds to the proteins hydrophobic regions and causes the protein to unfold its polypeptide chains

by disrupting non-covalent bonds, denaturing them and causing the molecules to lose their native shape. The anionic SDS binds to the main peptide chain at a ratio of one SDS anion for every two amino acid residues. This results in a negative charge on the protein that is proportional to the mass of that protein (approximately 1.4 g SDS/g protein). β-Mercaptoethanol ensures that a protein solution contains monomeric protein molecules by breaking disulphide bonds instead of disulfide linked dimers or higher order oligomers.

When samples were ready to be harvested, the medium was removed by aspiration and 250µl of 1X Laemmli Lysis buffer was added to the wells and mixed thoroughly. The samples were placed in eppendorfs and were boiled after harvesting for three minutes to eliminate any secondary and tertiary protein structures.

2.1.8 Sodium dodecylsulfate polyacrylamide gel electrophoresis:

The SDS-PAGE technique (Laemmli 1970) was used to separate proteins in lysed cell protein samples. Gels were mixed with varying concentrations of ingredients to achieve larger or smaller pore size depending on degree of protein separation required (Table 3). The 10cm x 8cm glass plates used for this procedure were first washed with water and then EtOH. The plates were assembled using gasket, spacers and 4 clamps. A mark was made on the plates about 1-2cm below where the end of the teeth of the comb lies. The resolving gel solutions were poured into 10cm x 8cm gel cassettes (stopping at the mark made). 500µl of water saturated iso-butanol was added to overlay the gel. After one hour the gels were polymerized, the iso-butanol was washed out of the cassette with distilled water. The stacking gel solution was poured into the glass plates to overlay the resolving gel and a comb was placed between the plates. Once the stacking gel had set the vertical plates were clamped into electrophoresis rig and 1X electrode buffer (Table 5) was added to the rig chambers to immerse the top and bottom of the gel. The combs were removed and bubbles that impede electric current were expunged from the buffer using a syringe. Once the bubbles had been removed a molecular weight marker was prepared using 3µl of precision plus protein dual colour standard per 10µl 1x laemmli buffer. The samples were boiled for three minutes prior to loading and the marker was loaded into the first

lane of the gel, samples were then loaded into the subsequent lanes. Gels were typically run at 170 volts to separate proteins.

Ingredients for SDS-PAGE:

Table 3: preparation of separating gel

Separating gel	7.5 %	10 %	12.5 %	17.5 %
Water (ml)	2.75	2.22	1.55	----------
1 M Tris pH 8.7(ml)	3.75	3.75	3.75	3.75
30 % Acrylamide (ml)	2.5	3.34	4.15	5.85
2 % Bis-Acrylamide (ml)	0.95	0.67	0.5	0.365
20 % SDS (µl)	50	50	50	50
10 % APS (µl)	33.35	33.35	33.35	33.35
TEMED (µl)	8.35	8.35	8.35	8.35

Table 4: preparation of stacking gel

Stacking gel	5%
Water (ml)	3.15
1 M Tris pH 6.8(ml)	0.625
30 % Acrylamide (ml)	0.850
2 % Bis-Acrylamide (ml)	0.350
20 % SDS (µl)	25
10 % APS (µl)	25
TEMED (µl)	12.5

Table 5: preparation of 10X electrode buffer

10 X electrode buffer
0.25 M Tris
2.5 M Glycine
1 % SDS

2.1.9 Western blotting:

Following electrophoresis the gels were removed from the glass plates and equilibrated in 1X transfer solution (Table 6). The composition of 1X and 5X transfer buffer is outlined below in table 6. The gels were transferred to transfer buffer-wetted whatmann paper and nitrocellulose sheets were placed on the gel. A further buffer wetted sheet of whatmann was placed over the nitrocellulose sheet and the sandwich was then clamped between two sponges which were themselves stabilized between two plastic scaffolds. The sandwich was then placed into the electrophoresis chamber and it was filled with enough 1X transfer buffer to cover the assembly. Typically the electrophoresis was run at 57 volts for 1 hour but longer times were employed to transfer larger proteins such as eIF4G.

Table 6: preparation of transfer buffer

1X transfer buffer	***5 X wet western transfer buffer***
20 % 5 X wet western transfer buffer	124 mM Tris
20 % methanol	960 mM Glycine
60 % UHP water	0.05 % SD

2.1.10 Western blot probing:

Primary antibody treatment:

After transfer electrophoresis the nitrocellulose blot was washed with TBS-Tween 0.1% for 1 minute. The blot was then blocked with 5% marvel low fat milk (Cadbury ltd) in TBS-Tween for 1 hour. The blot was then washed three times (x3) with TBS-T for 5 minutes (while rocking). The primary antibodies were diluted from ranges 1:1000 -1/10,000 (see table 2.1.13) in 3% Fraction V BSA/TBS-Tween/0.02% Sodium Azide The primary antibody was added to the blot and rocked at room temperature for 1 hour or over night at 4ºC. The primary antibody was then removed and the blots were washed three times (x3) with TBS-T for 5 minutes (while rocking).

2.1.12 Enhanced chemiluminescence detection:

Protein bands were developed using an enhanced chemiluminesence Kit (Pierce ECL western blotting prod #32106). After the wash step, blots were incubated with horseradish peroxidise conjugated secondary antibody diluted 1/6000 in TBS-T 5% Marvel milk and rocked at room temperature for 1 hour. After the secondary antibody step the membranes were washed with 1X TBS-Tween as before, the fluid was then removed and membranes were placed on cellophane. Equal volumes of ECL reagents were then mixed and placed on the membranes for 1 minute. The ECL solution was then removed and the membranes were enclosed in cellophane. The membrane was exposed to autoradiographic film (Kodak Biomax XAR-5 165 1454) in autoradiographic cassettes. The exposed film was developed for 5 minutes in developer (Kodak, LX 24) diluted 1: 6.5 in water. The film was then washed in water and placed in fixer solution (Kodak FX-40) diluted 1:5 in water for 5 minutes. The film was transferred to water, washed and air dried.

2.1.13 Western blot antibodies:

Antibody	*Host*	*Supplier*	*Dilution*
PML	Rabbit	Abcam (ab53773)	1/1000
ICP 4	Mouse	Abcam (ab6514)	1/1000
P44/42 **MAP Kinase**	Rabbit	Cell Signalling #9102	1/1000
p70 S6 Kinase	Rabbit	Cell Signalling #9202	1/1000
4E-BP1	Rabbit	Cell Signalling#9452	1/1000
Phospho-MEK1/2	Rabbit monoclonal	Cell Signalling#9154	1/1000
Phospho - P44/42	Rabbit polyclonal	Cell Signalling #9102	1/1000
Phospho- P38 MAP	Mouse monoclonal	Cell Signalling #9216	1/1000
P38 MAP Kinase total	Rabbit polyclonal	Cell Signalling #9212	1/1000
HSV-1 ICP0	Mouse monoclonal	Abcam (ab6513)	1/1000
HSV1 + HSV2 ICP5	Mouse monoclonal	Abcam (ab6508)	1/1000
HSV-1 ICP22	Rabbit polyclonal	Gift , John Blaho	1/3000
Caspase 3	Rabbit polyclonal	Cell Signalling #9662	1/1000
Caspase 7	Rabbit polyclonal	Cell Signalling #9492	1/1000
Caspase 9	Rabbit polyclonal	Cell Signalling #9502	1/1000
Cleaved Caspase 3	Rabbit polyclonal	Cell Signalling #9661	1/1000
Cleaved Caspase 7	Rabbit polyclonal	Cell Signalling #9491	1/1000
Cleaved Caspase 9	Rabbit polyclonal	Cell Signalling #9501	1/1000
Parp	Rabbit polyclonal	Cell Signalling #9542	1/1000
Cleaved Parp	Rabbit polyclonal	Cell Signalling #9541	1/1000
Heat shock protein 70	Rat polyclonal	Cell Signalling #2402	1/1000

Heat shock protein 27	Mouse monoclonal	Cell Signalling #4872	1/1000
Pabp	Rabbit polyclonal	Gift (Prof Simon Morley University of Sussex)	1/1000
eIF-4E	Mouse monoclonal	BD Labs #610269	1/1000
Us 11	Mouse monoclonal	Gift (Richard Roller)	1/1000
4E-BP-1	Rabbit polyclonal	Cell Signalling # 9644	1/1000
eIF-4G	Rabbit polyclonal	Gift (Ian mohr)	1:3000 1:10,000
Total eIF2α	Rabbit polyclonal	Cell Signalling #9722	1/1000
Phosphoylated eIF2α	Rabbit polyclonal	Cell Signalling #9721	1/1000
eIF3A	Rabbit polyclonal	Cell Signalling #2538	1/1000
Ribosomal protein RpS3	Rabbit polyclonal	Cell Signalling #2579	1/1000

2.1.14 Metabolic labelling of cells

For each well of six-well plates, the medium was changed to 1 ml of DMEM without Methionine or Cysteine (catalog no. D0422; Sigma-Aldrich) containing HEPES, pH 8, sodium pyruvate, L-glutamine, penicillin-streptomycin and 77 µCi of [^{35}S]-Methionine/Cysteine (catalog no. NEG072; Perkin Elmer) for 1 hour at 37°C. The cells were then lysed in 250µl 1x Laemmli Lysis buffer. The samples were placed in eppendorfs and were boiled.

[^{35}S] Sample Analysis:

[^{35}S] samples were Resolved/ on 12.5% SDS-PAGE gels as outlined in 2.1.8.

Gel fixation and drying:

Gels containing samples that had been labelled with [^{35}S] -Methionine/-Cysteine were placed in destain solution (Table 8) for 20 minutes at room temperature.

Table 8: preparation of destain solution

Destain solution
25 % Methanol
10 % Acetic Acid
65 % UHP water

The gel was dried on cardboard at 80°C for two hours under vacuum with a Speed Gel SG 200 gel dryer to remove the water in the gel. The dried gel was then exposed to X-ray film at -80°C for the appropriate exposure time and developed using Kodak developer/fixer solutions.

2.1.15 TCA precipitation and filter binding assay to quantitate [^{35}S] incorporation into protein:

In 1.5ml eppendorf tubes 67µl of 3% H202 + 10µl of 10N (10M) NaOH + 20µl of [^{35}S] labelled SDS lysate in Laemlli buffer were mixed. This step eliminates charged tRNAs.

The tubes were mixed and incubated for 10 minutes at 37°C. Subsequent to incubation 5µl of 10mg/ml V BSA stock was added to the mixture along with 1mls of ice cold 10% TCA/4mM L-Cys/4mM L-Met. This step allows for L-Cys/L-Met to compete with free radiolabelled Methioine and Cysteine.

The mixture was vortexed and incubated on ice for 15-30 min. As incubation was progressing, 25mm GF/C circles of Whatman-Glass Microfibre filters were soaked in 10% TCA/10mM L-cys/10mM L-met in a 10cm² plate. The filters were then placed on the millipore vacuum unit. Once the unit was secured and attached to a vacuum pump the samples were poured into appropriate wells. The sample tubes were washed twice with 1ml cold 10% TCA/10mM L-cys/10mM L-met and poured into appropriate wells. The vacuum was then applied and shut off once the samples had passed through each filter. Each well was then washed with 10% TCA solution and the vacuum was re-applied. This step was repeated twice more. The vacuum was then shut off and each well was filled with ice-cold EtOH and the vacuum was applied. The previous step was repeated one more time. The vacuum unit was then disconnected from the vacuum and disassembled, the filters were removed and placed

on aluminium foil and placed on an inverted Styrofoam rack. When filters had dried they were placed in labelled scintillation tubes with 4mls of Econo Safe liquid scintillation fluid (RPI) and allowed to incubate for 20 minutes. Once incubation was complete the tubes were placed in a LS 6500 Liquid Scintillation Counter and measured for [^{35}S] using program three in the count protocol list.

2.1.16 Isoelectric Focusing:

Isoelectric focusing (IEF) is a technique for separating different molecules by their electric charge differences. It is a type of zone electrophoresis, and takes advantage of the fact that a molecule's charge changes with the pH of its surroundings. This description of the procedure was kindly given to us by Professor Simon Morley (University of Sussex) and is based on the vertical slab version of IEF published by Jagus *et. al.* (Dev. Gen. 14: 412-423) using a Bio-Rad Protean II minigel aparatus (0.75mm spacers).

Firstly a 50ml stock of incomplete gel mix was made, and stored at 4°C.

Table 9: preparation of incomplete gel mix

42.8ml pure water	24.13ml H^2O
4.86g Acrylamide	12.17ml 40% Acrylamide
274.3mg Bis-Acrylamide	13.7ml 2% Bis-Acrylamide
1.71g CHAPS	1.71g of CHAPS

The above mixture was then filtered using a 0.22µM filter (large syringe and pressure, not vacuum)

To make and pour the gels:

The gel was mixed as outlined in the table 10 below, leaving out APS and TEMED. The mixture was gently heated in a 37°C waterbath to dissolve the urea. Once Urea was dissolved, the APS and TEMED was added. The gel was then poured right to the top of plates, the combs were added and gels were allowed to set.

Table 10: preparation of iso-electric focusing gels

1 Gel (6mls)	2 Gels (12mls)
3.5ml Incomplete gel mix	7ml incomplete gel mix
3.24g urea	6.48g urea
0.45ml ampholines	0.9ml ampholines
20µl 10% APS	40µl 10% APS
10µl TEMED	10µl TEMED

Sample buffer and sample preparations:

7X sample buffer was made as described in the table 11 below. 1ml aliquots were stored at -20°C and reused). For a 5ml stock the following were mixed as described below in table 11.

Table 11: preparation of 7X sample buffer

21% (v/v) ampholines pH range 3-10 (same as for gels)	1.05ml
14% (v/v) ß-mercaptoethanol	0.7ml
35% (w/v) CHAPS	1.75g
H^2O	3.0ml

For sample preparation 1X sample buffer was made as described below.

Table 12: preparation of 1X sample buffer

143µl of 7X sample buffer
0.54g urea (gives 9M final)
550µl MilliQ water.

Running Buffers were created as described below in table 13

Table 13: preparation of electrode buffer

Cathode (outer chamber)	0.05M Histidine (= 3.88g/500ml)
Anode (inner chamber)	0.01M Glutamic Acid (= 0.73g/500ml)

500ml of each buffer was prepared fresh. The buffers were chilled as the high voltages used during IEF can increase buffer temperature and warp the gels .

Running the Gels:

Due to the numerous voltage changes used in the IEF procedure, the Bio-Rad programmable powerpack was used for this procedure.
Once the gels had set the combs were removed and wells were washed out thoroughly with water. 20-25µl of 1X sample buffer was then added to each well. The wells were then carefully overlayed with 10µl 6M urea followed by glutamic acid. The inner chamber of the IEF apparatus was filled with 0.01M Glutamic Acid and the outer chamber was filled with 250ml 0.05M Histidine. The apparatus was placed in a plastic container and surrounded with ice to maintain a low temperature.
The gels were then prefocused for a total of 1 hour on reverse polarity at the following voltage, 20min at 200v, 20min at 300v and 20min at 400v.

After prefocusing the wells were washed out thoroughly with water. Before the end of the prefocus stage the samples to be analysed were prepared by boiling and allowed to cool. A 1:1 dilution of sample with 1x IEF sample buffer was prepared. The samples were then vortexed quickly to mix and 25µl of sample was loaded into to each well. The sample was overlayed with urea and glutamic acid as for prefocusing, and IEF was performed by increasing voltages in 50v increments every 20 minutes starting at 500 up to 750v, i.e. 500-550-600-650-700-750v, each one for 20 minutes. The samples were then ran at 1000v for a further 20 minutes. Again, as for prefocus, IEF was run on reverse polarity. After electrophoresis the gel was transferred to nitrocellulose and probed with anti-eIF4E antiserum as described for Western Blotting.

2.1.17 Cap pulldown protocol

For cap pulldown procedure the lysis buffer prepared as follows

NP-40 Lysis Buffer - For every 10mls of buffer**:**

Table 13: preparation of NP-40 Lysis Buffer

500µl	1M HEPES/KOH pH 7.4 containing 40mM EDTA
400µl	2.5M NaCl
80µl	0.25M Na_3VO_4
250µl	1M Glycerophosphate
125µl	20% NP40
3.3µl	1.5M $MgCl_2$
8.56ml	Sterile loop water (ddH^2O)

The buffer was mixed well on a rocker to make sure NP-40 was evenly mixed and 10mls from each stock of buffer had one protease inhibitor tablet (Complete® Mini-tablets) dissolved in it .

For the cap pulldown everything was kept on ice or at 4°C throughout the procedure.

To perform the cap pulldown the cells were washed in PBS, then 700µl of NP-40 Lysis Buffer (NLB) was added to cells and cells were scraped off the plate and transferred to eppendorff tubes then were incubated on orbital shaker at 4°C for 30-40 minutes.

The cells were then centrifuged at 10,000 x g for 10 min at 4°C. The supernatant was removed and placed in a fresh eppendorf containing 1.3µl RNase A and 8µl 100mM $CaCl_2$. The eppendorfs were then rocked at room temperature for 20 minutes. After RNase treatment the samples were chilled on ice, supernatants from each sample were

then placed in fresh tube containing 40µl Sepharose 4B, prewashed in NLB. The samples were mixed by inversion and rocked at 4°C for 40 minutes. Once pre clear had completed the samples were centrifuged for 1 minute at 10,000 x g, input samples (27µl to which 33µl 2x sample buffer was added) were then taken from the supernatent. The rest of the pre-cleared sample was added to fresh eppendorfs containing 40µl washed 7-Methyl GTP Sepharose.

The eppendorfs were then mixed by inversion and placed on rocker for 1 hour at 4°C. After this step each tube was centrifuged and the supernatant removed. 800µl of fresh NP-40 Lysis Buffer containing 8µl 100mM GTP was then placed in eppendorfs containing 7-Methyl GTP Sepharose bound to sample and the eppendorfs were mixed by inversion and rocked at 4°C for 1 hour. After the GTP step, the eppendorfs were centrifuged for 1 minute at 10,000g and the supernatents removed. The beads were then washed 3 times in at least 500µl NP40 lysis buffer and boiled for 3 min in 40µl Laemmli buffer. The samples were vortexed gently to mix before boiling then stored at -20°C as usual for protein samples.

2.1.18 Immunofluorescence protocol

For Immunofluorescence, cells were seeded on coverslips in six well plates and the appropriate experiment performed. For sample preparation medium was aspirated from cells and cells were washed with 1X PBS. The cells were then fixed with 2mls 3.7% formaldehyde per well for 15 minutes. The formaldehyde was aspirated and the cells were washed with 1X PBS four times. The cells were then stored at 4ºC until the day of immunofluorescence. On the day of immunofluorescence the storage PBS was removed from the cells and 100µl of 0.1% Triton X-100 in 1X TBS was added for 30 minutes at room temperature to permeabilize cells. The Triton was aspirated and the cells were briefly washed twice with TBS and incubated with TBS for 5 minutes. The cells were then blocked with 3% BSA in TBS for 30 minutes at room temperature. The blocking solution was aspirated and primary antibody (diluted in TBS-T plus 3% BSA) was added to each sample and incubated for 1 hour at Room Temp. The primary antibody was removed and cells were washed three times with TBS-T for five minutes each wash. The TBS-T was removed by aspiration and the sample was incubated with the appropriate fluorophore-conjugated secondary antibody in TBS-T plus 3% BSA for 40 minutes. After secondary binding the antibody was removed by aspiration and the sample was washed three times with TBS-T. The nuclei were stained with Hoechst for 5 minutes and washed twice with TBS-T, then briefly incubated for 5 minutes in TBS-T. The TBS-T was then removed and the mounting medium applied to glass slides, on which sample coverslips were then placed.

2.1.19 Phalloidin staining:

0.1 mg per ml Stock phalloidin solutions were made using DMSO and stored at -20ºC. Cells to be analysed were washed with PBS and fixed with 3.7% formaldehyde for 15 minutes. The cells were then washed three times with PBS. On the day of analysis the PBS was removed from the cells and a grease pen was used create grease rings in each well. 100µl of PBS was added to the centre of each grease ring to prevent culture dehydration. The PBS was aspirated and 100µl of 0.1% Triton X-100 in 1X PBS was added to permeabilize samples for 30 minutes at room temperature. The Triton was aspirated and the cells were briefly washed twice with PBS and incubated with TBS for 5 minutes. Each sample was then incubated with 1/100 dilution of the 0.1 mg Stock phalloidin in PBS and incubated at room temperature for 40 minutes. The samples nuclei were then stained with Hoechst for 5 minutes and washed twice with PBS and then briefly incubated for five minutes in PBS. The PBS was then removed and mounting medium applied, then coverslips where placed on samples.

Section 3.0: Results

3.0 Results

Introduction to results

3.1 Establishing quiescent infection in vitro

Latent HSV-1 infection is very poorly understood. The majority of models developed to study latency are based in animals which imposes serious limitations on the types of studies that can be conducted. In addition, questions relating to how well mouse and rabbit models reflect the true nature of human infection has led to doubt over results garnered from such experiments.

In vitro HSV-1 latency models termed quiescence models have been developed using non-human neurons but are relatively inefficient. Systems have also been developed in human cells, particularly primary human fibroblasts. The success in using these cells appears to lie in their reduced metabolic state in culture that may resemble the metabolic state in neurons more closely than transformed cell types. In addition, these models employ infection at elevated temperature and experiments by Crouch and Rapp have shown that HSV-1 is sensitive to temperatures above 40.5°C during the initial stages of lytic infection (Crouch, Rapp 1972). However, to date, establishing HSV-1 quiescence in human cells has required deletion of the early viral gene encoding ICP0, to prevent the establishment of a lytic infection.

In an attempt to develop a quiescent model in human cells using wild type virus, we endeavoured to create a non permissive environment for productive infection (outlined in Figure 3.1.1). The parameters tested included serum starvation to further reduce the metabolic state of the host cell combined with heat shock to induce protective heat shock proteins that might mimic the function of LATs, which induce Hsp70, in the absence of their expression in fibroblast systems. Finally, infecting cells at elevated temperature was also expected to prevent entry into the lytic phase of replication and promote the establishment of a quiescent infection. We then tested whether virus could be recovered from the non-productive state in a controlled manner by supplying exogenous viral genes or through spontaneous reactivation events.

Fig 3.1.1 Flow chart for the method of establishing quiescence

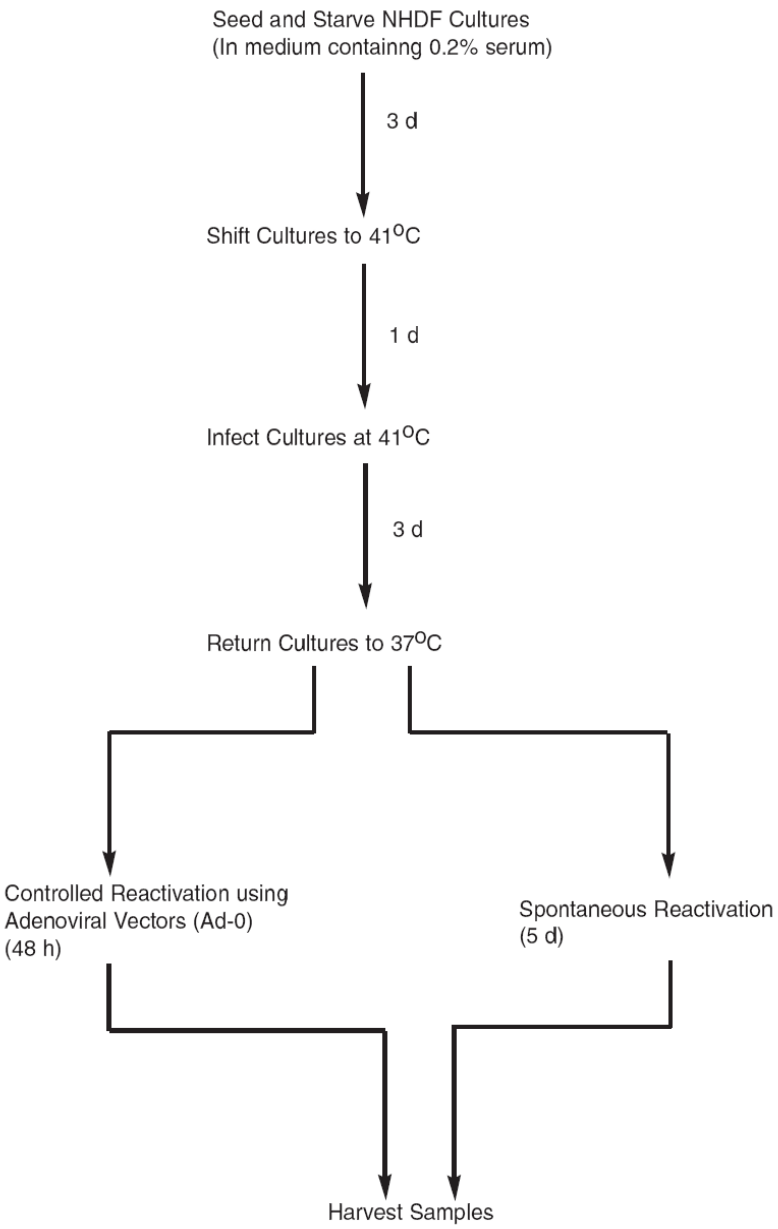

Figure 3.1.1 Flow chart illustrating the steps in establishing a HSV-1 quisescent infection. Firstly confluent cultures of low passage primary NHDFs were starved in 0.2% FBS DMEM for 3 days and subsequently subjected to temperature elevation at 41°C for 30 hours. Cultures were then infected at 41°C with HSV-1 at a m.o.i of 0.5-1 and the cultures were maintained at 41°C for either 3 or 6 days to repress viral replication. To reactivate the virus, cultures were placed in the 37°C incubator subsequent to replenishment in fresh 5% FBS DMEM and either allowed to spontaneously reactivate over a 5 day period or alternatively the virus could be controllably reactivated by transduction with Adeno virus vectors encoding the HSV-1 gene ICP0. To harvest samples the cultures were lysed in 1x Laemmli buffer.

Figure 3.1.2

Western blot analysis of Heat shock protein 70 and 27 abundance in NHDFs exposed to either 37ºC or 41ºC

Firstly we examined the ability of the culture conditions to elicit changes in expression of Heat shock proteins 27 and 70.

NHDF cells were serum starved and incubated for 30 hours at either 37 °C or 41°C then mock-infected or infected with wild type HSV-1 Kos at 0.5 to 1 PFU per cell. Total cell extracts were prepared by laemmli buffer lysis at 12 hours and 24 hours post-infection and resolved by SDS-PAGE, then transferred to nitrocellulose membranes and probed with either Heat shock protein 27 or 70 antisera. The Hsp70 blot is representative of results 12 hours post-infection while Hsp27 and PABP blots are representative of results 24 hours post-infection.

Importantly, the infection had no effect on Hsp 27 or 70 expression. Significantly, heat shock protein induction in cultures was specific, as no change in the representative cellular antigen loading control PABP was observed.

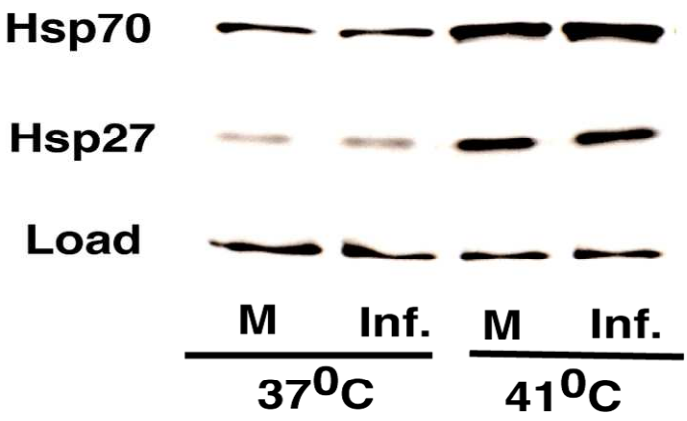

Figure 3.1.2 Western blot of heat shock protein (Hsp) expression within mock infected and infected cultures at either 37°C or 41°C. PABP was probed for as load control.

Figure 3.1.3

Immunofluorescense analysis of ICP4 production in temperature elevated cells 12 hours post infection

To validate that cells could be infected and to quantify the amount of infected cells within conditioned cultures at 12 hours post infection, the expression of the IE gene product ICP4 was analysed by indirect immunofluorescence. NHDF cells were grown to confluence on glass coverslips and subsequently serum starved. The cells were then incubated for 30 hours at 41°C and infected with HSV-1 for 12 hours. The slides were fixed and probed with ICP4 antiserum. ICP4 was detected with FITC-labelled anti-mouse secondary. Hoechst was employed to counterstain nuclei.

At 12 hours postinfection, approximately 60% of cells in cultures infected at 41°C expressed the viral IE gene product ICP4. A similar amount of ICP4 staining was observed in cells infected at 37°C at this stage post-infection (not shown) which confirmed that the selective pressures of elevated temperature and serum starvation had no effect on viral entry into the cell or the expression IE gene product ICP4 (see also figure 3.1.7).

Figure 3.1.3

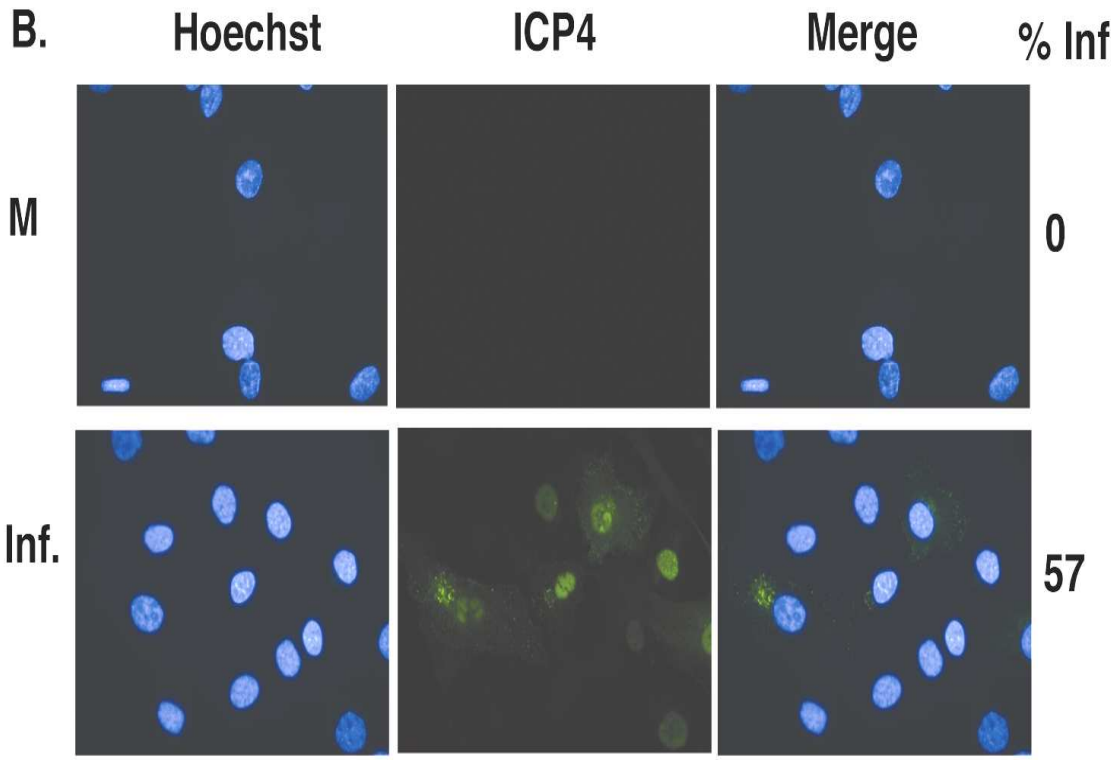

Figure 3.1.3 Immunofluorescence of ICP4 production in temperature elevated cells 12 hours post infection. The Images were taken at 63x magnification and a representative field shown. The percentage of antigen-positive cells in each field is shown to the right of the panel.

Figure 3.1.4

Immunofluorescense analysis of ICP4 production in temperature elevated cells 24 hours post infection

To quantify the amount of cells within conditioned cultures harbouring virus at 24 hours post infection, NHDF cells were grown to confluence on coverslips, serum starved and infected at 41°C or 37°C for 24 hours. The cells were processed as in Figure 3.1.3. The percentage of antigen-positive cells in each field is also shown to the right of the panel.

Approximately 60% of the culture infected at elevated temperature stained positive for ICP4 at 24 hours post infection, indicating that virus replication and secondary spread to neighbouring cells was inhibited. Conversely virus in cells infected at 37°C replicated and spread to 100% of the culture by 24 hours postinfection.

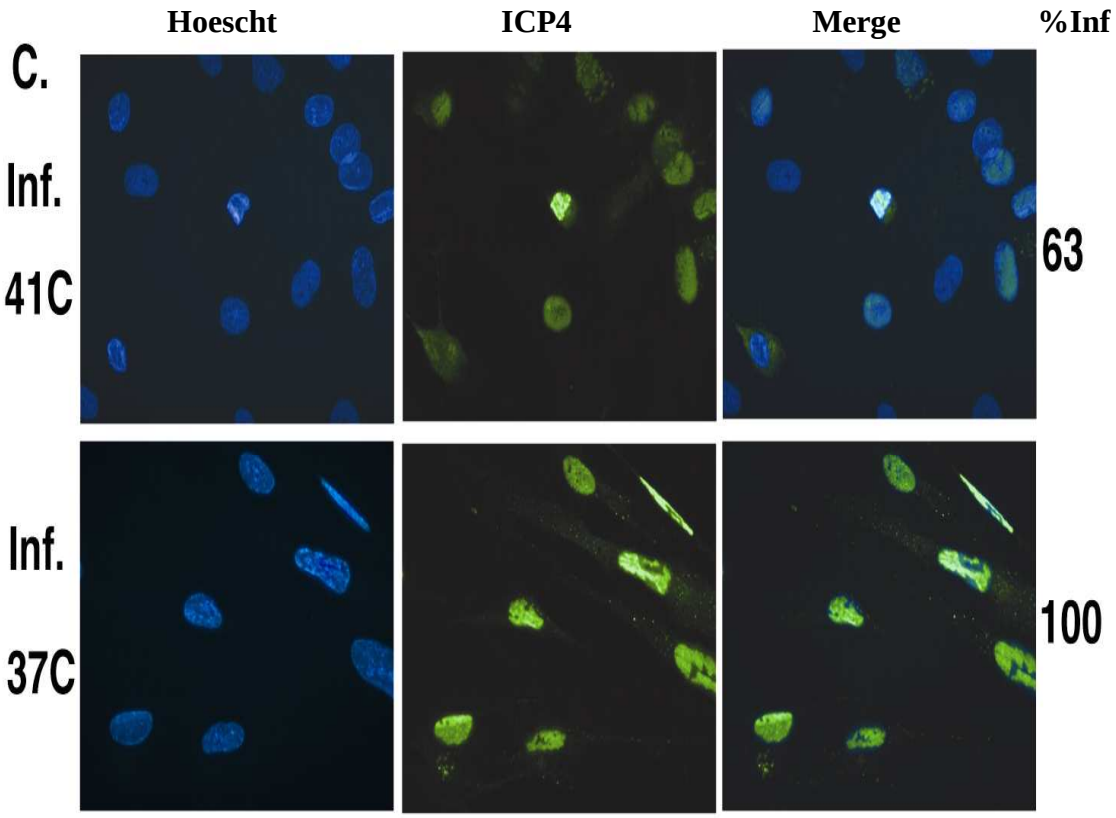

Figure 3.1.4 Immunofluorescence of ICP4 production in temperature elevated cells 24 hours post infection. The Images were taken at 63x magnification and a representative field shown. The percentage of antigen-positive cells in each field is shown to the right of the panel.

Figure 3.1.5

Viral titers from cultures infected at 37º or 41ºC

In order to confirm that the cultures infected at elevated temperatures were not producing viable infectious virus, a quantification of plaque forming virus being produced in 35mm dishes of infected NHDF cultures at either 37°C or 41°C was performed. Samples were taken at one, two and three days post-infection (d.p.i).
Amounts of virus were determined by titration on Vero cells and calculated as pfu/culture. The numbers illustrated on the diagram are representative of three independent experiments.

As expected, viral replication was uninhibited in cultures infected at 37ºC over the time points indicated. It was discovered that minimal amounts of infectious virus were detectable in cultures infected at 41°C over the first 48 hours and virus became completely undetectable by 72 hours postinfection, suggesting that as long as cultures were maintained at 41°C, the infection was maintained in a non-productive state.

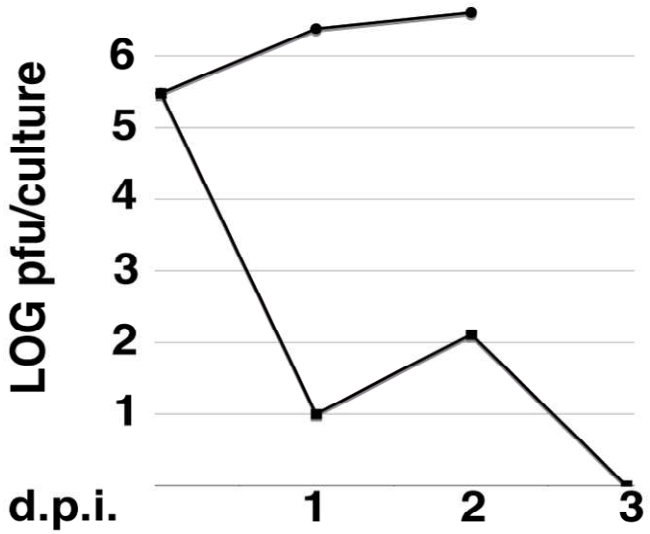

Figure 3.1.5 Viral titers from NHDF cultures infected at 37º or 41ºC. Supernatents for titering experiments were taken at 24 hour intervals over a three day period. The amount of plaque-forming virus was titered on permissive Vero cells. The circle points represent virus particle numbers in supernatents from 37°C infections while the squares represent virus partice numbers in supernatents from 41°C infections at the indicated time points in days postinfection (d.p.i).

Figure 3.1.6

Metabolic labelling of infected NDHF cultures

As illustrated in Figure 3.1.2 and 3.1.3, approximately 60% of the culture could be initially infected and this number remains static at 24 hours post infection. As the majority of the culture can be infected it is possible to observe population wide changes in protein production during entry into a viral non-productive state. Therefore an examination of the global patterns of protein synthesis in uninfected and infected cells was conducted by metabolic labelling.

NHDFs were grown to confluence and serum starved. Cells were incubated at 41°C for 30 hours and either Mock-infected (M) or infected with HSV-1 (Inf) at either 37°C (B) or 41°C (A). At 1 hour prior to the indicated sampling times in hours post-infection (h.p.i.), cultures were incubated with [^{35}S]-Methionine/Cysteine and total protein was harvested by lysing cells in 1x Laemmli buffer. Samples were resolved by SDS-PAGE and dried gels were exposed to X-ray film.

Cells infected at 41°C differentially expressed a small number of proteins at 12 hours post infection. (A) When compared to lytically infected cultures, (B) these proteins have similar size and comigration with viral proteins normally produced in productive infection and appear to represent viral polypeptides. By 24 hours postinfection a reduction in synthesis of these proteins was observed. By 48 hours postinfection viral protein production at 41°C was undetectable. During infection at 37°C, HSV-1 shuts off host protein synthesis and directs the cell toward synthesising viral proteins. Compared to cultures infected at 37°C (B), many of the viral proteins normally associated with productive infection were not detectable in cells infected at the elevated temperature (A), suggesting that they were either not produced or were made at very low levels. In addition, at no point did infection at the elevated temperature alter host cell protein synthesis patterns or elicit the shutoff of host translation associated with lytic replication

Figure 3.1.6

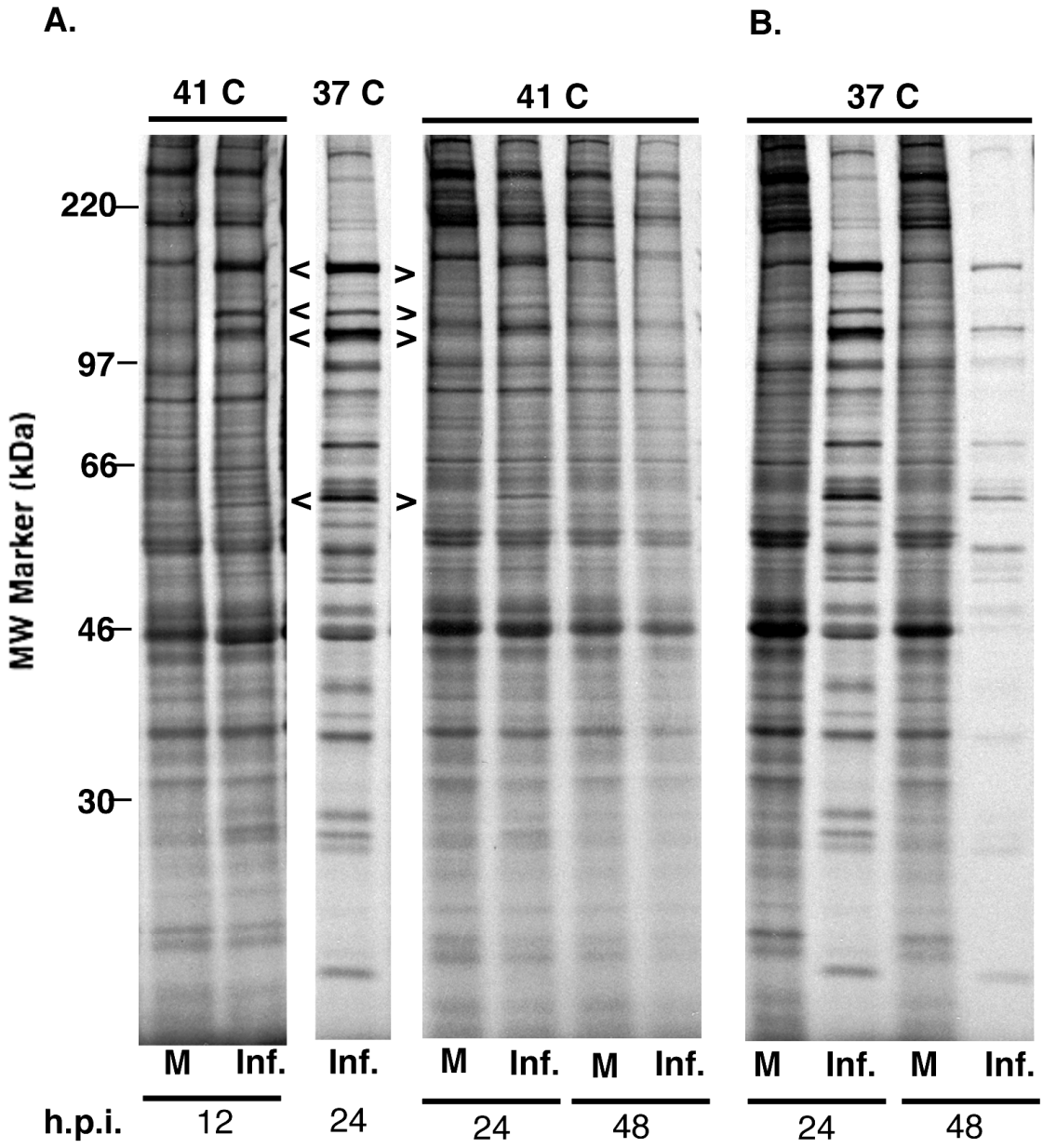

Figure 3.1.6 Metabolic labelling of infected NHDF cultures incubated at 41°C for 30 hours and either mock infected (M) or infected with HSV-1 (Inf). At 1 hour prior to the indicated sampling times in hours postinfection (h.p.i.), cultures were incubated with [^{35}S]-Methionine/Cysteine, and total protein was harvested by lysing cells in Laemmli buffer. Samples were resolved by SDS-PAGE and 50µl of sample was loaded into each well. Molecular weight standards (in kDa) are shown to the left of the panel. The arrows indicate suspected viral proteins produced during non-productive infection at 41°C. For comparative analysis, the pattern of proteins expressed in cultures harbouring a lytic infection (from image B) is shown between the 12 hour and 24 hour 41ºC time points.

Figure 3.1.7

Western blot analysis of viral protein production during lytic and non-productive infection in NHDF cultures

To verify and further investigate the production of viral proteins in cells infected at elevated temperature, western blotting with antiserum directed against an array of viral IE proteins was performed. Serum starved NHDF cultures were mock infected or infected at 37°C or 41°C. Whole cell extracts were prepared at the indicated times in hours post-infection (h.p.i) by Laemmli lysis. Samples were resolved by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was probed with antisera against IE gene products ICP0, ICP4 and ICP22 or the late gene product, Us11.

Both cultures infected at 37°C and 41°C expressed the 175-kDa viral IE protein ICP4. While production increased over time in 37°C samples, it declined in 41°C samples confirming results from the immunofluorescence studies shown in Fig. 3.1.3. The 66-kDa IE protein ICP22 was also expressed in both 37°C and 41°C. However cells infected at 41°C produced unprocessed ICP22 unlike cells infected at 37°C, which was heavily post-translationally modified, detected as multiple immunoreactive species. This ICP22 modification is an indicator that the infectious cycle is progressing, and unprocessed ICP22 is indicative of infection that is stalled at an early stage. The *trans*-activating protein ICP0 was produced at 41°C but accumulated at greatly reduced levels compared to cultures infected at 37°C. The “late” protein Us11, which is made late in infection, was produced in 41°C cultures at minute amounts relative to 37°C cultures, which further confirms that viral infection was not progressing when cells were held at 41°C.

Fig 3.1.7

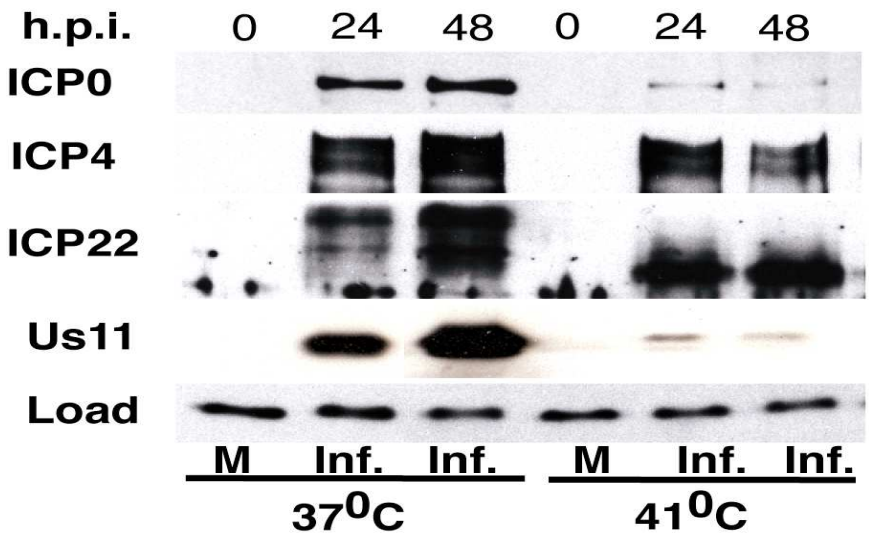

Figure 3.1.7 Western blot analysis for production of HSV-1 viral proteins: ICP0, ICP4, ICP22 and Us11 during lytic 37°C and non-productive 41°C infection in NHDF cultures. Blots were also probed with antiserum against total p38 MAPK to demonstrate even loading of samples.

Figure 3.1.8

Immunofluorescence analysis of leaky late protein ICP5 production during lytic and non-productive infection in NHDF cultures

The production of small amounts of virus and Us11 in cultures infected at 41°C indicated that a low level of lytic replication was occurring. In order to further validate if this phenomena is indeed occurring and quantify the numbers of cells supporting lytic replication, we examined expression of the late protein ICP5 by indirect immunofluoresence. Serum-starved NHDF cultures grown on glass coverslips were mock infected or infected at either 41°C or 37°C for 24 hours. Cells were then fixed and probed with ICP5 antiserum. Cells were counterstained with Hoechst to visualise nuclei.

At 24 hours post-infection, cultures infected at 37°C expressed extremely high levels of ICP5, whereas an average of only 15% of cells infected at 41°C faintly expressed this late antigen at very low levels.

Figure 3.1.8

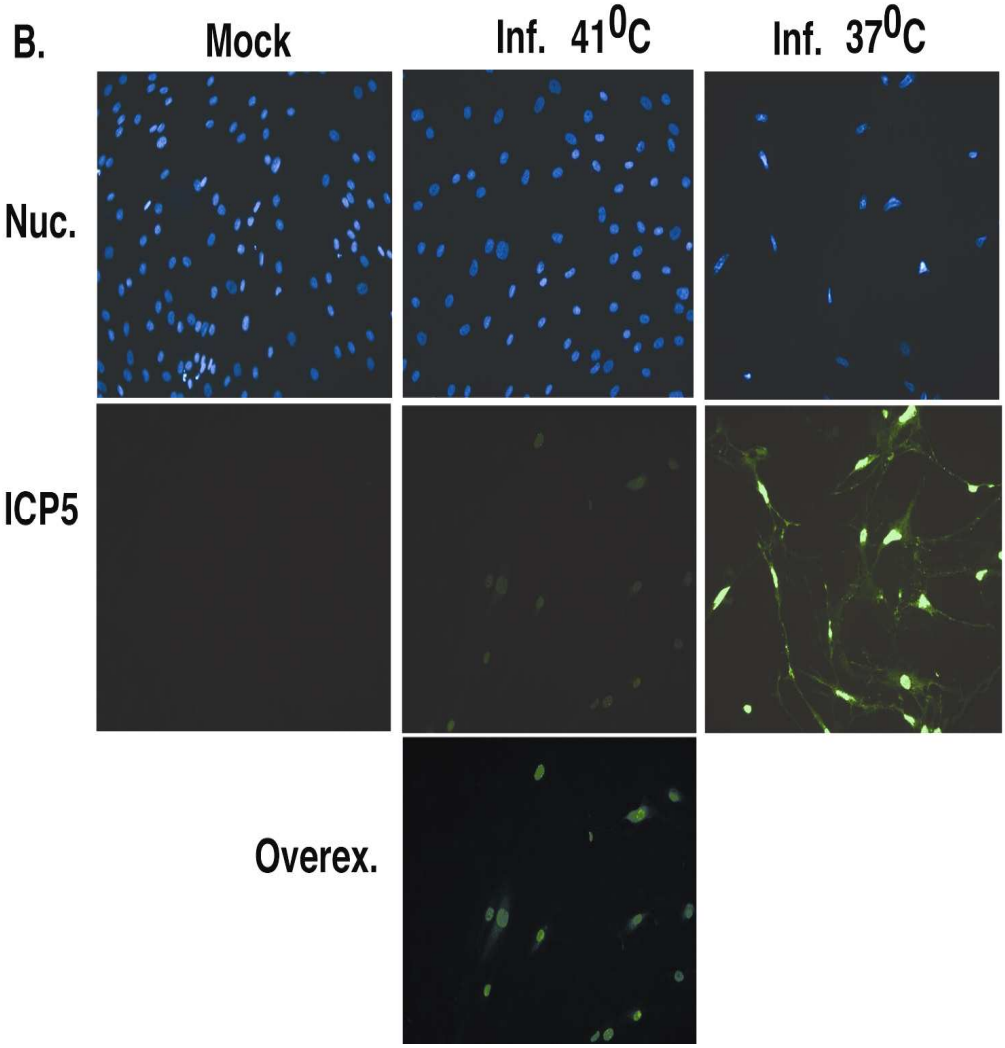

Figure 3.1.8 Immunofluorescence of ICP5 production in cultures infected at either 37°C or 41°C at 48 hours post infection. The images were captured at 20x magnification and represent a typical field of view. Due to low antigen expression at 41°C, a weak fluorescent signal was observed when using the same exposure times as those used for 37°C samples. Consequently, overexposed (Overex) images of ICP5 staining were taken at 41°C to illustrate the number of antigen-positive cells (lower image).

Figure 3.1.9

Phase contrast image capture of lytic and non-productive infection in NHDF cultures

In order to assess the degree of cytopathic effect (CPE) in cultures either infected at 41°C or 37°C, serum-starved NHDF cultures were mock infected or infected for 48 hours and then photographed by phase-contrast microscopy.

As expected, virus in cultures infected at 37°C lytically replicated and cells exhibited significant CPE by 48 hours postinfection, characterised by changes in cell morphology and detachment from one another. In contrast, cultures infected at 41°C remained healthy with the exception of some cells exhibiting low level CPE. These cells are probably representative of cells harbouring low level lytic replication and will consequently die or recover, possibly to then harbour a quiescent infection.

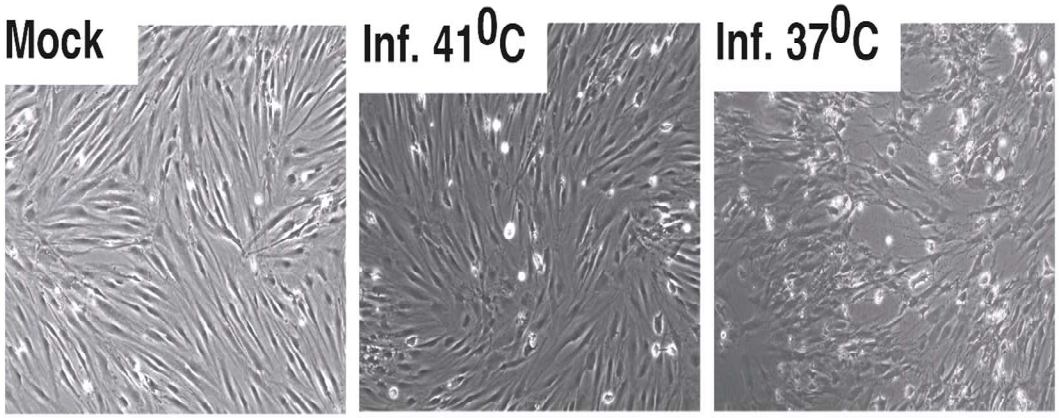

Figure 3.1.9 Phase contrast microscopy of Serum-starved NHDF cultures that were either mock infected or infected at 41°C or 37°C for 48 hours.

Figure 3.1.10

Patterns of protein synthesis during prolonged quiescent infection

The stability of non productive infection was assessed at 3 and 6 days postinfection (d.p.i.) by examining the pattern of protein synthesis in either mock-infected (M) or infected (Inf) NHDF cultures. Cultures were metabolically labelled using [^{35}S]-Methionine/Cysteine for 1 hour at both 3 and 6 d.p.i. Whole-cell extracts were resolved by SDS-PAGE, fixed and the dried gels were then exposed to X-ray film. The migration of molecular weight markers (in thousands) is shown to the left of the panel.

As illustrated, the pattern and rates of protein synthesis were indistinguishable from those of mock-infected cells at both 3 and 6 days, suggesting a lack of viral gene expression and demonstrating again that at 41°C the virus cannot establish productive replication.

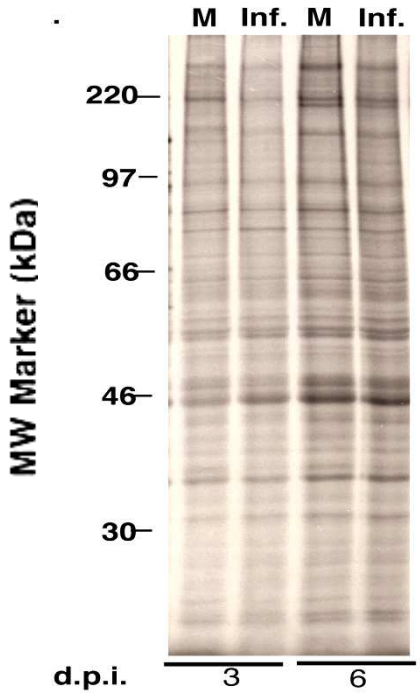

Figure 3.1.10 Metabolic labelling of mock infected (M) and infected NHDF cultures incubated at 41°C for either 3 or 6 days. At 1 hour prior to the indicated sampling times in days postinfection (d.p.i.), cultures were incubated with [^{35}S]-Methionine/Cysteine, and total protein was harvested by lysing cells in Laemmli buffer. Samples were resolved by SDS-PAGE where 50µl of sample was loaded into each well. Molecular weight standards (in kDa) are shown to the left of the panel.

Figure 3.1.11

Comparative phase contrast images of mock vs quiescently infected cultures at 6 days post infection

To assess the morphologies of cultures infected at 41°C, NHDF cultures were either mock-infected (M) or infected (Inf) at 41°C and phase-contrast images were taken after 6 days at 41°C.

It was found that the morphology of the culture infected and maintained at 41°C was identical to that of the mock infected culture, and CPE was not apparent.

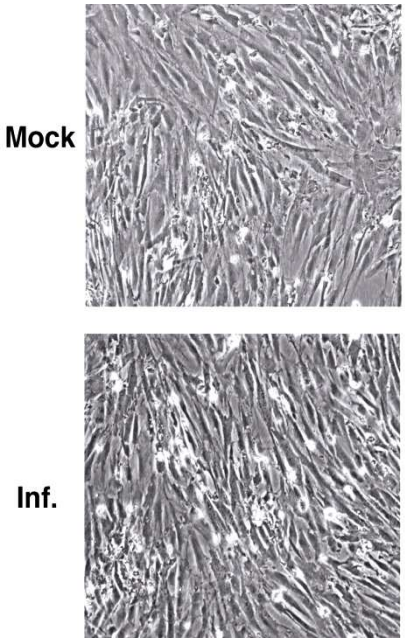

Figure 3.1.11 Phase contrast microscopy of Serum-starved NHDF cultures that were either mock infected or infected at 41°C for 6 days.

Figure 3.1.12

Levels of viral antigen production during quiescence over a six day period

Considering the patterns of proteins synthesised and morphology of cultures that were either uninfected or infected at 41°C resembled each other, it was decided to analyze production of Us11 and ICP4 in 41°C cultures harvested at 1 day and 6 days post infection in order to decipher if any viral proteins associated with lytic replication were being produced 6 days postinfection at 41°C.

To accomplish this, whole-cell extracts from NHDF cultures that were either mock infected (M) or infected (Inf) for 1 or 6 days at 41°C. Samples were resolved by SDS-PAGE and probed with antiserum against ICP4 or Us11. Images of ICP4 are taken from the same blot and exposure as presented in Fig 3.1.7 to allow direct comparison of expression levels at days 1 and 6 post infection. To illustrate that Us11 becomes completely undetectable over time the image was intentionally overexposed.

As expected, the low level expression of Us11 visible on overexpressed blots in samples became undetectable in samples over time, whereas low levels of ICP4 remained visible even at 6 days post infection. The presence of ICP4 signifies that either ICP4 is synthesized at minute quantities or was proteolytically stable in cells that harbour HSV-1. This result agrees with previous reports showing that low levels of ICP4 transcript are detected in mouse ganglia latently infected with HSV-1 (Kramer, MF 1995).

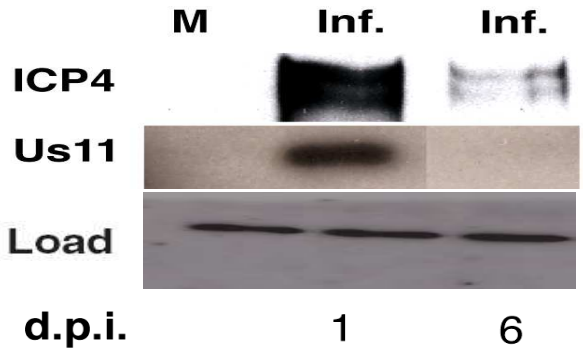

Figure 3.1.12 Western blot analysis for production of HSV-1 viral proteins: ICP4 and Us11 at 1 and 6 days postinfection in cultures maintained at 41°C. Blots were also probed with antiserum against eIF4E to demonstrate even loading of samples.

Figure 3.1.13

Western blot analysis of viral antigen production in reactivated cultures vs lytically infected cultures

For a non-productive infection to be considered quiescent, reactivation of the virus to a state of replication must be possible. To investigate whether the infection was quiescent, NHDF cultures were infected and maintained at 41°C for 6 days to establish quiescence. Cultures were subsequently returned to 37°C and transduced for 48 hours with an adenoviral vector encoding HSV-1 ICP0, a key regulator of reactivation from latency in vivo and quiescence in vitro

Levels of viral antigen production in reactivated cultures were compared to cultures infected at 37°C and it was found that at 48 hours post transduction with Ad-0, quiescently infected cultures had levels of the late viral protein Us11 that were comparable to those observed in cells infected at 37°C for either 1 or 2 days.

For this experiment, the same amount of virus was used to infect cells at 37° C as was used during a quiescent infection i.e m.o.i 0.5-1.

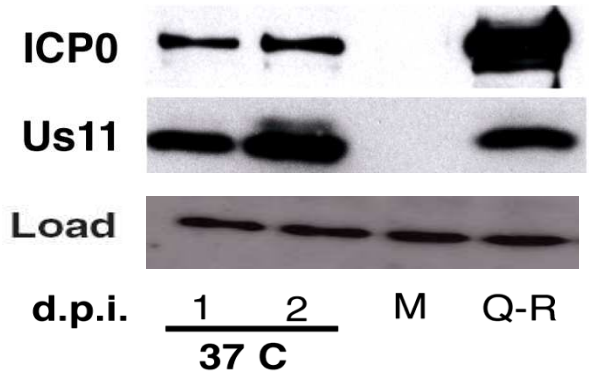

Figure 3.1.13 Western blot analysis for production of HSV-1 viral proteins: ICP0 and Us11 from NHDF cells infected for 24 or 48 hours (d.p.i. 1 and 2) at 37°C, mock-infected cell extracts (M), or cell extracts from NHDF cells quiescently infected for 6 days and then reactivated for 48 hours (Q-R) using an adenovirus encoding HSV-1 ICP0 (Ad0) were resolved by SDS-PAGE, and membranes were probed with antiserum against ICP0 or Us11. Blots were also probed with antiserum against eIF4E to demonstrate even loading of samples.

Figure 3.1.14

Recovery of infectious virus from quiescently-infected NHDF cultures

To quantify the amount of recoverable virus from quiescence , NHDF cells were grown in 35-mm dishes and infected at 41°C. At 3 or 6 days postinfection (d.p.i) quiescent virus was reactivated by transduction with adenovirus encoding HSV-1 ICP0 and allowed to reactivate for 48 hours. Titers of infectious virus were quantified on Vero cells.

Its was found that the yields of infectious virus from ICP0-transduced cultures that had been quiescently infected for either 3 or 6 days were equivalent to those from cells infected at 37°C and harvested at 48 hours postinfection (see fig 3.1.5)

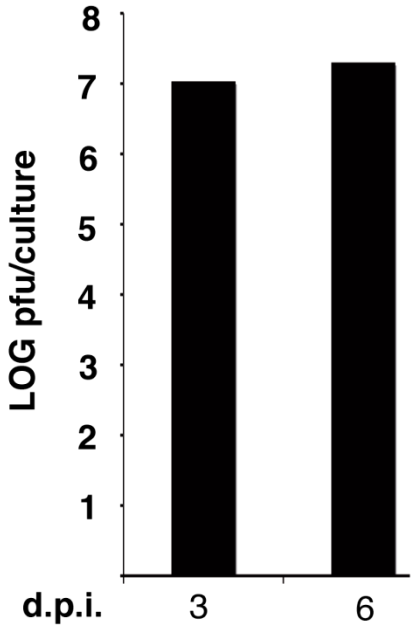

Figure 3.1.14 Viral titers from cultures infected at 41ºC for either 3 or 6 days and subsequently reactivated for 48 hours by transducing cultures with adenovirus encoding HSV-1 ICP0. Titers of infectious virus were determined on Vero cells. Titers are representative of multiple independent experiments.

Figure 3.1.15

Efficient quiescent infection requires non-dividing cells and temperature optimization for different HSV-1 strains.

In establishing our system, one of the selective pressures employed to prevent viral replication was serum starvation. To demonstrate its importance and quantify the levels of viral replication in unstarved cells infected at 41°C, NHDF cultures were maintained in 5% FBS and were either mock infected or infected with wild-type KOS for 72 hours at 41°C. Cultures were photographed by phase-contrast microscopy.

A low level of virus activity was detectable in cultures at 72 hours post infection as evident by small areas of CPE and notable changes in the morphology and detachment of cells. This higher level of CPE present in unstarved cells implies that cultures contain an amalgam of nondividing and dividing cells, where the dividing cells possibly support productive infection.

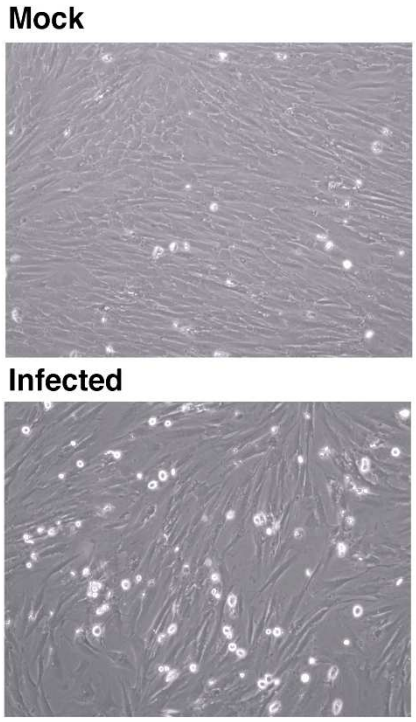

Figure 3.1.15 Phase contrast microscopy of unstarved NHDF cultures that were either mock infected or infected at 41°C for 3 days.

Figure 3.1.16

Serum starvation is required for efficient quiescent infection

To further explore the importance of serum starvation, unstarved and starved cells were infected with wild-type KOS for 72 hours at 41°C. Total protein was then solubilised after 72 hours at 41°C, or alternatively cultures were reactivated by transduction with adenovirus encoding ICP0 (R) and lysed 48 hours later. Samples were resolved by SDS-PAGE, and membranes were probed with antiserum against either IE protein ICP4 or late protein Us11.

Although viral replication was largely inhibited by temperature elevation in unstarved cultures, the presence of Us11 in unreactivated cultures above levels in starved cultures infected at the same time indicates low level viral replication was occurring. The increased expression of Us11 in adenovirus transduced cultures further supported the idea that cycling cells harboured a mixture of quiescent and lytic infection.

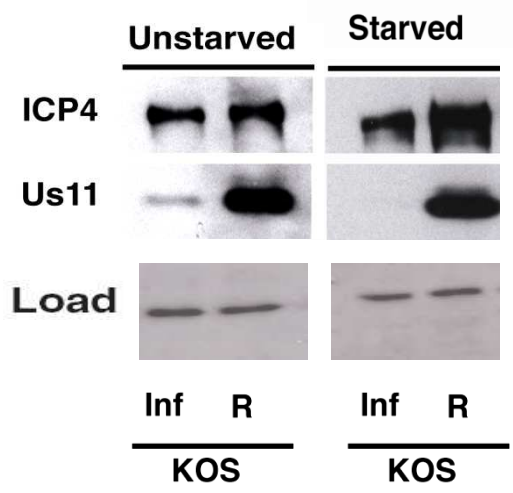

Figure 3.1.16 Western blot analysis of production of HSV-1 viral proteins ICP4 and Us11 in unstarved and starved NHDF cultures that were preincubated at 41°C and then infected with wild-type KOS for 72 hours at 41°C (Inf). Total protein was then solubilized, or alternatively cultures were transduced with adenovirus encoding ICP0 (R) and then lysed 48 hours later. The control probed to ensure even loading of samples was eIF4E.

Figure 3.1.17

Efficient quiescent infection requires temperature optimization for different HSV-1 strains

To address whether inhibition of replication by serum starvation and temperature elevation could be achieved using alternative strains of HSV-1, serum-starved NHDF cultures were infected (inf) with wild-type HSV-1 strains KOS or Patton (Patt) for 72 hours at 41°C. Total protein was then solubilised, or alternatively cultures were transduced with adenovirus encoding ICP0 (R) and lysed 48 hours later. Samples were resolved by SDS-PAGE, and membranes were probed with antiserum against either IE protein ICP4 or late protein Us11.

It quickly became clear that the replication of the Patton strain of HSV-1 was less sensitive to the elevated temperature of 41°C as extensive CPE was observed (not shown). This was reflected in abundant production of Us11 in Patton infected cultures.
Characteristic of the Patton strain, its Us11 gene product is larger than that of Kos.

Having found that 41°C was incapable of inhibiting the Patton strain, serum-starved NHDF cultures were infected with wild-type HSV-1 Patton at 42°C and processed as described above. At 42°C, non productive infection was achieved using the Patton strain, as shown by the absence of Us11 expression at elevated temperature. The infection was indeed quiescent as the Patton strain was controllably reactivated to a productive state by transduction with Ad-0, illustrated by a lytic pattern of Us11 expression at 48 hours post Ad-0 transduction.

Fig 3.1.17

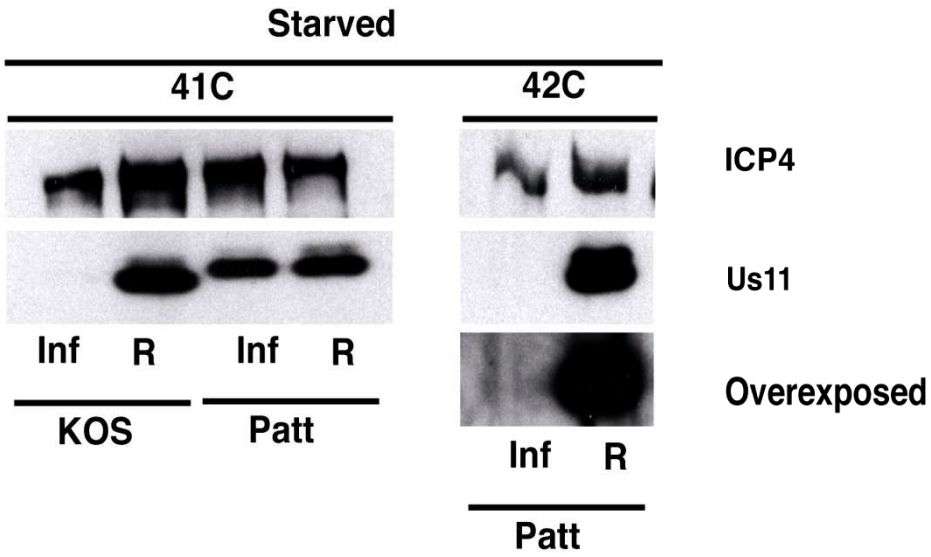

Figure 3.1.17 Western blot analysis of production of HSV-1 viral proteins ICP4 and Us11 in starved NHDF cultures that were preincubated at 41°C and then infected with wild-type KOS and or Patton (Patt) for 72 hours at either 41°C or 42°C (Inf). Total protein was then solubilised or alternatively cultures were transduced with adenovirus encoding ICP0 (R) and then lysed 48 hours later. The lower panel shows an extensive overexposure demonstrating the lack of detectable Us11 protein in quiescently infected cultures.

Figure 3.1.18

Recovery of Patton strain from quiescently infected cultures maintained at 42°C

The levels of infectious virus produced in cultures infected with patton was assessed 3 days post infection at 42°C and two days post reactivation with Ad-0.

Titers were determined by titration on Vero cells. Titers were representative of at least three independent experiments. At the 42°C three day time point the culture harboured a quiescent infection (Q) as no infectious virus was detected , whereas the virus could be reactivated (Q-R) to a productive state by transduction with Ad-0 as evident by extensive virus production at 48 hours post reactivation. This indicated that the Patton strain of HSV-1 was also repressible using this tissue culture model.

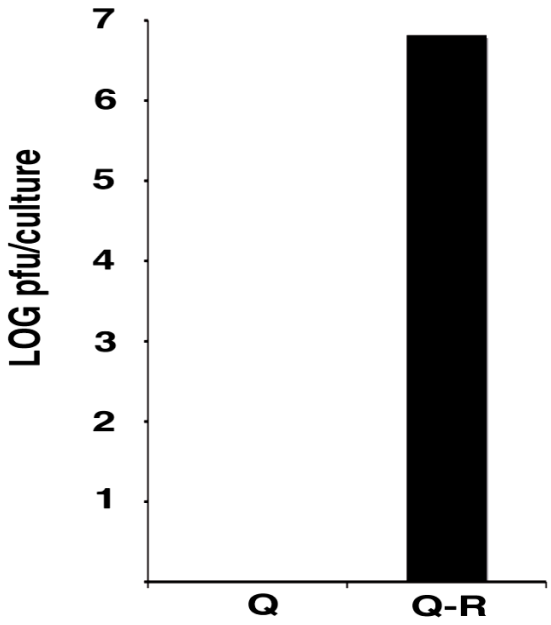

Figure 3.1.18 Viral titer experiment analyzing the amount of infectious virus contained in NHDF cultures quiescently infected (Q) with HSV-1 Patton at 42°C 72 hours post infection or quiescently infected cultures reactivated (Q-R) by transduction with adenovirus encoding ICP0 were determined by titration on Vero cells. Titers are representative of a number of independent experiments.

Figure 3.1.19

HSV-1 ICP0 specifically reactivates virus from quiescently-infected NHDF cells

To address if the reactivation of virus from a quiescent state was due to the specific addition of ICP0, serum-starved NHDF cultures were mock infected (M) or infected (Inf) at 41°C for 72 h to establish quiescence. Cultures were returned to 37°C and either mock transduced with growth medium (med) or adenoviral vectors encoding HSV-1 ICP4 (Ad-4) or HSV-1 ICP0 (Ad-0) as indicated. At 47 hours post-reactivation cultures were metabolically labelled for 1 hour using [^{35}S] Methionine/Cysteine, whole-cell extracts were resolved by SDS-PAGE and fixed, dried and gels were then exposed to X-ray film. The migration of molecular weight markers (in thousands) is indicated to the left of the panel.

The reactivation of virus was found to be specific to Ad-0 transduction as neither Ad-4 nor growth medium could induce a viral pattern of protein synthesis. Also, the addition of Ad-0 to mock-infected cultures proved that the appearance of a viral pattern of protein synthesis was not due to a HSV-1 contaminated Ad-0 vector.

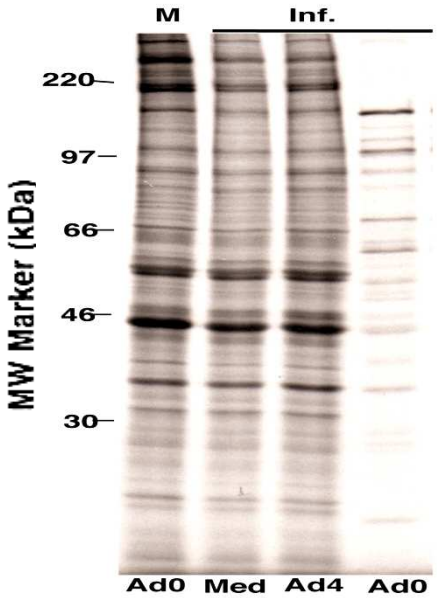

Fig 3.1.19 Metabolic labelling of mock infected (M) and infected NHDF cultures incubated at 41°C for 6 days and either mock transduced with growth medium (med) or adenoviral vectors encoding HSV-1 ICP4 (Ad-4) or HSV-1 ICP0 (Ad-0) for 48 hours. At 1 hour prior to the sampling time, cultures were incubated with [^{35}S] methionine-cysteine, and total protein was harvested by lysing cells in Laemmli buffer. Samples were resolved by SDS-PAGE where 50µl of sample was loaded into each well. Molecular weight standards (in kDa) are shown to the left of the panel.

Figure 3.1.20

ICP0 mediated reactivation induces CPE characteristic of productive infection

To evaluate the cytopathic effect (CPE) present in cultures 48 hours posttransduction with the indicated adenoviral vectors described in fig 3.1.19, a series of phase contrast images were taken.

As expected, quiescently infected cultures that were transduced with an adenoviral vector encoding ICP4 had a morphology largely indistinguishable from uninfected NHDFs, although a small percentage of cells appeared to be rounding up and possibly harbouring productive infection at 48 hours after return to 37°C. This may represent small numbers of cells that had spontaneously reactivated from quiescence.

Cells that had been mock infected and transduced with Ad-0 presented no CPE whereas cells that were quiescently infected and subsequently transduced with Ad-0 exhibited extensive CPE, indicating efficient viral reactivation and replication.

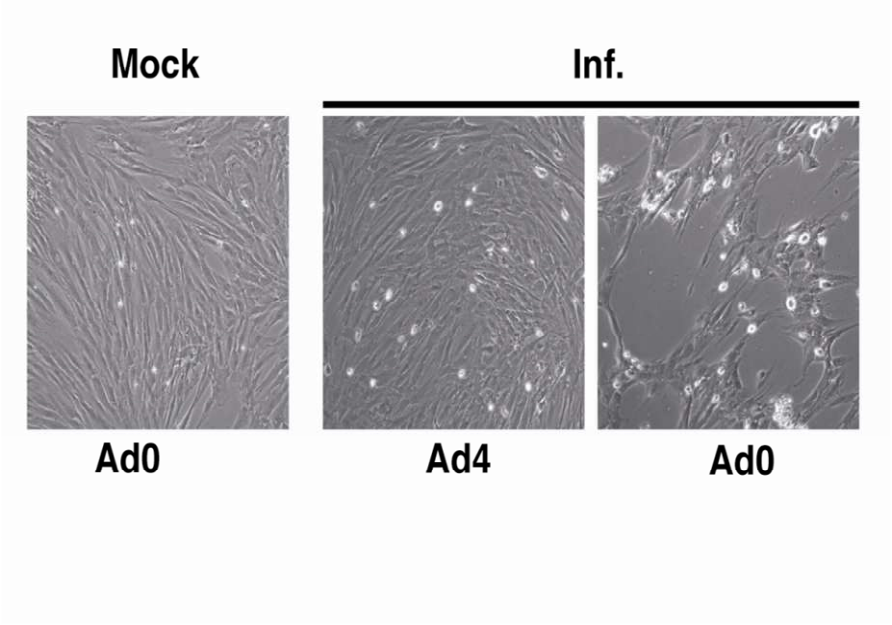

Figure 3.1.20 Phase contrast microscopy of mock infected and quiescently infected cultures that were transduced either with Ad-0 or Ad-4. Images were captured 48 hours after transduction at 10x magnification.

Figure 3.1.21

Human serum prevents secondary virus spread and illustrates the efficiency of virus reactivation from quiescently-infected NHDFs

In order to quantify the amount of the virus in cultures reactivating from a quiescent state it was necessary to prevent reactivating virus from spreading and infecting uninfected cells within the culture. This was achieved by the addition of 5% human serum containing neutralizing antibodies which prevent secondary spread. Titering experiments confirmed the efficiency of 5% human serum against preventing HSV-1 lytic infection (not shown) and as a consequence this concentration of human serum was used for all subsequent experiments when required.

To observe patterns of proteins synthesised in cultures reactivating from quiescence in the presence of human serum, serum-starved NHDFs were infected at 41°C for 72 hours and then returned to 37°C and transduced with the indicated adenoviral vectors encoding ICP4 (Ad-4) or ICP0 (Ad-0). An additional Ad-0 transduced culture was maintained in the presence (+) of 5% human serum after the adenoviral vector was removed. At 47 hours post-reactivation, cultures were metabolically labelled; whole-cell lysates were resolved by SDS-PAGE and fixed, dried gels were exposed to X-ray film. Migration of molecular weight standards (in thousands) is indicated to the left of the panel.

The presence of human serum during reactivation resulted in a pattern of protein synthesis containing a mixture of host cell and viral proteins, suggesting that human serum reduced the secondary spread of virus in reactivated cultures and that a large proportion of the culture initially infected reactivated efficiently in response to Ad-0 transduction.

Figure 3.1.21

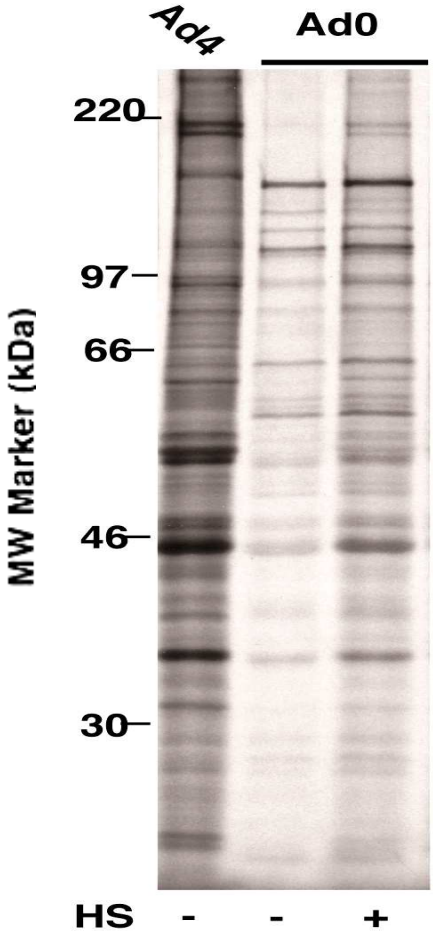

Figure 3.1.21 Metabolic labelling of mock infected (M) and quiescently infected cultures transduced either with Ad-0 or Ad-4 for 48 hours in the presence of 5% FBS DMEM or in the presence of 5% Human serum DMEM (H.S). At 1 h prior to sampling, cultures were incubated with [^{35}S]-Methionine/Cysteine, and total protein was harvested by lysing cells in Laemmli buffer. Samples were resolved by SDS-PAGE where 50µl of sample was loaded into each well. Molecular weight standards (in kDa) are shown to the left of the panel.

Figure 3.1.22

Expression of Us11 in samples reactivated in the presence of Human serum

To further confirm secondary spread inhibition by human serum, NHDF cultures were either mock infected (M) or infected (Inf) at 41°C for 72 hours and then returned to 37°C and mock transduced with growth medium (Med) or transduced with the indicated adenoviral vectors in the presence or absence of 5% human serum (HS). At 48 hours posttransduction whole-cell extracts were resolved by SDS-PAGE, and membranes were probed with antiserum against Us11.

The levels of Us11 expression were reduced in cultures controllably reactivated in the presence of 5% human serum relative to cultures reactivated in the presence of 5% FBS DMEM and this was more apparent on the normal exposure of Us11 blots.

As outlined previously, low levels of Us11 expression are indicative of spontaneous reactivation occurring in quiescently infected cultures that are transferred to 37°C and supplemented with 5% FBS for 48 hours. However, when quiescent cultures are transferred to 37°C in the presence of human serum, Us11 production becomes undetectable by western blot, again highlighting the effectiveness of human serum in preventing secondary spread from occurring.

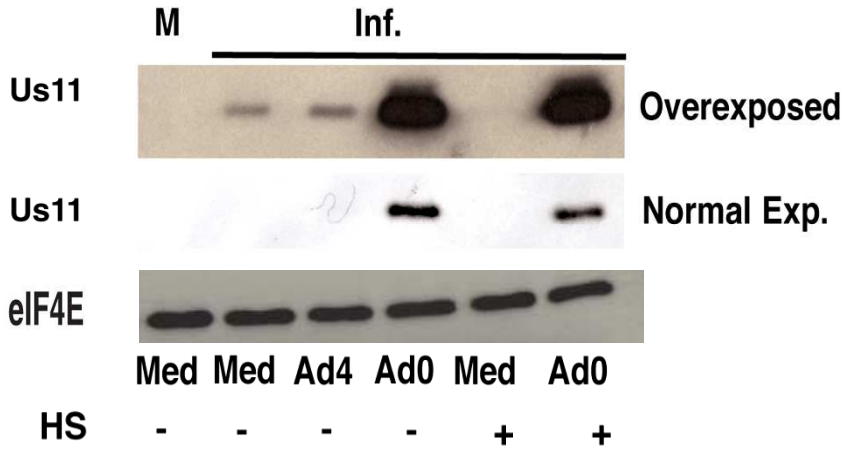

Figure 3.1.22 Western blot analysis of production of HSV-1 viral protein Us11 in mock infected and quiescently infected NHDF cultures that were transduced with either medium, Ad-0 or Ad-4 vectors in the presence or absence of human serum. Samples were reactivated for 48 hours and then lysed in 1x Laemmli buffer. An Overexposure was taken to illustrate low level production Us11 production in cells spontaneously reactivating.

Figure 3.1.23

Quantification of virus reactivation from quiescence

The quantify the amount of the culture reactivating from quiescence in the presence of human serum, NHDF cells were grown on glass coverslips and infected at 41°C for 72 hours. Cells were returned to 37°C and mock transduced (Med) or transduced with adenovirus encoding ICP0 (Ad-0) in the presence or absence of 5% human serum.

When virus was reactivated in the presence of 5% FBS DMEM approximately 95% to 100% of the culture stained positive for ICP5. This percentage dropped to around 60% when reactivated in the presence of human serum. Quiescently infected cultures that were not reactivated with ICP0 but instead maintained in the presence of human serum showed that about 2% of cells underwent spontaneous reactivation in the first 48 hours upon return of the culture to 37°C.

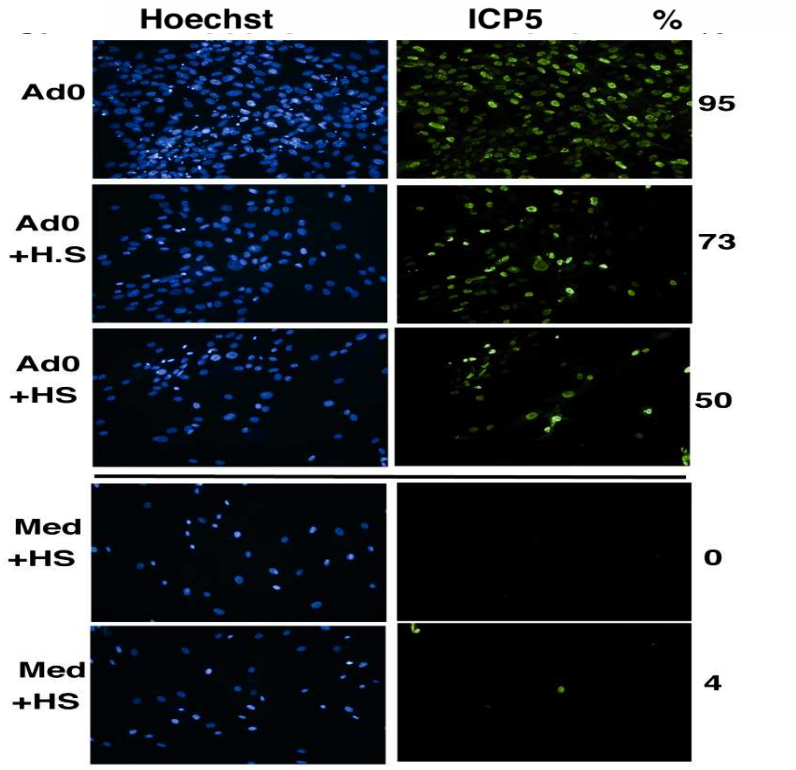

Figure 3.1.23 Immunofluorescence of ICP5 production in cultures 48 hours post Ad-0 or medium alone transduction in the presence or absence of 5% Human serum (HS). Cultures were fixed and probed with the antiserum against ICP5. Nuclei were counterstained with Hoechst, and images were captured at a 20x magnification. Two representative fields are shown for HS experiments. Percent reactivation is presented to the right of the immunofluorescent images. For Ad-0 + human serum and medium +human serum reactivation experiments two images were taken to represent each culture.

Figure 3.1.24

Enlargement of PML structures in NHDF cells infected at elevated temperature

During the establishment of quiescence it was observed that only small amounts of the immediate early protein ICP0 was being produced relative to immediate early proteins ICP4 and ICP22. ICP0 is a ubiquitin ligase which functions to disrupt the repressive ND10 structures within cell nuclei thus promoting the establishment of a lytic infection. (Clements, Stow 1989; Cai, Schaffer 1992; Maul, Guldner & Spivack 1993; Maul, Everett 1994; Chee *et al*., 2003). PML is a key ND10 component that is dispersed during lytic infection but recruited to viral genome and enlarged during non-productive infection.

To observe PML morphology and localization within nuclei of cells during the establishment of quiescence, a series of indirect immunofluorescence experiments were performed. Serum-starved NHDF cultures were grown on glass coverslips and mock infected or infected at 41°C or 37°C for 10 hours and then fixed.

The indirect immunofluorescence experiments showed that PML structures exhibited a normal pattern of nuclear speckling in uninfected cells at 41°C. The dispersal of these structures was observed in infected cells undergoing lytic replication at 37°C, as identified by costaining for the viral antigen ICP4. Conversely, cells infected at 41°C contained enlarged PML aggregate structures. This phenomenon occurred only in infected cell nuclei that co-stained for viral antigen ICP4 while neighbouring uninfected cells had normal PML morphology. This suggested that failure to produce sufficient ICP0 to disperse repressive PML was likely to play a role in the establishment of a non productive infection.

Figure 3.1.24

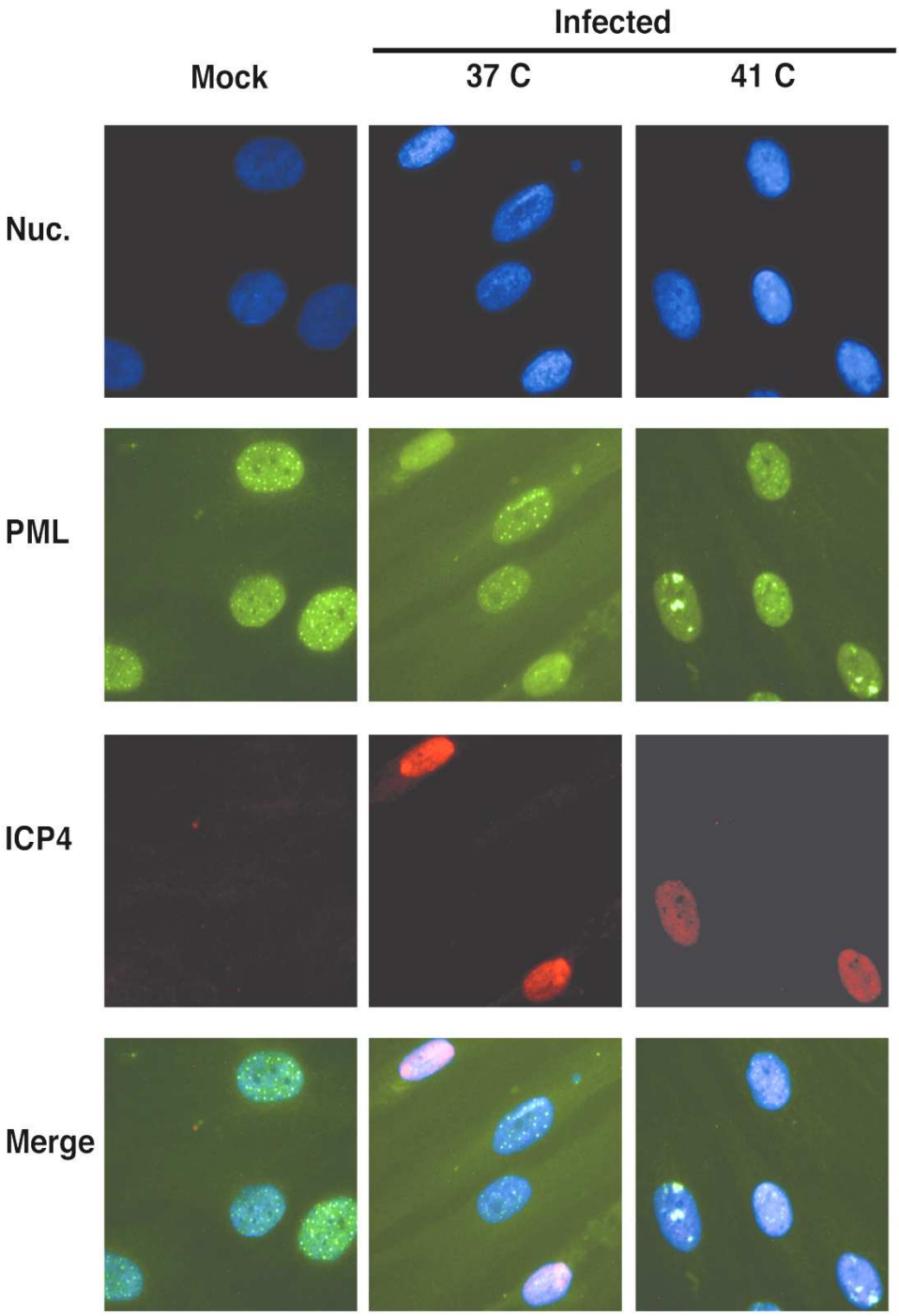

Figure 3.1.24 Immunofluoresence of enlarged PML structures in NHDF cells infected at 37°C and at elevated temperature 41°C. Cultures were co-probed with antiserum against human PML (green) and HSV-1 ICP4 (red); proteins were then detected using the appropriate FITC-conjugated or TRITC-conjugated secondary antibodies. Nuclei were visualised by Hoechst counterstain and images were captured at 63x magnification.

Figure 3.2

The role of host kinases during HSV-1 reactivation.

The development of this model which facilitates quiescent infection of primary human diploid fibroblasts allows the opportunity to directly compare HSV-1 lytic replication and HSV-1 reactivation in the same cell type. As such, this approach can be used to illuminate the mechanistics of the various stages in the life cycle of HSV-1.

Whereas previous reports have shown that the activity of host MEK-ERK signaling pathways is stifled during lytic infection within a number of cell lines (McLean, Bachenheimer 1999; Walsh, Mohr 2004; Sloan *et al*. 2006; Santamaría *et al*. 2009), little is known about the signaling pathways required by HSV-1 during reactivation from quiescence. To address this issue we first decided to characterize the kinetics of reactivation from a quiescent state over the 48 hour reactivation period.

Figure 3.2.1

The pattern of protein synthesis during reactivation

Serum starved NHDFs were mock-infected (M) or infected with HSV-1 KOS (m.o.i. 0.5-1) at 41°C for 6 days to establish quiescence (Q). Cultures were returned to 37°C and either mock-transduced (-) or transduced (+) with Adeno viral vectors encoding the immediate early trans activating protein ICP0 to initiate reactivation of quiescent virus (R).

During the first 24 hours of reactivation there were no significant differences in patterns of proteins synthesized between mock, quiescent and reactivating samples. By 34 hours post reactivation the synthesis of a small number of higher molecular weight proteins was observed and these proteins co-migrated with polypeptides abundantly produced in reactivating cultures at the 48 hour time point.

Figure 3.2.1

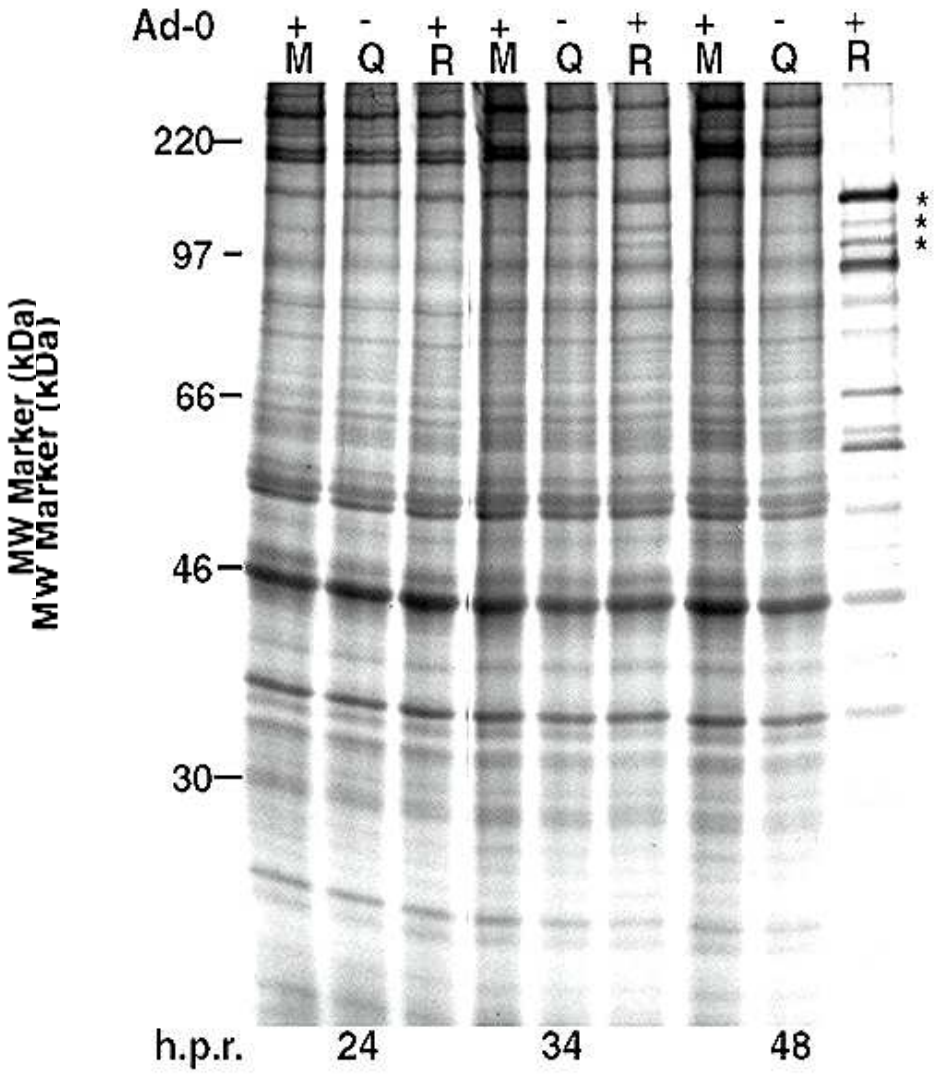

Figure 3.2.1 Metabolic labelling of mock (M) quiescently infected (Q) and reactivated (R) NHDF cultures. At 1 hour prior to the indicated sampling times in hours post-reactivation (h.p.r.), cultures were incubated with [^{35}S]-Methionine/Cysteine, and total protein was harvested by lysing cells in Laemmli buffer. Samples were resolved by SDS-PAGE where 50µl of sample was loaded into each well. Molecular weight standards (in kDa) are shown to the left of the panel. The asterisk indicates suspected viral proteins produced during reactivation.

Figure 3.2.2

Kinetics of viral protein production in reactivating cultures

To further explore the kinetics of viral protein production in reactivating cultures, quiescently-infected cultures were established and reactivated as described in figure 3.2.1 and whole cell extracts were prepared at the indicated time points. Samples were resolved by SDS-PAGE and membranes were probed with the indicated antibodies for immediate early (IE) proteins ICP0, ICP4 and ICP22, the leaky-late protein ICP5 which is produced both prior to and following viral DNA synthesis and finally the late protein Us11, which is only produced after viral DNA synthesis.

The presence of low level ICP4 and ICP5 was detected by western blot in samples from unreactivated quiescently infected cultures that were mock transduced with medium. This production of ICP4 and ICP5 does not necessarily signify lytic replication as production of a number viral genes, including those of ICP4, have been reported in latently-infected neurons in vivo as well as in tissue culture models (Deatly *et al*., 1987; Kramer, Coen 1995; Chen *et al*., 1997).

The production of the lytic gene transactivator ICP0 was not evident in quiescently infected cultures that were mock transduced with medium. Furthermore, ICP0 was not efficiently produced in mock infected cultures transduced with Ad-0 as the ICP0 gene encoded by this Adenoviral vector is under the control of its own promoter (Zhu *et al*., 1990). As ICP4 binds to the ICP0 promoter to regulate its expression (Zhu, Cai & Schaffer 1994) the low levels of ICP4 that exist within quiescently infected cultures may play an important role in promoting efficient expression of exogenous ICP0 upon trans-gene delivery by the adenoviral vector.

By 34 hours post-transduction ICP0 was produced and significant accumulation of ICP4 and ICP5, as well as production and processing of ICP22, detected as higher-migrating species in SDS-PAGE gels was evident in Ad-0-reactivated cultures. Uninfected cultures transduced with Ad-0 showed no expression of these antigens while quiescent cultures that were transduced with medium alone showed the same levels of ICP4 and ICP5 expression over the 48 hour time period. These results suggest that the significant reactivation events began somewhere between 24-34 hours post-transduction, coincident with robust expression of ICP0. Low levels of Us11

were detectable at 34 hours in reactivated cultures and combined with the accumulation of processed ICP22 suggested that reactivation was progressing into a fully productive infection. Indeed, robust production of all viral antigens examined along with characteristic host Shutoff of protein synthesis (Fig 3.2.1) was evident by 48 hours post-transduction of quiescently infected NHDFs.

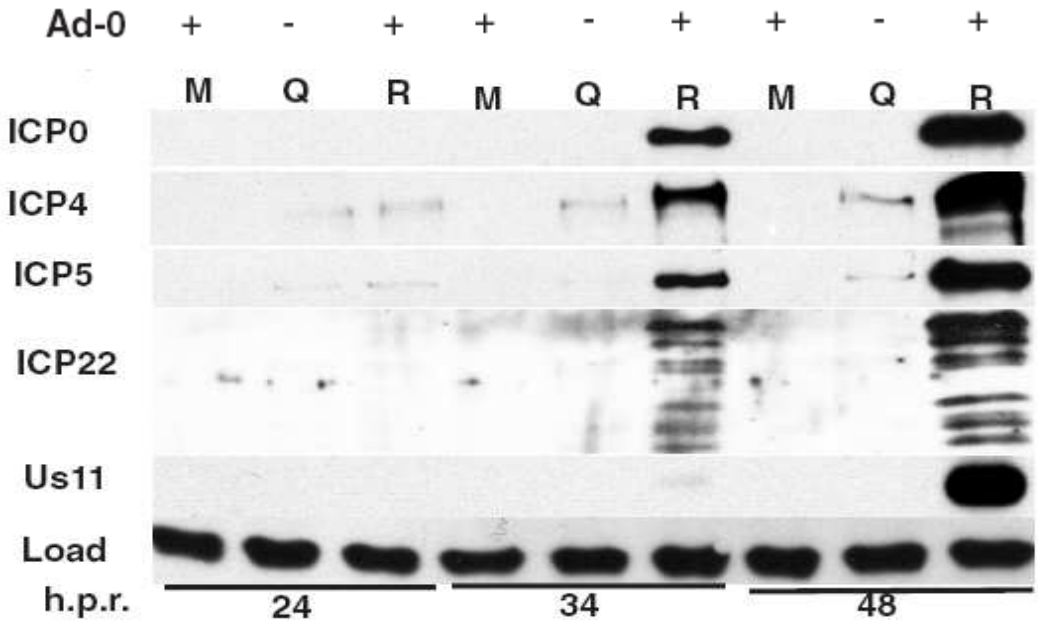

Figure 3.2.2 Western Blot analysis of the production of HSV-viral proteins ICP0, ICP4, ICP22, ICP5 and Us11 in mock (M), quiescently infected (Q) and reactivated (R) NHDF cultures at the indicated sampling times in hours post-reactivation (h.p.r.). To ensure even loading of samples eIF4E was probed for as a loading control.

Figure 3.2.3

Analysis of viral antigen expression in quiescent and reactivating cultures by indirect imuunofluorecence

Both ICP4 and ICP5 were expressed at low levels in unreactivated quiescent NHDFs but accumulated rapidly in reactivating cultures at 34 hours post-transduction. To address whether these expression patterns were either the result of high-level expression in a small subset of cells or low level expression within the majority of the culture, the production of both ICP5 and ICP4 was assessed in reactivated cultures by indirect immunofluorescence.

NHDFs were mock-infected (M) or quiescently infected (Q), then mock-infected or infected with Ad-0 to reactivate quiescent virus (R). At 30 hours post-transduction with either medium control or Ad-0, cultures were washed, fixed in formaldehyde and probed with antiserum against ICP4 or ICP5. Nuclei were counterstained with Hoescht and images were captured at 63x magnification using a Leica DFC 500 microscope.

It was discovered that neither ICP5 nor ICP4 were visible in either mock or quiescently infected cells, suggesting that their detection in quiescently-infected samples by western blotting represented low-level expression which was below the sensitivity of indirect immunofluorescence. Conversely, cultures that were transduced with Ad-0 had approximately 40% of cells staining positive for both viral antigens by 30 hours post-transduction. The absence of significant viral antigen accumulation over the first 24 hours but accumulation of early and mid-phase viral gene expression in approximately 40% of the culture by 30 hours post-transduction suggested that reactivation occurred in a synchronous manner between 24-30 hours coinciding with robust expression of ICP0 and further reactivation of additional virus likely occurred over time.

Figure 3.2.3

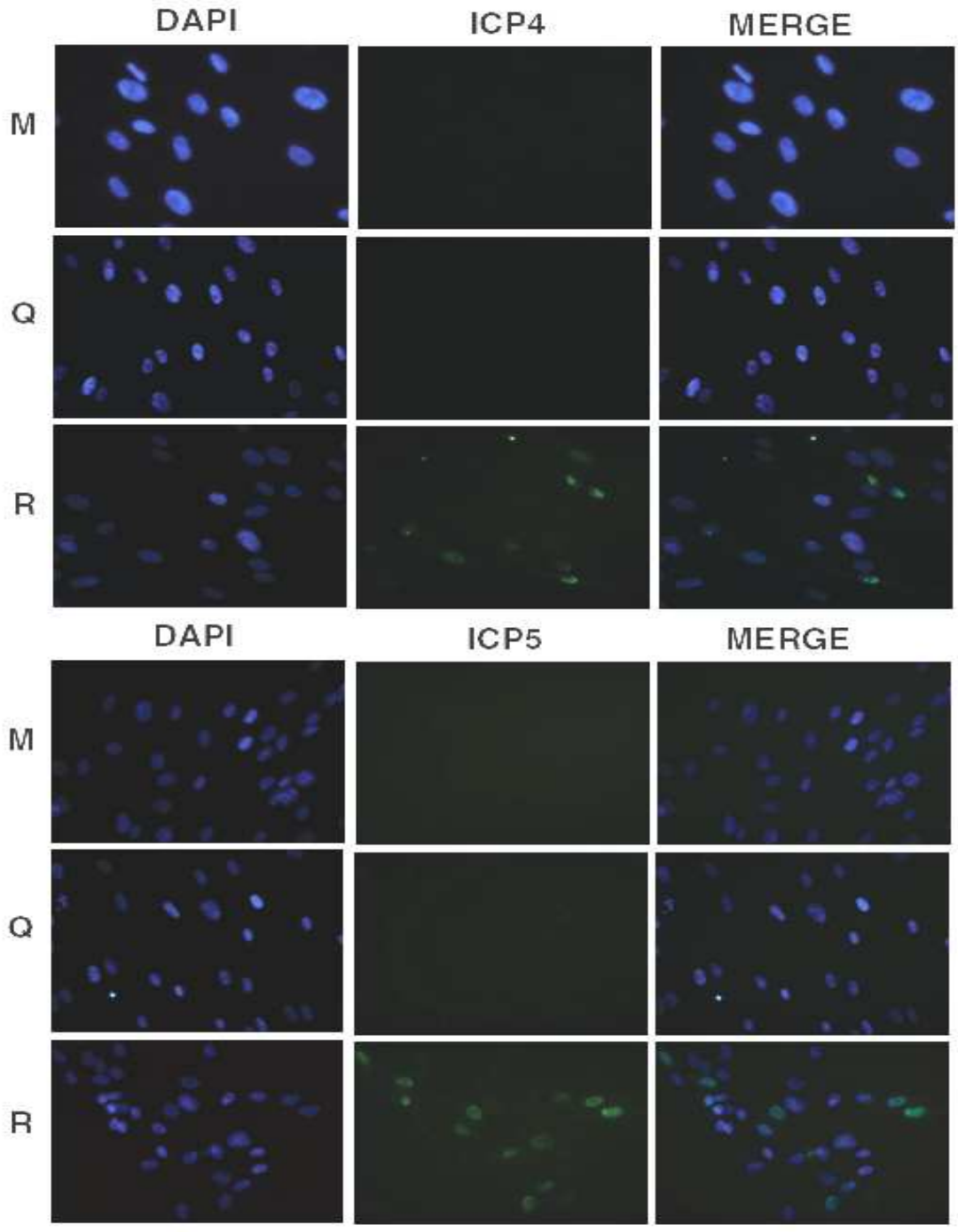

Figure 3.2.3 Immunofluorescence of HSV-1 viral proteins ICP4 and ICP5 in mock (M), quiescently infected (Q) and reactivated (R) NHDF cultures at 30 hours post-reactivation. Cultures were probed with antiserum against HSV-1 ICP4 (green) and ICP5 (green); proteins were then detected using the appropriate FITC-conjugated conjugated secondary antibody. Nuclei were visualised by Hoechst counterstain. Images were captured at 63x magnification.

Figure 3.2.4

The pattern of ERK and p38 activity in reactivating cultures

Having established the kinetics of reactivation and discovered that a significant proportion of the culture can be controllably reactivated in a synchronous manner, changes in the activity of signalling pathways could now be measured during the various stages of reactivation. With this in mind, we decided to study the phosphorylation patterns of both the ERK and p38 signaling pathways in either mock-infected (M), quiescently infected (Q), or reactivated (R) cultures.

Serum-starved and temperature-elevated NHDF cultures were mock infected or infected and maintained for 6 days at 41°C. At 6 days post infection at 41°C mock-infected (M) or quiescently-infected (Q) NHDFs were returned to 37°C and mock-infected (-) or infected (+) with Ad-0 to reactivate quiescent virus (R). Whole cell extracts were prepared at the indicated times, in hours post-reactivation (h.p.r.). Samples were analyzed by Western blotting with antibodies towards total or phosphorylated forms of ERK or p38.

The levels of total p38 remained unchanged in mock, quiescent and reactivated cultures. Phosphorylated p38 remained identical in both mock and quiescent NHDFs at each point in the time course. Quiescent cells that were transduced with AD-0 had no change in p38 phosphorylation over the first 24 hours. At 34 hours post-transduction, p38 phosphorylation increased. This coincided with the onset of reactivation as evident by the expression of viral proteins seen in Fig 3.2.2. As the reactivation progressed, p38 phosphorylation continued to increase. Similar to p38, abundance of total ERK remained unchanged in samples over the course of reactivation. Interestingly, quiescent cultures displayed a modest reduction in the levels of phosphorylated ERK relative to mock cells at each time point. This slight reduction in ERK activity suggested that quiescence had a negative affect on mitogenic signaling. Over the first 24 hours of reactivation from quiescence, changes in ERK phosphorylation were not observed. At 34 hours post reactivation a modest but reproducible increase in ERK phosphorylation was observed relative to quiescent cultures. By 48 hours post reactivation ERK phosphorylation had declined below that

of either mock or quiescent NHDFs, signifying that the virus entered a more lytic-like state.

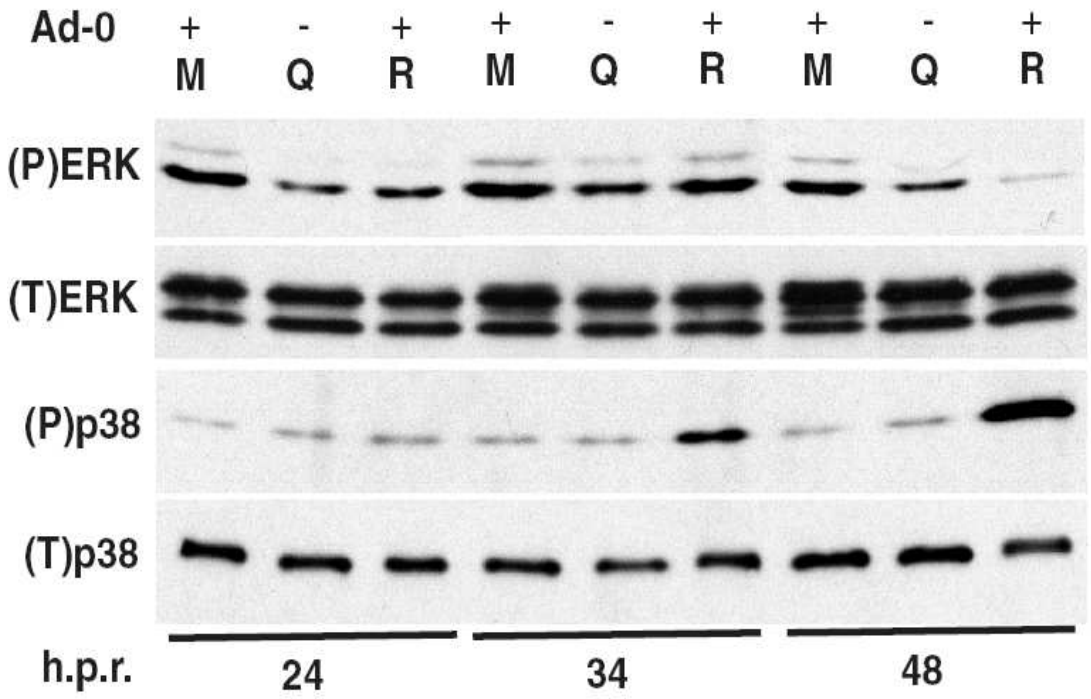

Figure 3.2.4 Western Blot analysis of NHDFs of signalling pathways activated in mock (M), quiescently infected (Q) and reactivated (R) NHDF cultures. Whole cell extracts were prepared at the indicated times in hours post-reactivation (h.p.r.). Samples were resolved by SDS-PAGE and probed with antibodies towards total or phosphorylated forms of ERK or p38.

Figure 3.2.5

ERK stimulation is specific to HSV- 1 reactivation

The transient stimulation of ERK phosphorylation during reactivation from quiescence was not expected and contrasted with previous reports that described ERK inhibition during lytic replication (Gillis, Okagaki & Rice 2009)(Zachos, Clements & Conner 1999)(Hargett, McLean & Bachenheimer 2005). To elucidate whether the stimulation of ERK activity observed was a consequence of the employed culture conditions, NHDFs were mock-infected at 41°C for 6 days and returned to 37°C. Cultures were then mock-infected (-) or infected (+) with Ad-0 for 2 hours, then washed and mock-infected (M) or infected (I) with HSV-1 KOS at m.o.i 1 for 48 hours. Whole cell extracts were prepared and analyzed by western blotting with the indicated antisera. The cellular antigen eIF4E was probed for as a loading control.

It was discovered that ERK activity was robustly suppressed as the virus replicated, as illustrated by the accumulation of the leaky-late and late viral proteins ICP5 and Us11. At no time during the infection was ERK stimulation observed when timepoints were examined (not shown). Additionally, the mock infected cells that were maintained for 6 days at 41°C, returned to 37°C and transduced with Ad-0 prior to lytic infection with HSV-1 showed no stimulation of ERK activity which confirmed that the process of Ad-0 transduction was not the cause of ERK stimulation, nor did it interfere with HSV-1 mediated ERK suppression.

In summary, the culture conditions employed were not a contributory factor in the activation of ERK observed during reactivation.

Figure 3.2.5

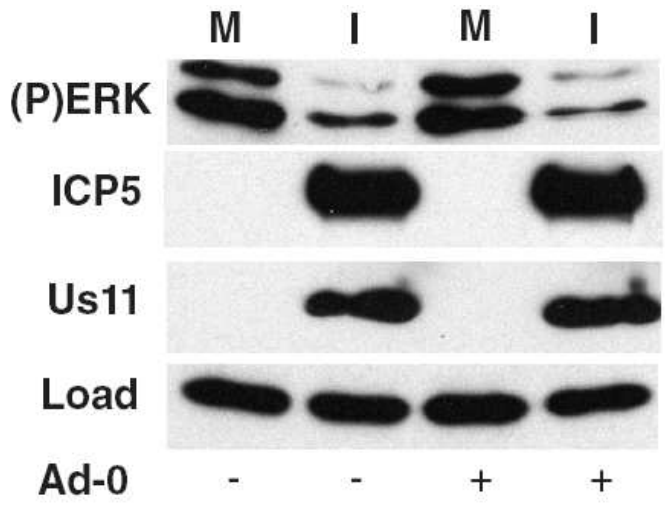

Figure 3.2.5 Western blot of phosphorylated ERK (pERK) and HSV-1 viral proteins ICP5 and Us11 in NHDFs that were mock-infected at 41°C for 6 days, returned to 37°C and subsequently mock-infected (-) or infected (+) with Ad-0 for 2 hours, then washed and mock-infected (M) or infected (I) with HSV-1 KOS at m.o.i 1 for 48 hours. The cellular antigen eIF4E was probed for as a loading control.

Figure 3.2.6

U0126 inhibits ERK activation during reactivation

The modest stimulation of ERK during the initial stages of reactivation led us to examine the potential role(s) of both MEK-ERK and p38 during early stages of reactivation from quiescence. Quiescently-infected NHDFs were returned to 37°C and mock-transduced to maintain quiescence (Q) or infected with Ad-0 to reactivate virus (R) in the presence of equal volumes of DMSO (solvent control), U0126 (20μM) or SB203580 (40μM). 34 hours later whole cell lysates were prepared and analyzed by western blotting with the indicated antibodies. The concentrations of U0126 (20μM) or SB203580 (40μM) were chosen from experiments outlined in (Walsh, Mohr 2004).

Again, the ERK activity in reactivated cultures was found to be modestly stimulated relative to quiescent cultures and it was observed that U0126 robustly inhibited ERK activity in reactivating cultures. Importantly, both U0126 and SB203580 had no influence on the initial expression of adeno-viral derived ICP0 at this early point in reactivation, nor did they affect total ERK levels.

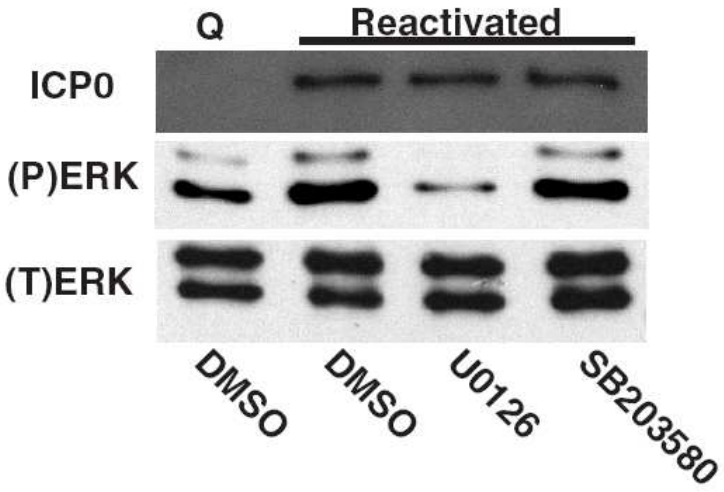

Figure 3.2.6 Western blot of total or phosphorylated forms of ERK and HSV-1 viral protein ICP0 in quiescently-infected NHDFs mock-transduced to maintain quiescence (Q) or infected with Ad-0 to reactivate virus (R) in the presence of equal volumes of DMSO, U0126 (20μM) or SB203580 (40μM).

Figure 3.2.7

Inhibition of MEK-ERK signalling reduces HSV-1 antigen accumulation during reactivation

To observe what effects MEK-ERK inhibition had on viral antigen accumulation during reactivation, quiescently-infected NHDFs were mock-reactivated (Q) or reactivated with Ad-0 (R) in the presence of equal volumes of DMSO, U0126 (20μM) or SB203580 (40μM).

The presence of U0126 caused a significant reduction in the expression of immediate early, leaky late and late viral antigens at all time points taken whereas SB203580 had no significant effect on antigen accumulation.

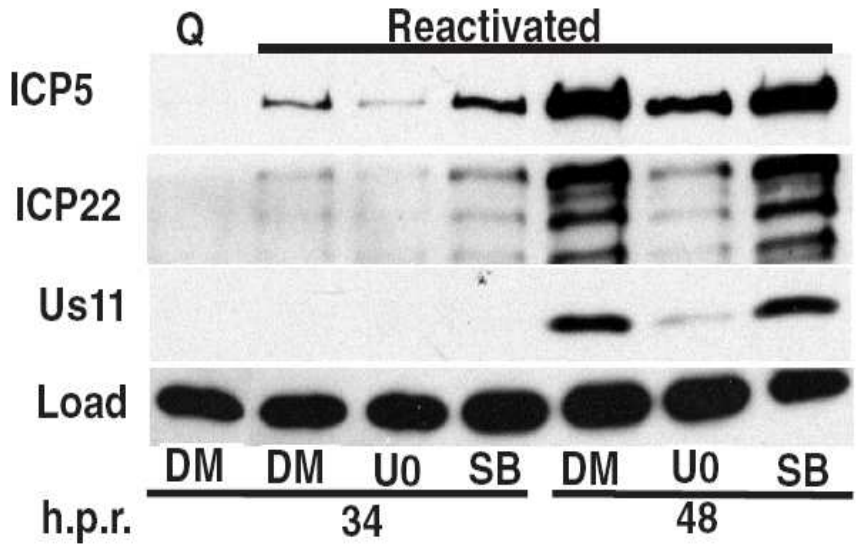

Figure 3.2.7 Western blot of HSV-1 viral proteins ICP5, ICP22 and Us11 in quiecently-infected NHDFs mock-transduced to maintain quiescence (Q) or infected with Ad-0 to reactivate virus (R) in the presence of equal volumes of DMSO (solvent control), U0126 (20μM) or SB203580 (40μM). Whole cell extracts were prepared at the indicated times in hours post-reactivation (h.p.r.) and eIF4E was used as a loading control (Load).

Figure 3.2.8

Inhibition of MEK-ERK signalling reduces HSV-1 reactivation

To further confirm the requirement for ERK activity during HSV-1 reactivation from quiescence, quiescently-infected NHDFs were reactivated (R) with Ad-0 in the presence of equal volumes of DMSO or U0126 (20μM).

The numbers of cells staining positive for both ICP5 and ICP4 was notably reduced in cultures reactivated in the presence of U0126. Additionally, ICP5 and ICP4 staining intensities in antigen-positive cells were reduced in the presence of U0126.

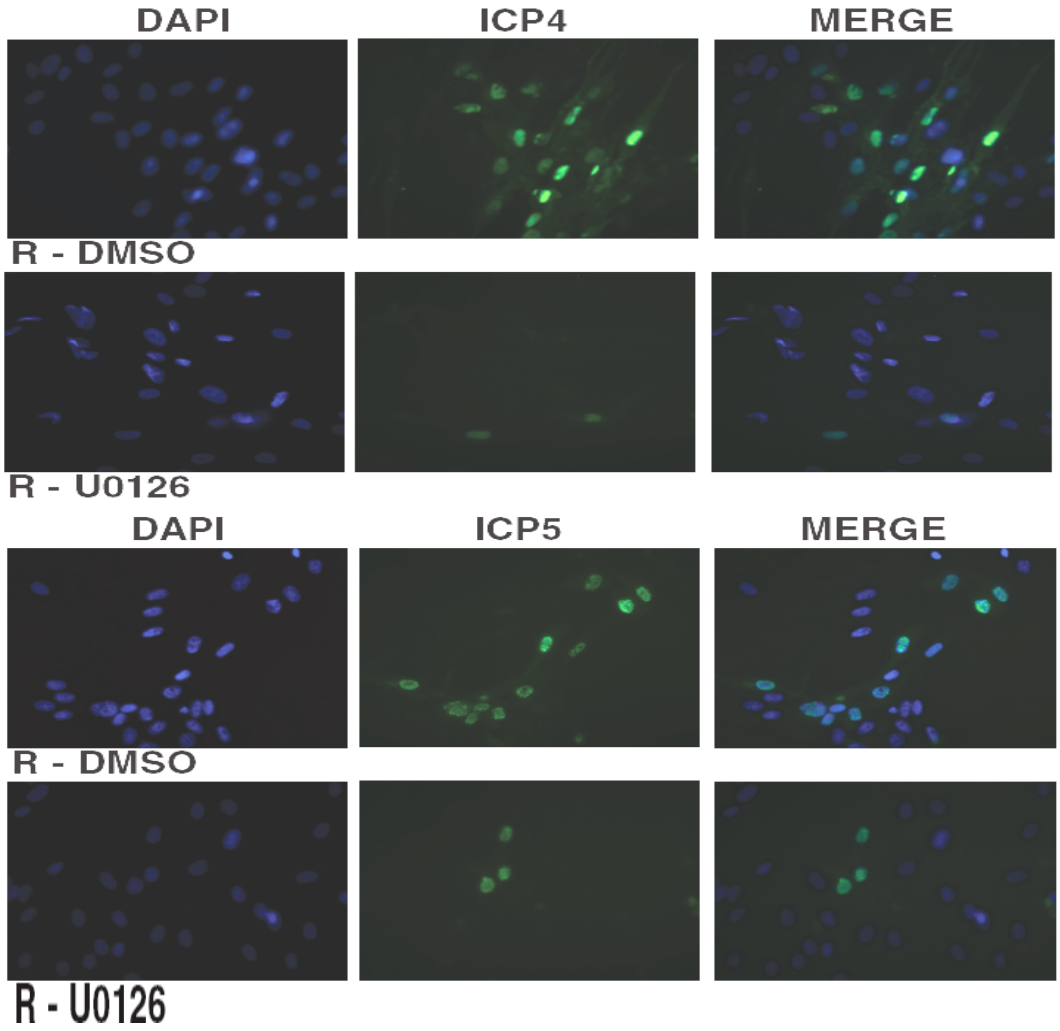

Figure 3.2.8 Immunofluoresence of HSV-1 viral proteins ICP4 in reactivated (R) NHDF cultures at 34 hours post-reactivation in either the presence of either DMSO and U0126 (20μM). Cultures were probed with antiserum against HSV-1 ICP4 (green) proteins were then detected using the appropriate FITC-conjugated conjugated secondary antibody. Nuclei were visualised by Hoechst counterstain. Images were captured at 20x magnification.

Figure 3.2.9

The effect of U0126 on yields of infectious virus produced in reactivating cultures

To quantify the affects of U0126 on yields of infectious virus produced in reactivating cultures, NHDFs were quiescently-infected in 35mm dishes and reactivated by Ad-0 transduction for 48 hours in the presence of DMSO, U0126 (20μM) or SB203580 (40μM). Culture supernatants were serially diluted, plated on permissive Vero cells and titers of infectious virus were calculated as p.f.u./culture supernatant.

It was found that SB203580 had no effect on reactivation from quiescence as the level of infectious progeny within supernatants were identical to those taken from cultures reactivated in the presence of DMSO. In contrast to SB203580, U0126 caused a 20 fold reduction of virus production compared to DMSO treated samples.

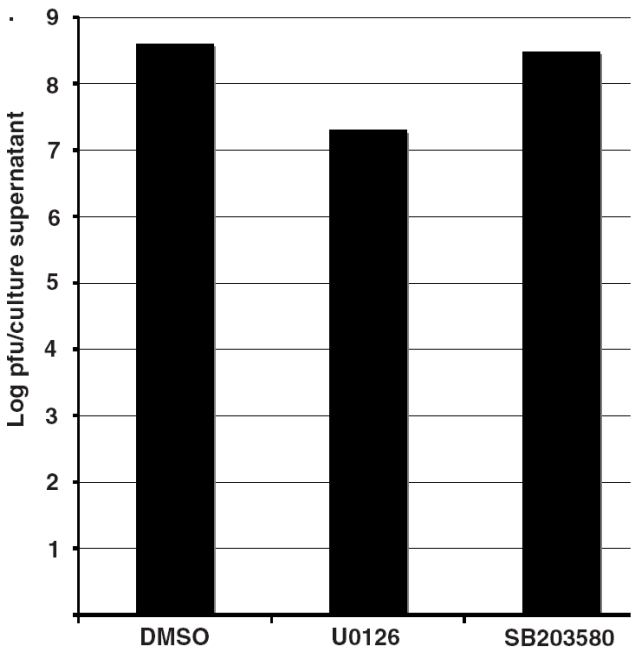

Figure 3.2.9 Viral titers of HSV-1 produced over a 48 hour period of reactivation in the presence of either DMSO, U0126 (20μM) or SB203580 (40μM). Titers are represented as Log/pfu/culture supernatant and are the average of a number of independent experiments.

Figure 3.2.10

The reactivation of HSV-1 in the presence inhibitors and Human serum

To confirm that the inhibition of HSV-1 replication previously observed was due to U0126 inhibiting reactivation and not secondary viral spread, the reactivation of virus was performed in the presence of human serum to inhibit viral secondary spread. Mock-infected (M) or quiescently-infected NHDFs were mock-reactivated (Q) or reactivated with Ad-0 (R). After 2 hours the adenoviral vector was removed and cultures were washed and maintained in medium containing 5% human serum along with either DMSO, U0126 (20μM) or SB203580 (40μM). Samples were analyzed by western blotting with antibodies toward ICP4, ICP5, Us11 or eIF4E.

Again it was found that U0126 inhibited reactivation from quiescence as evident by a reduction in production of ICP4, ICP5 and Us11 at both the 34 hour and 48 hour time points. Furthermore, inhibition of p38 had no effect on antigen accumulation, with the exception of modest effects on ICP5.

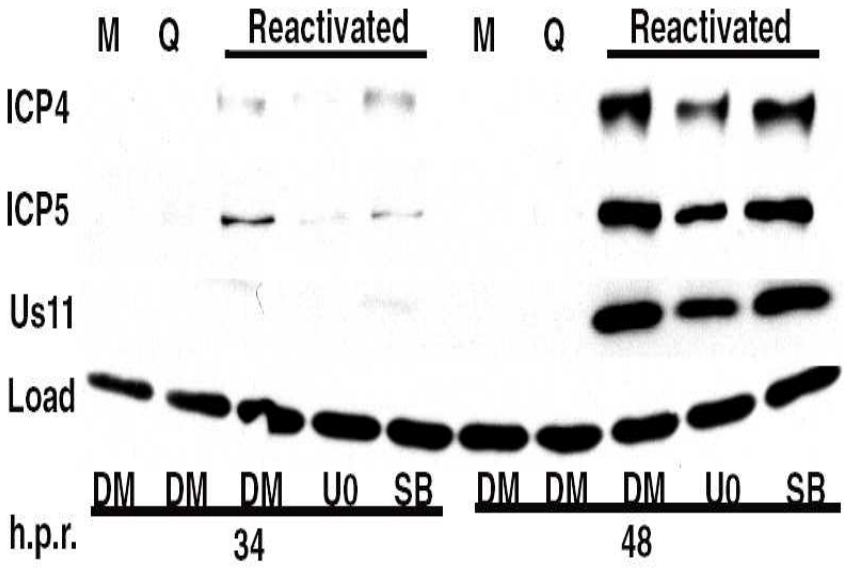

Figure 3.2.10 Western blot of HSV-1 viral proteins ICP4, ICP5 and Us11 in quiescently-infected NHDFs mock-transduced to maintain quiescence (Q) or infected with Ad-0 to reactivate virus (R) in the presence 5% human serum and equal volumes of either DMSO (solvent control), U0126 (20μM) or SB203580 (40μM). Whole cell extracts were prepared at the indicated times in hour post-reactivation (h.p.r.) and eIF4E was probed for as a loading control (Load).

Figure 3.2.11

Inhibitors of ERK phosphorylation do not affect lytic replication

These findings suggested a role for MEK-ERK in virus reactivation. However, to exclude the possibility that this was due to metabolic changes in the cell due to culture conditions that might influence the processes involved in HSV-1 infections we analysed whether ERK activity was required for lytic replication in cells that had been mock-quiescently infected. NHDFs were mock-infected for 6 days at 41°C. Upon return to 37°C, cultures were mock-infected (M) or infected (Inf) at moi 0.5-1 in the presence of DMSO or U0126 (20μM). At 48 hours post infection, whole cell lysates were prepared and analyzed by western blotting with the indicated antibodies.

The inhibition of ERK did not affect the replication or spread of HSV-1, as viral antigen accumulation was identical in cultures infected either in the presence of DMSO or U0126. This data correlates with findings from a previous report (Walsh, Mohr 2004), which demonstrated that ERK inhibition does not affect lytic HSV-1 replication and suggests that the reduction of viral reactivation from quiescence observed in U0126 treated cultures is the result of the drug affecting the reactivation process directly.

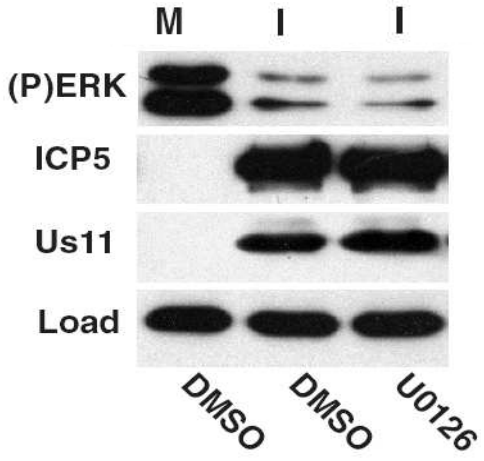

Figure 3.2.11 Western blot of HSV-1 viral proteins ICP5 and Us11 in NHDFs that were mock-infected for 6 days at 41°C and returned to 37°C, cultures were then mock-infected (M) or infected (I) at moi 0.5-1 in the presence of DMSO or U0126 (20μM). At 48 hours post infection whole cell lysates were prepared and analyzed by western blotting with the indicated antibodies. eIF4E was probed for as a loading control.

Figure 3.2.12

The effects of U0126 on spontaneous reactivation of quiescent HSV-1

As spontaneous reactivation occurs naturally within all in-vitro models (Feldman *et al*., 2002; Margolis *et al*., 2007; Knickelbein *et al.,* 2008) and possibly reflects the natural dynamic state of HSV-1 in vivo, it was important to assess whether ERK inhibition affected this process. This also allowed us to exclude effects of ERK inhibition on Ad-0 mediated reactivation. To observe whether ERK inhibition impacted spontaneous reactivation, mock-infected (M) or quiescently-infected NHDFs were returned to 37°C and allowed to spontaneously reactivate for 5 days in the presence of either DMSO, U0126 (20μM) or SB203580 (40μM).

Whole cell extracts were prepared and analyzed by western blotting with antisera against ICP0, Us11 or cellular eIF4E as a loading control. The Us11 blots were purposely overexposed to detect the low levels of Us11 in drug-treated reactivating cultures. The same blot and exposure was used but an empty dividing lane to allow excessive overexposure (O.E) was cropped out.

Spontaneous reactivation of HSV-1 in cells treated with either U0126 or SB203580 showed that both U0126 and SB203580 significantly reduced the expression of viral antigen accumulation with U0126 having a greater inhibitory capacity than SB203580.

The fact that SB203580 inhibited spontaneous reactivation was not surprising as spontaneous reactivation is asynchronous in nature, where virus reactivates at different times resulting in areas within the culture where low level spread is occurring. As discussed previously low level spread is inhibited by the SB203580 and this is likely the reason why SB203580 reduces spontaneous reactivation.

Figure 3.2.12

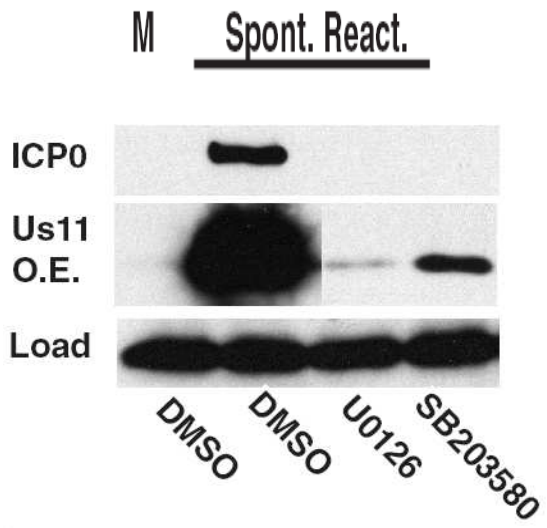

Figure 3.2.12 Production of HSV-1 viral proteins ICP0 and Us11 in NHDFs that were either mock or quiescently-infected, then returned to 37°C and allowed spontaneously reactivate in the presence of either DMSO, U0126 (20μM) or SB203580 (40μM). At 5 days post initiation of spontaneous reactivation, whole cell lysates were prepared and analyzed by western blotting with the indicated antibodies. eIF4E was probed for as a loading control. Us11 blot was intentionally overexposed to detect signal in the U0126 treated sample.

Figure 3.2.13

Inhibition of infectious virus production during spontaneous reactivation by U0126 or SB203580

To quantify the affects of both U0126 and SB203580 on yields of infectious virus produced during spontaneous reactivation, supernatants from 35mm dishes of quiescently infected cultures allowed to spontaneously reactivate for 5 days in the presence of the indicated inhibitors were taken. The supernatents were then serially diluted, plated on permissive Vero cells and titers of infectious virus were calculated as p.f.u./culture. Titers are representative of at least three independent experiments.

It was found that both U0126 and SB203580 greatly reduced spontaneous reactivation relative to DMSO, with U0126 being the more potent inhibitor of the two. These findings reflected the accumulation of viral antigens in whole cell extracts analysed by western blotting (Figure 3.2.12).

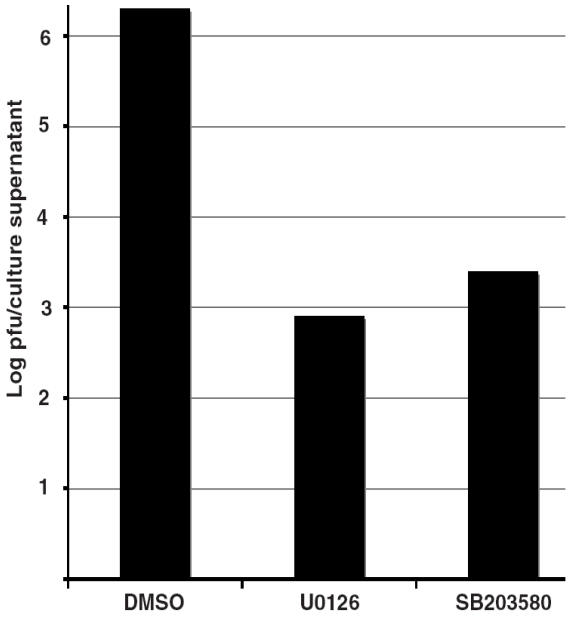

Figure 3.2.13 Viral titers of HSV-1 produced over a 5 day period of spontaneous reactivation in the presence of either DMSO, U0126 (20μM) or SB203580 (40μM). Titers are represented as Log/pfu/culture supernatant and are the average of three independent experiments.

Figure 3.2.14

MEK-ERK and p38 inhibition potently blocks spontaneous reactivation

To determine the pattern of virus reactivation, mock infected or quiescently-infected NHDFs were returned to 37°C and allowed to spontaneously reactivate for 5 days in the presence of equal volumes of DMSO, U0126 (20μM) or SB203580 (40μM). Cells were washed in PBS, fixed and analyzed by indirect immunofluorescence using anti-ICP5 antiserum.

It was discovered that although large areas of DMSO-treated cultures were antigen positive, ICP5 expression was not evident in cultures reactivated in the presence of U0126 or SB203580. This suggested low levels of reactivation occured below the detection limits of immunofluorescence.

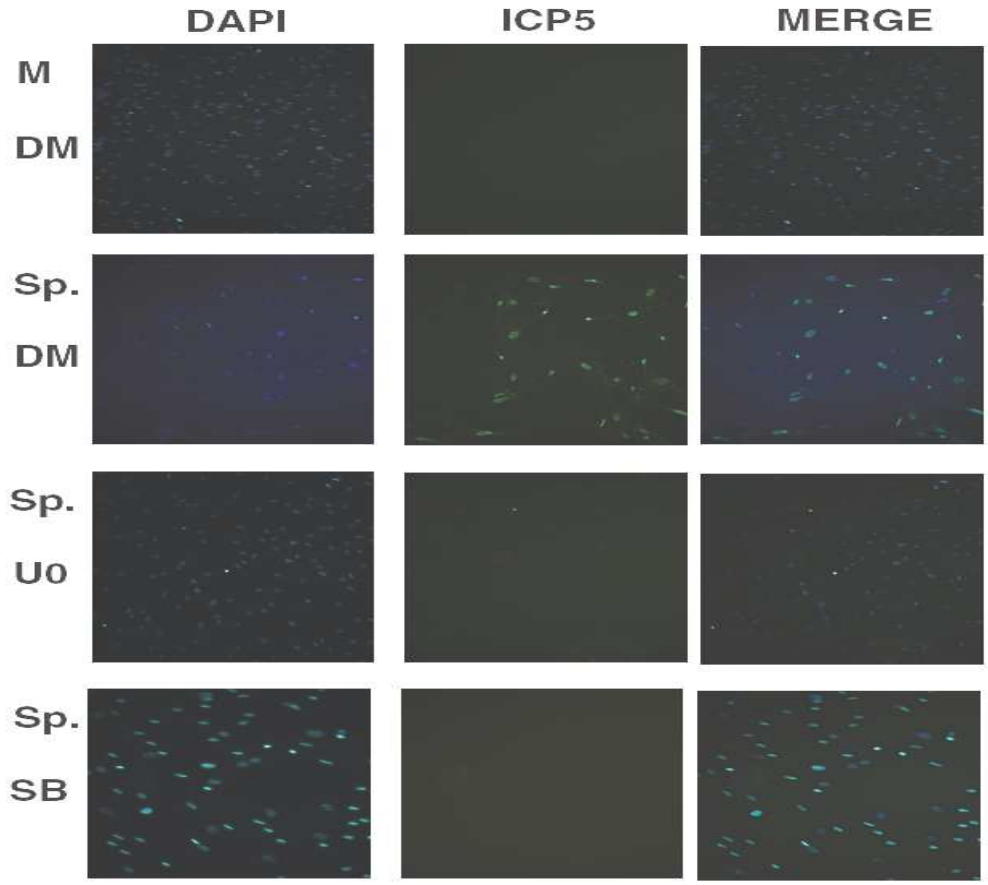

Figure 3.2.14 Immunofluorescence of HSV-1 viral protein ICP5 production in spontaneously reactivated (Sp.) NHDF cultures treated with either DMSO, U0126 (20μM) or SB203580 (40μM) at 5 days post reactivation. Cultures were probed with antiserum against HSV-1 ICP5 (green), proteins were then detected using the appropriate FITC-conjugated conjugated secondary antibody. Nuclei were visualised by Hoechst counterstain. Images were captured at 20x magnification.

Figure 3.2.15

U0126 does not affect low multiplicity lytic infection and spread of HSV-1

To ensure that the previous effects of ERK inhibition on spontaneous reactivation were not due to effects on lytic virus spread, NDHFs were mock-infected at 41°C for 6 days then returned to 37°C and infected with HSV-1 at m.o.i. 0.025 in the presence of DMSO or U0126 (20μM). After 3 days whole cell extracts were prepared and analyzed by western blotting using the indicated antibodies.

It was confirmed that U0126 had no effect on lytic replication and secondary spread as the accumulation of both ICP5 and Us11 was unaffected by ERK inhibition, suggesting that effects of U0126 were due largely to the inhibition of reactivation events.

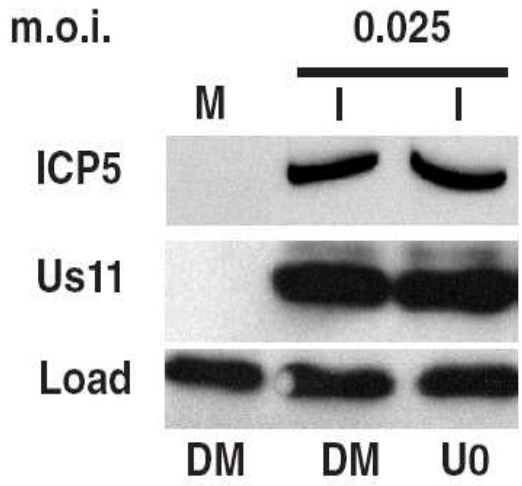

Figure 3.2.15 Western blot of HSV-1 viral proteins ICP5 and Us11 in NHDFs that were mock-infected for 6 days at 41°C and returned to 37°C, cultures were then mock-infected (M) or infected (Inf) at moi 0.025 in the presence of DMSO or U0126 (20μM). At 72 hours post infection whole cell lysates were prepared and analyzed by western blotting with the indicated antibodies. eIF4E was probed for as a loading control.

Figure 3.2.16

Inhibitors of Mnk and mTOR cause a reduction of HSV-1 reactivation from quiescence

eIF4E is a major constituent of the eIF4F complex and mediates the binding of capped mRNA. Considering that the ERK and p38 signalling pathways directly control the phosphorylation of the Mnk-1 kinase known to phosphorylate eIF4E, which results in an increase of HSV-1 protein synthesis during lytic replication (Walsh, Mohr 2004; Duncan, Peterson & Sevanian 2005), it was decided to investigate the roles of both Mnk and mTOR activity during reactivation of HSV-1 from quiescence. The drug concentrations used were chosen from experiments outlined in (Walsh, Mohr 2004).

It was discovered that both Rapamycin and CGP treatment resulted in the suppression of virus reactivation from quiescence, reducing the accumulation of all viral antigens examined.

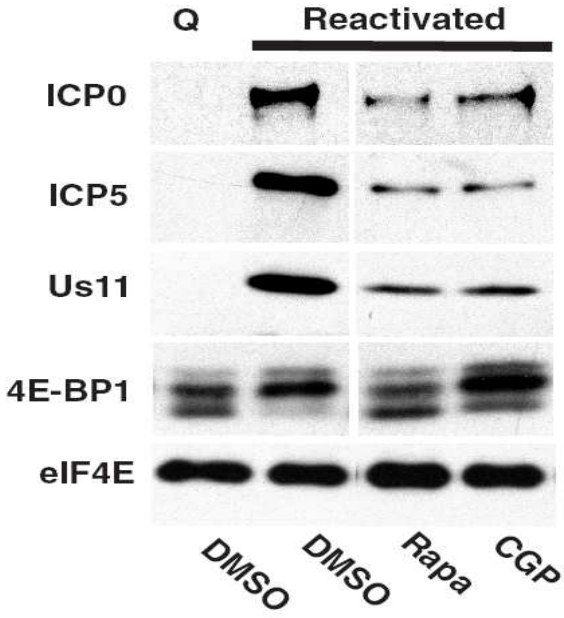

Figure 3.2.16 Western blot of HSV-1 viral proteins ICP0, ICP5, Us11 and cellular proteins eIF4E and 4E-BP1. Quiescently-infected NHDFs that were mock-reactivated (Q) or reactivated with Ad-0 (Reactivated) in the presence of equal volumes of DMSO, the Mnk1 inhibitor CGP (30μM) or the mTORC1 inhibitor Rapamycin (125nM). Whole cell extracts were prepared at 48 hours post-reactivation. The Us11 blots were intentionally overexposed to detect the low levels of Us11 in drug-treated reactivating cultures. The same blot and exposure is used but an empty dividing lane to allow excessive overexposure was cropped out.

Figure 3.2.17

Inhibitors of Mnk and mTOR cause a reduction of spontaneous reactivation from quiescence

To observe whether Mnk and mTOR inhibition impacted spontaneous reactivation, mock-infected (M) or quiescently-infected NHDFs were returned to 37°C and allowed to spontaneously reactivate for 5 days in the presence of either DMSO, CGP57380 (30μM) or Rapamycin (125nM). Whole cell extracts were prepared and analyzed by western blotting with antisera against ICP4, ICP5, Us11, p70S6K and eIF4E as a loading control.

It was discovered that the presence of either CGP57380 (30μM) or Rapamycin (125nM) significantly reduced spontaneous reactivation, with Rapamycin having a stronger effect.

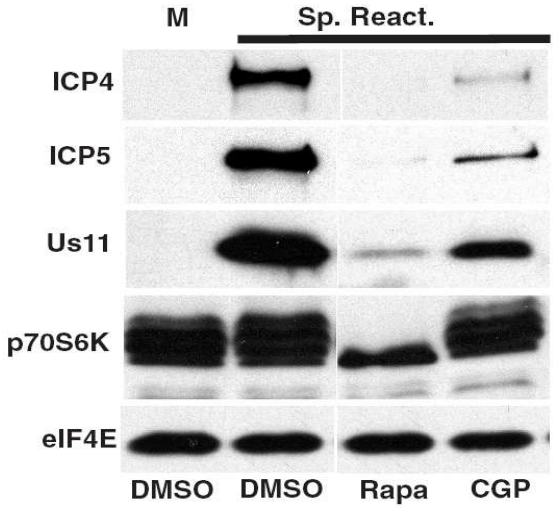

Figure 3.2.17 Western blot HSV-1 viral proteins ICP4, ICP5, Us11 and cellular proteins eIF4E and p70S6K. Mock infected and quiescently-infected NHDFs were spontaneously reactivated in the presence of equal volumes of DMSO, the Mnk1 inhibitor CGP (30μM) or the mTORC1 inhibitor Rapamycin (125nM). Whole cell extracts were prepared at 5 days post reactivation. eIF4E was probed for as a loading control while p70S6K was probed for to show Rapamycins capacity to inhibit the mTORC1 pathway.

Figure 3.1.18

The effect of Rapamycin and CGP on yields of infectious virus produced in spontaneously reactivating cultures

To quantify the affects of both Rapamycin and CGP57380 on infectious virus produced during spontaneous reactivation, supernatants from 35mm dishes of quiescently infected to spontaneously reactivate for 5 days in the presence of the indicated inhibitors were serially diluted, plated on permissive Vero cells and titers of infectious virus were calculated as p.f.u./culture supernatant.

It was found that both Rapamycin and CGP57380 significantly reduced spontaneous reactivation relative to DMSO with Rapamycin being the more potent inhibitor of the two. This was in agreement with their effects or antigen accumulation (Figure 3.2.17) and suggested that the active eIF4F is required by HSV-1 during spontaneous reactivation from quiescence.

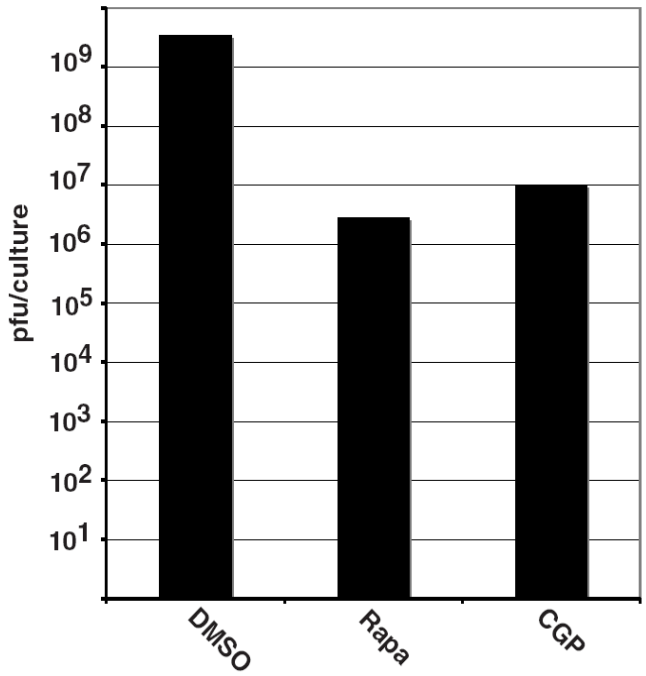

Figure 3.1.18 Viral titers of HSV-1 produced over a 5 day period of spontaneous reactivation in the presence of either DMSO, Rapamycin or CGP57380. Titers are represented as Log/p.f.u/culture supernatant and are the average of three independent experiments.

Figure 3.3

Inhibition of translation in primary human fibroblasts by 4EGi-1

Considering that the inhibition of eIF4F regulators, MNK and mTOR both reduced reactivation from quiescence, it was deemed plausible that inhibition of eIF4F activity may be a viable therapeutic target for inhibition during HSV-1 lytic replication or reactivation from quiescence. The small molecule inhibitor 4EGi-1 has been reported previously to prevent eIF4E- eIF4G binding and therefore is thought to inhibit eIF4F assembly (Moerke *et al*. 2007). We therefore tested its effects on virus reactivation and replication.

Figure 3.3.1

The effects of 4EGi-1 on host translation rates

To investigate whether this drug could inhibit HSV-1 replication, the levels of 4EGi-1 needed to inhibit translation within NHDF cultures were optimised. NHDFs were treated with increasing concentrations of 4EGi-1 for 3 hours. Cells were then metabolically labelled with [^{35}S]-Methionine/Cysteine for 1 hour in the presence of 4EGi-1. Whole cell extracts were prepared in 1x Laemmli and resolved by SDS-PAGE. The gels were then fixed dried and exposed to x-ray film. Migration of molecular weight standards is indicated to the left of the panel.

It was found that a gradual decrease of translation rates occurred with increasing concentrations of 4EGi-1, the most effective concentrations being between 30 μM and 50μM. Cells were found to be stressed at 60 μM and above (not shown).

Figure 3.3.1

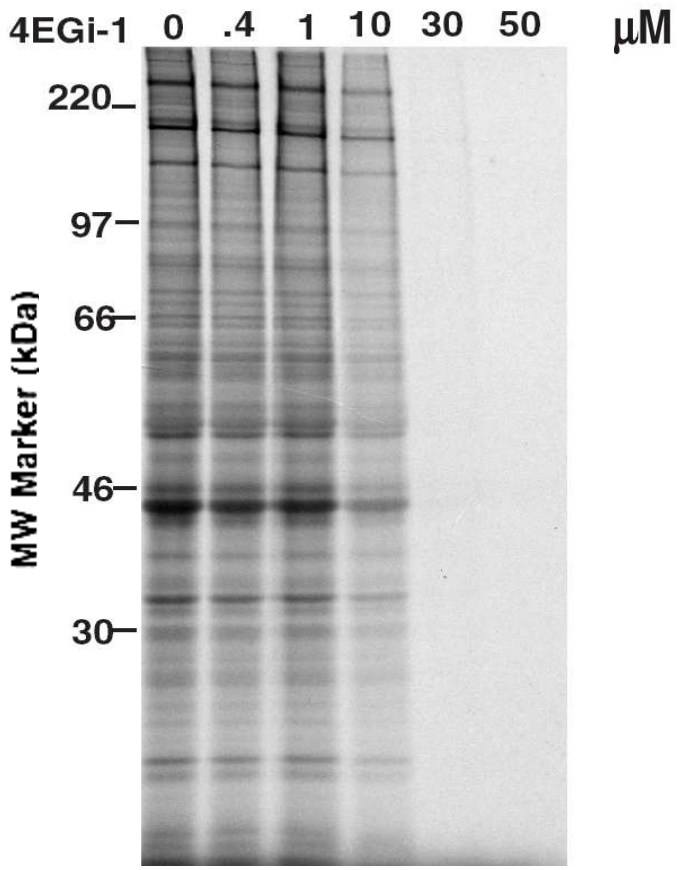

Figure 3.3.1 Metabolic labelling of NHDFs treated with DMSO or increasing concentrations of 4EGi-1 for 4 hours. At 1 hours prior to sampling, cultures were incubated with [^{35}S]-Methionine\Cysteine. Total protein was harvested by lysing cells in Laemmli buffer. Samples were resolved by SDS-PAGE where 50μl of sample was loaded into each well. Molecular weight standards (in kDa) are shown to the left of the panel.

Figure 3.3.2

Cellular effects of 4EGi-1 at 4 hours post treatment

While characterization of the effect of 4EGi-1 on eIF4F complex formation has been described previously, little is known about the effects of the drug on initiation factor abundance or the activity of cell signalling pathways which impart translational control. To investigate these questions, NHDFs were treated for 4 hours with DMSO or 40μM 4EGi-1; whole cell extracts were then analyzed by western blotting with the indicated antibodies.

The total levels of 4E-BP1 were examined using non-resolving 7.5% gels while phosphoylated 4E-BP1 was resolved using 17.5% gels. The eIF4E phosphorylation profile in cells treated with 4EGi-1 was elucidated by iso-electric focusing and membranes were probed with anti-eIF4E antibody. Migration of the phosphorylated (p-4E) and hypophosphorylated (4E) forms of eIF4E is indicated to the left of the blot.

The steady state levels of eIF4E, PABP, eIF4G and 4E-BP1 were unaffected by treatment with 4EGi-1. In addition, the phosphorylation profiles of ERK, a MAPK substrate, or stress-activated targets such as p38 or eIF2α were unaffected. The mTOR substrates, p70S6K and 4E-BP1 were slightly stimulated. In addition, the phosphorylation profiles of eIF4E in DMSO and 4EGi-1 samples were identical. This suggested that the translational repression observed in NHDFs treated with 40μM 4EGi-1 was not linked to changes in either the abundance of key translation initiation factors or the activity of signal pathways that regulate their function.

(A) Western blotting of total protein levels **(B) Iso-electric focusing**

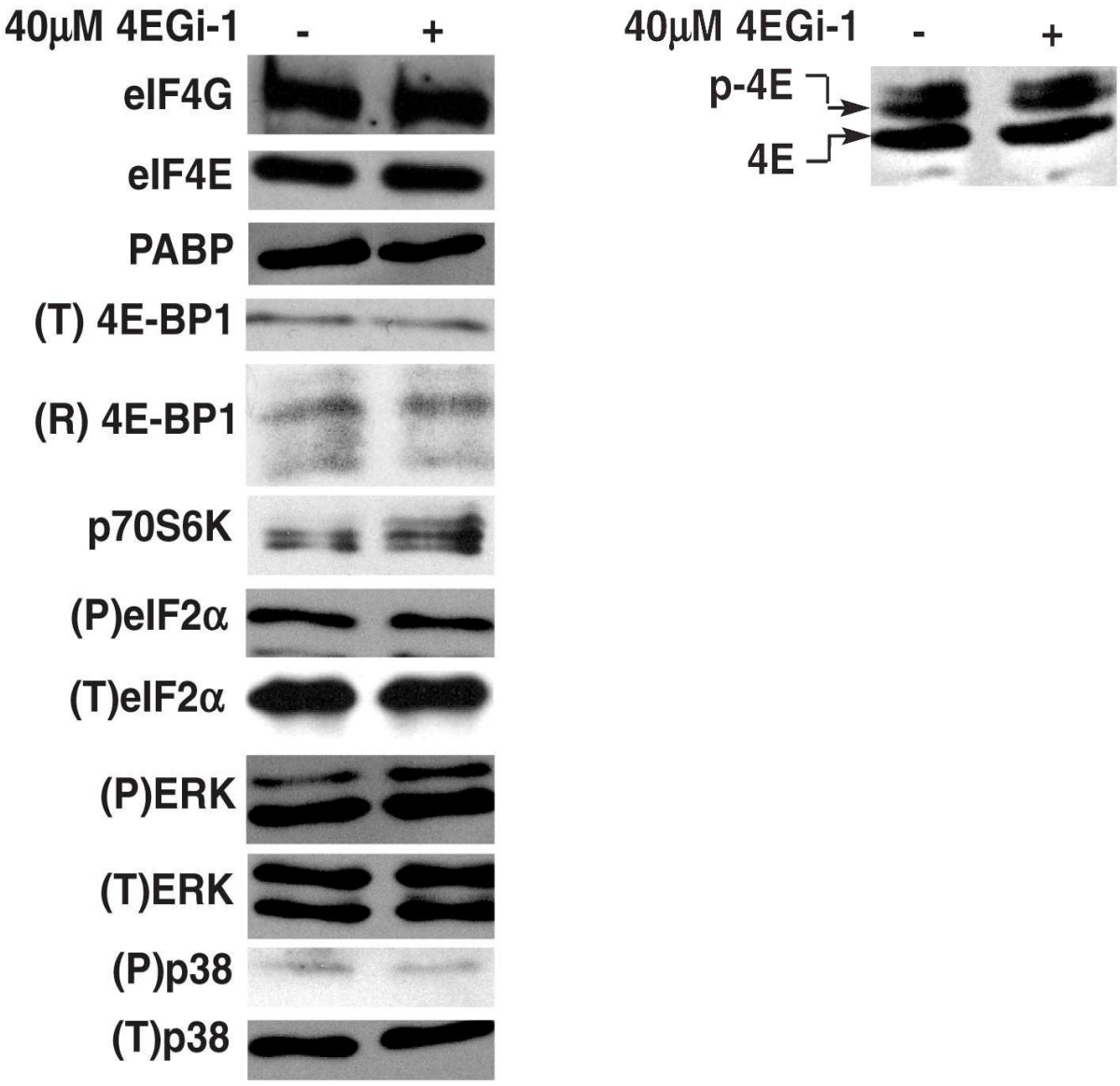

Figure 3.3.2 (A) Western blot of translation factor abundance and cell signalling pathway activities in NHDFs that were treated for 4 hours with DMSO or 40μM 4EGi-1.Whole-cell extracts were analysed with the indicated antibodies. 17.5% gels were used to probe and resovle (R) hyper and hypo phosphorylated forms of 4E-BP1. Samples described for the right hand panel (B) were fractionated by isoelectric focusing and membranes were probed with anti-eIF4E antibody. Migration of the phosphorylated (p-4E) and hypophosphorylated (4E) forms of eIF4E are indicated.

Figure 3.3.3

The effects of 4EGi-1 on the composition of initiation complexes in NHDFs

It has been reported previously that 4EGi-1 significantly disrupted eIF4F complex formation in transformed cell lines at concentrations of 100μM or above (Moerke *et al.*, 2007). As outlined previously it was found during our investigations that NHDFs were stressed at concentrations above 60μM but translation was inhibited at comcentrations between 30μM-50 μM.

As primary human cells are of lower metabolic activity and contain considerably smaller amounts of translation initiation factors than transformed cells, we examined the effects of 40μM 4EGi-1 on the composition of initiation complexes.

Confluent NHDFs were treated for 4 hours with DMSO or 40μM 4EGi-1. It has been shown that 4EGi-1 binds to eIF4E reversibly. For this reason, cell extracts were prepared by freeze-thaw in the absence of detergent and 4EGi-1 was added to all buffers at every step of the assay. Soluble cell extracts were precleared with sepharose 4B and subsequently subjected to 7-Methyl-GTP chromatography.

The abundance of initiation factors and phosphorylation profiles of 4E-BP1 and eIF2α present in the input samples were similar, proving again that 4EGi-1 had no significant effect on steady state levels of initiation factors or the activity regulating kinases. However, it was discovered that 4EGi-1 had no inhibitory effect on the eIF4E:eIF4G interaction, in line with a previous report showing that higher concentrations are needed to disrupt eIF4F (Moerke *et al*., 2007. A slight increase in eIF4E-4E-BP1 binding was observed in 4EGi-1 treated cells, which was also observed in the initial report characterising 4EGi-1. Interestingly, large amounts of total and phosphorylated eIF2α were found in initiation complexes from 4EGi-1-treated cultures. To confirm that whole initiation complexes were being pulled down with the cap, eIF3A and Ribosomal Protein S3 (RPS3) were also examined. While 3A was unchanged, binding of RPS3 increased, suggesting that 4EGi-1 increased ribosomal complexes with inactive eIF2.

Figure 3.3.3

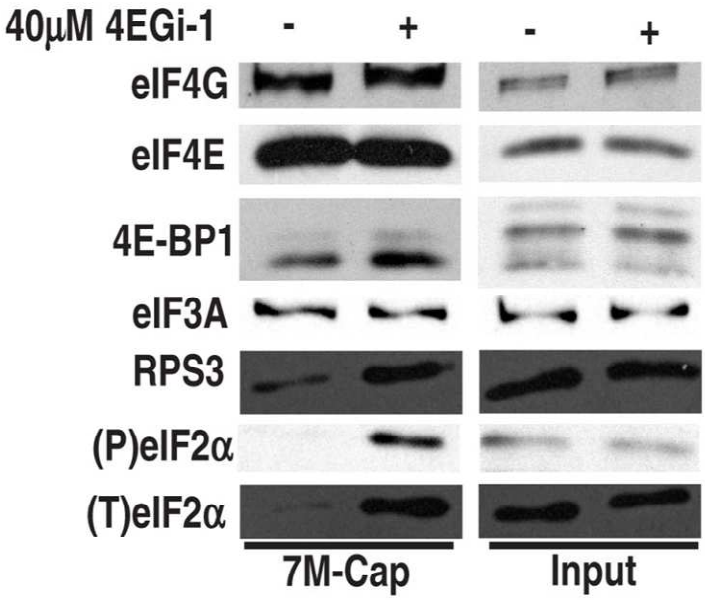

Figure 3.3.3 NHDFs treated for 4 hours with DMSO or 40μM 4EGi-1. Soluble extracts were subjected to to 7-Methyl-GTP chromatography. 7M-Cap-bound and input samples were then resolved by SDS-PAGE. Membranes were probed with the indicated antibodies. 4E-BP1 was examined using 17.5% gels to resolve phosphorylated species.

Figure 3.3.4

The effects of 4EGi-1 on the composition of initiation complexes in HeLa cells

To determine if the effects of 4EGi-1 on the composition of initiation complexes was unique to NHDFs, a repeat of the previous experiment was conducted on HeLa cells. As HeLa cells are transformed with a higher metabolic acitivy, a higher concentration of 4EGi-1 was chosen to suppress tranlation. Consequently HeLa cells were treated for 4 hours with DMSO or 60μM 4EGi-1 then processed and analyzed as described in (Figure 3.3.3).

The treatment of HeLa cells with 4EGi-1 had similar effects to those observed in NHDFs, as the levels of translation factors as well as the phosphorylation of 4E-BP1 and eIF2α in input samples remained identical to DMSO treated cells.

It was again discovered that 4EGi-1 had no inhibitory effect on the eIF4E:eIF4G interaction and that a small increase in 4E-BP1 binding to eIF4E was observed in 4EGi-1-treated cultures. The large amounts of phosphorylated eIF2α that were found in NHDF initiation complexes from 4EGi-1-treated cultures were also found in 4EGi-1treated Hela cells.

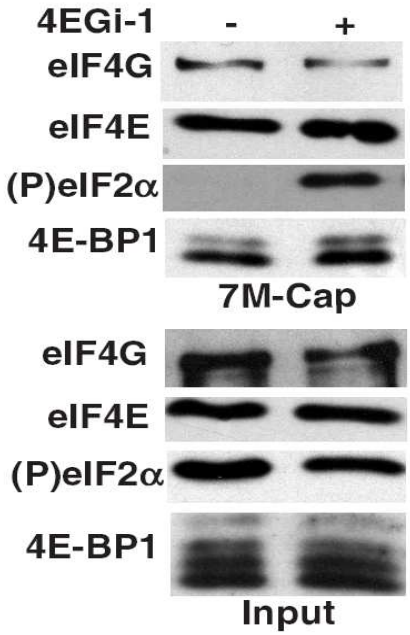

Figure 3.3.4 HeLa cells were treated for 4 hours with DMSO or 60μM 4EGi-1 and soluble extracts subjected to 7-Methyl-GTP chromatography. 7M-Cap-bound and input samples were then resolved by SDS-PAGE. Membranes were probed with the indicated antibodies. 4E-BP1 was examined using 17.5% gels to resolve phosphorylated species.

Figure 3.3.5

Reversible accumulation of phosphorylated eIF2α in eIF4F complexes observed with alternatively sourced 4EGi-1

To decipher if phosphorylated eIF2α disassociates from the cap complex in vitro when the drug is removed during the cap pull-down assays a series of experiments were performed. HeLa cell cultures were treated with 60μM 4EGi-1 for 4 hours. Cell extracts were prepared by freeze-thaw in the absence of detergent. Soluble cell extracts were precleared with sepharose 4B and subsequently subjected to 7-Methyl-GTP Chromatography. For the 4EGi-1 samples the drug was added to all buffers at every step of the assay. For the wash out sample, drug was added to all buffers up until the final wash steps post the cap binding step. In addition, to determine if the effects observed with 4EGi-1 were the result of compound impurities it was decided to repeat the HeLa cap pulldown experiment with 4EGi-1 purchased from a different company (Santa Cruz).

When samples were analysed western blotting, the treatment of HeLa cells with Santa Cruz 4EGi-1 had similar effects to those observed with the Calbiochem (CB) 4EGi-1 experiments; the levels of translation factors as well as the phosphorylation of eIF2α in input samples remained identical to DMSO treated cells. It was again discovered that 4EGi-1 had no inhibitory effect on the eIF4E:eIF4G interaction. The large amounts of phosphorylated and total eIF2α in addition to Ribosomal Protein S3 that were found in NHDF initiation complexes from Calbiochem 4EGi-1-treated cultures (Figure 3.3.3) were also in Santa cruz 4EGi-1-treated HeLa cells, suggesting that the phenomena of eIF2α accumulation in initiation complexes was not an anomaly of the Calbiochem 4EGi-1 compound. Interestingly, when the drug was removed (R) during the wash steps a reduction in phosphorylated and total eIF2α, in addition to Ribosomal Protein S3 bound to the complex was observed suggesting that the effect of the drug was reversible.

Figure 3.3.5

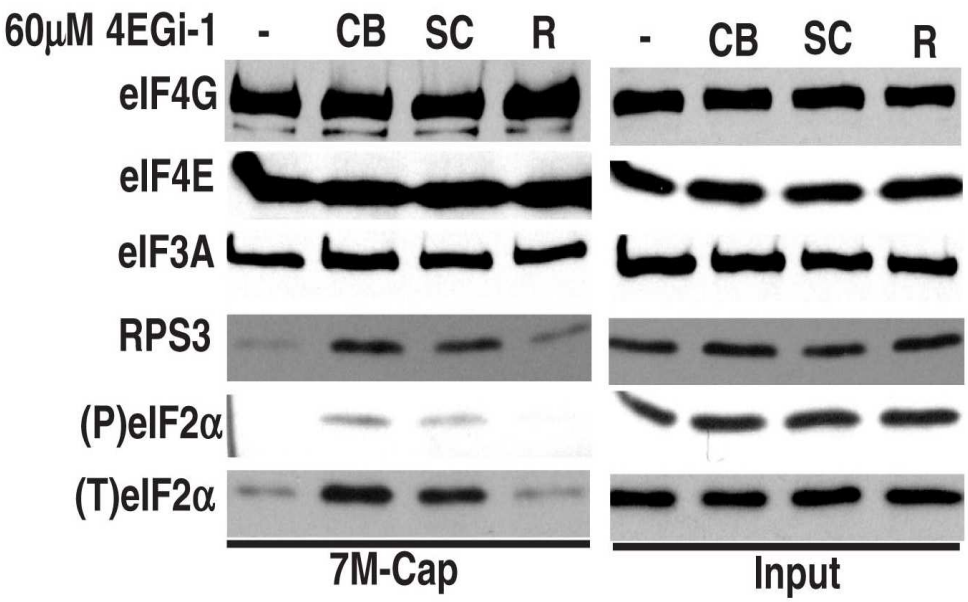

Figure 3.3.5 HeLa cells were treated with DMSO or 60μM 4EGi-1 for 4 hours. Soluble extracts were then subjected to 7-Methyl-GTP chromatography. Two independent sources of 4EGi-1 were used, Calbiochem (CB) and Santa Cruz Biotechnology (SC). An additional sample was prepared from cells treated with 60μM 4EGi-1 but the inhibitor was removed (R) by omitting it from wash buffers at the end of the assay to examine reversibility. 7M-Cap-bound and input samples resolved by SDS-PAGE. Membranes were probed with the indicated antibodies. 4E-BP1 was examined using 17.5% gels to resolve phosphorylated species.

Figure 3.3.6

4EGi-1 reversibility upon removal from culture medium

To determine how quickly the affects of 4EGi-1 last in cells once the drug has been removed from the culture medium, a series of wash out experiments were performed. To do this, NHDF cultures were treated with either DMSO or 40μM 4EGi-1 for 4 hours and labelled with [^{35}S]-Methionine/Cysteine for 10 or 30 mins either in the presence or absence of drugs.

It was observed that translation rates recovered to near the levels observed in the DMSO treated samples even after 10 minutes of labelling when then inhibitor was removed (R), while after 30 minutes in the absence of 4EGi-1 the cells synthesise proteins as efficiently as cells treated with DMSO. These results indicate that once 4EGi-1 is removed from the culture medium the inhibitory affect the drug reverses almost instantaneously.

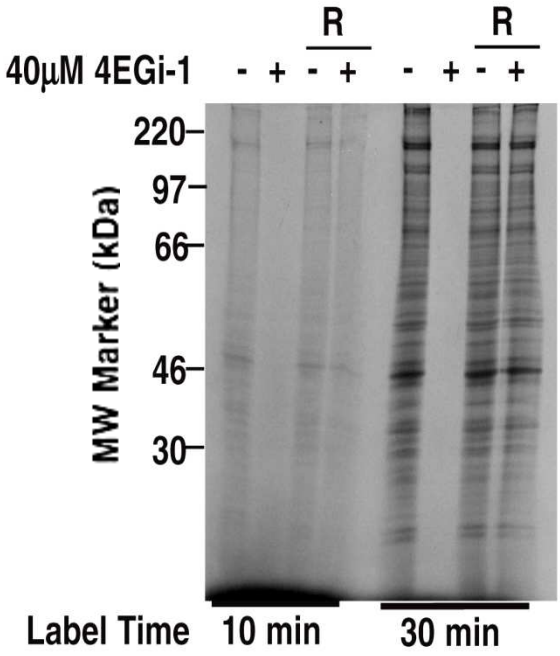

Figure 3.3.6 Metabolic labelling of NHDF cultures treated with either DMSO, 4EGi-1 40μM (NHDF) for 1 hour at either 10 or 30 minutes prior to sampling, cultures were incubated with [^{35}S]-Methionine\Cysteine in either the presence or absence 4EGi-1, total protein was harvested by lysing cells in Laemmli buffer. Samples were resolved by SDS-PAGE where 50μl of sample was loaded into each well. Molecular weight standards (in kDa) are shown to the left of the panel.

Figure 3.3.7

The effects of Torin1 on the composition of initiation complexes in NHDFs

Given that 4EGi-1 treatment had no effect on eIF4E:eIF4G interaction but still robustly inhibited translation, we decided to examine the contribution of eIF4F to rates of protein production by treating cells with 100nM Torin1, a catalytic site-specific mTOR inhibitor that inhibits 4E-BP1 phosphorylation (Thoreen *et al*., 2009). NHDFs were treated with DMSO or 100nM Torin1 for 1 day and processed as in (Figure 3.3.3). The Torin1 drug concentration used was chosen from experiments as outlined in (Thoreen *et al*., 2009).

The treatment of cells with Torin1 had no effect on protein abundance in input samples but caused a robust dephosphorylation of 4E-BP which resulted in increased 4E-BP binding to eIF4E on the cap. Incidentally, the increased binding of 4E-BP to eIF4E caused a large decrease in the amounts of eIF4G bound to eIF4E on the cap. Importantly, phosphorylated eIF2α was not found in NHDF initiation complexes from Torin1 treated cultures.

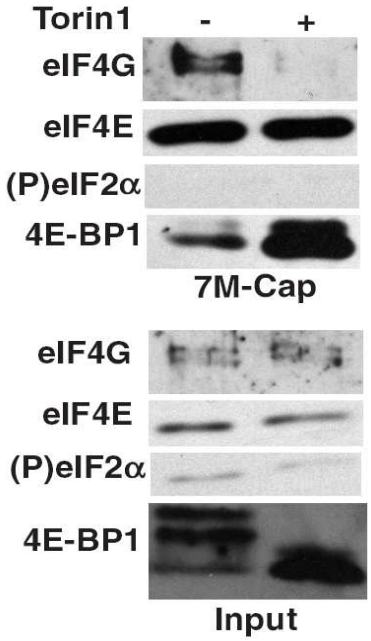

Figure 3.3.7 NHDFs were treated for 24 hours with DMSO or 100nM Torin1 and soluble extracts were subjected to 7-Methyl-GTP chromatography. 7M-Cap-bound and input samples were then resolved by SDS-PAGE. Membranes were probed with the indicated antibodies. 4E-BP1 was examined using 17.5% gels to resolve phosphorylated species.

Figure 3.3.8

The effects of 4EGi-1 and Torin1 on translation rates in human cells

Torin 1 was found to cause a robust increase in the abundance of hypophosphorylated 4E-BP1 in input samples, resulting in a large increase in the association of 4E-BP1 with eIF4E and a loss of eIF4G binding, whereas 4EGi-1 inhibited translation without causing eIF4F disruption. Therefore, a comparison of the effects of inhibitors on rates of translation in HeLa cells and NHDFs was performed.

NHDFs or HeLa cells were treated with DMSO, 40µM (NHDF) or 60µM (HeLa) 4EGi-1 or 100nM Torin1 for 1 day. Cultures were metabolically-labelled for 1 hour prior to sampling. Whole cell extracts were resolved by SDS-PAGE and fixed dried gels were exposed to x-ray film. MW standards are indicated to the left of the panel.

The autoradiogram illustrates how Torin1 affects the expression of specific proteins, in line with a role for eIF4F in stimulating translation of specific types of mRNAs. In contrast to 4EGi-1, Torin1 only had a modest effect on reducing global rates of protein synthesis in either HeLa or NHDFs.

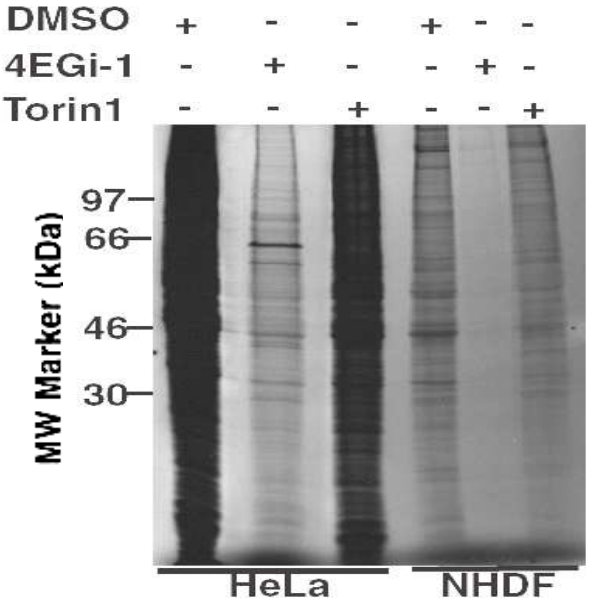

Figure 3.3.8 Metabolic labelling of HeLa and NHDF cultures treated with either DMSO, 4EGi-1 40µM (NHDF) 60µM (HeLa) or 100nM Torin1 for 1day. At 1 hour prior to sampling, cultures were incubated with [^{35}S]-Methionine\Cysteine in the presence of drugs, and total protein was harvested by lysing cells in Laemmli buffer. Samples were resolved by SDS-PAGE where 50µl of sample was loaded into each well. Molecular weight standards (in kDa) are shown to the left. As with all previous experiments, drugs were disolved in DMSO.

Fig 3.3.9

4EGi-1 toxicity in NHDF and Hela cells

It was noticed that HeLa cells treated with 4EGi-1 appeared visibly stressed after 24 hours, while NHDFs were indistinguishable from DMSO controls (not shown).

To verify if cellular stress was occurring upon 4EGi-1 treatment, HeLa and NHDF cultures were treated with DMSO or 40μM (NHDF) or 60μM (HeLa) 4EGi-1 for 1 day, then whole cell extracts were prepared and subjected to western blot analysis using anti Caspase-9 and Caspase 3 antibodies. The migration of full-length (FL) and cleaved (Cl) forms of Caspases is indicated to the left of the panel.

It was found that a decrease in full-length caspases and appearance of specific cleavage products was evident in HeLa cells but not in NHDFs.

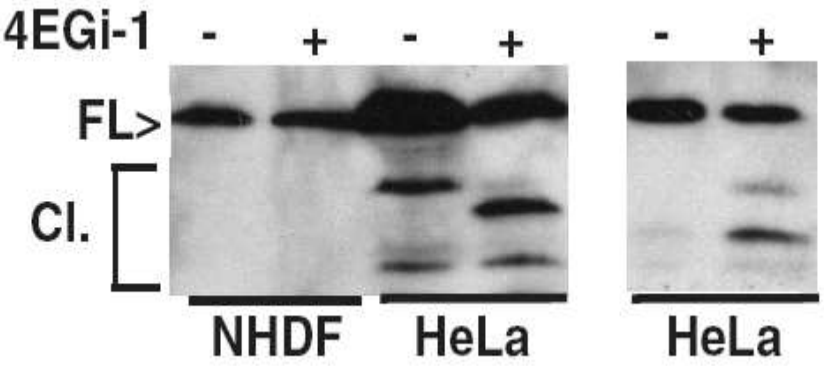

Fig 3.3.9 Western blot of full length (FL) and cleaved forms (Cl) of Caspase 3 and 9 in cultures of HeLa and NHDFs treated with DMSO, 40μM (NHDF) or 60μM (HeLa) 4EGi-1 for 1 day.

Figure 3.3.10

4EGi-1 reversibility after prolonged treatment of cells

To determine how quickly the affects of 4EGi-1 reverses once the drug has been removed from cells after prolonged treatment of cells, another series of wash out experiments were performed. NHDF cultures were treated with either DMSO or 40μM 4EGi-1 for 8 days, the drug was replaced after 4 days and 7 days. On the 8th day the cells were labelled with ^{35}S-Methionine/Cysteine in the absence of 4EGi-1 for 30 minutes.

It was observed that unlike the results garnered from short treatments where translation rates recovered to near the levels observed in the DMSO treated samples (Figure 3.3.6), after 30 minutes in the absence of 4EGi-1 translation recovered but to a lesser degree. This is most likely because the cells have been in a state of constant translational suppression for 8 days which resulted in a lowering of metabolic activity, including decreased levels of translation factors that would thus increase the translation recovery time upon drug removal.

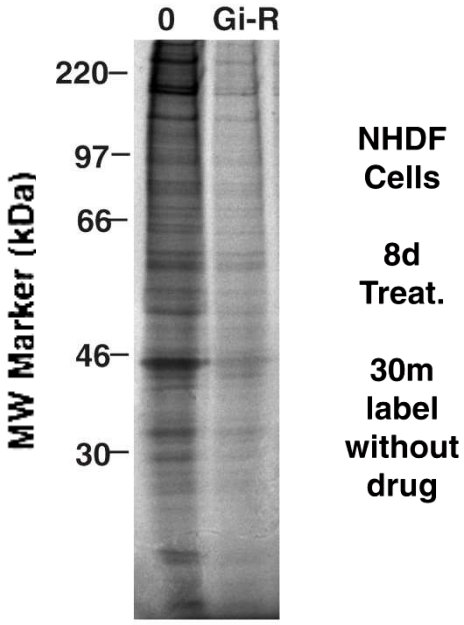

Figure 3.3.10 Metabolic labelling of NHDF cultures treated with either DMSO or 40μM 4EGi-1 for 8 days. At 30 minutes prior to sampling, cultures were incubated with [^{35}S]-Methionine/Cysteine in the absence of DMSO (0) or the absence of 4EGi-1 (Gi-R), and total protein was harvested by lysing cells in Laemmli buffer. Samples were resolved by SDS-PAGE where 50μl of sample was loaded into each well. Molecular weight standards (in kDa) are shown to the left of the panel.

Figure 3.3.11

4EGi-1 stability in culture

To elucidate the length of time 4EGi-1 is active in cultured NHDFs, cells were seeded at low density (3.5 x 10^4) on 35mm dishes. Upon seeding after cell attachment, four cultures were treated with 40μM 4EGi-1 and one culture was treated with DMSO alone. At 1 , 2 and 3 days post seeding, a culture was taken from the 37°C incubator and the drug removed (rem) from the cells by washing once with 5% FBS DMEM, and returning to culture in fresh 5% FBS DMEM containing DMSO. 4EGi-1 was not removed from the last culture. On day four each culture was trypsinised, resuspended in medium and 50μl of the cell solution was added to an equal volume of 8% trypan blue. The cells were then counted using a haemocytometer. The numbers shown are an average of two independent experiments.

It was found that in the absence of 4EGi-1 the cells increased in number to approximately 22.5 x 10^4 over a period of five days. When the drug was removed after one day the cells reached 21 x 10^4 per well. When drug was removed on day two the cells reached 6.5 x 10^4 per well and on day three the cells reached approximately 2.5 x 10^4 cells per well. Finally the 4EGi-1 culture which had no wash step was found to have 2.6 x10^4 cells per well. Taken together with the results from (Figure 3.3.5, 3.3.6) these results suggest that although 4EGi-1 is rapidly reversible upon removal but is highly stable in cultures for periods up to at least three days.

Figure 3.3.11

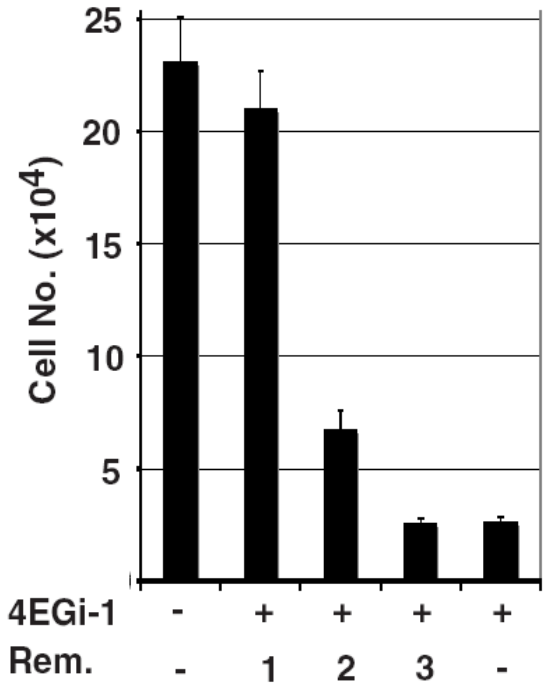

Figure 3.3.16 Cell counts taken from four NHDF cultures seeded at 3.5 x10^4 per 35 mm dish and treated with 40µM 4EGi-1. One culture was treated with DMSO alone. At 1 , 2 and 3 days post seeding, a culture was taken from the 37°C incubator and the drug removed (rem) from the cells by washing once with medium, and returning to culture in fresh 5% FBS DMEM containing DMSO. 4EGi-1 was not removed from the last culture. On day four each culture was trypsinised, resuspended in medium and 50µl of the cell solution was added to an equal volume of 8% trypan blue. The cells were then counted using a haemocytometer. The numbers shown are an average of two independent experiments.

Figure 3.3.12

The effects of extended exposure to 4EGi-1 on protein synthesis rates in NHDFs

To determine the effects of extend exposure of primary cells to 4EGi-1, we then quantified translation rates at timepoints over an eight day period. Previous studies have reported that 4EGi-1 is stable in culture for 3 to 7 days (Moerke *et al.*, 2007; Tamburini *et al.*, 2009) in line with our findings that it is stable for at least 3 days (Figure 3.3.11).

It was discovered that the rates of translation were reduced to 5% relative to DMSO control samples by 3-4 hours post-treatment, while continued exposure to 4EGi-1 further reduced rates to 0.5-1% of control samples at later points suggesting that 4EGi-1 can suppress translation for long periods of time.

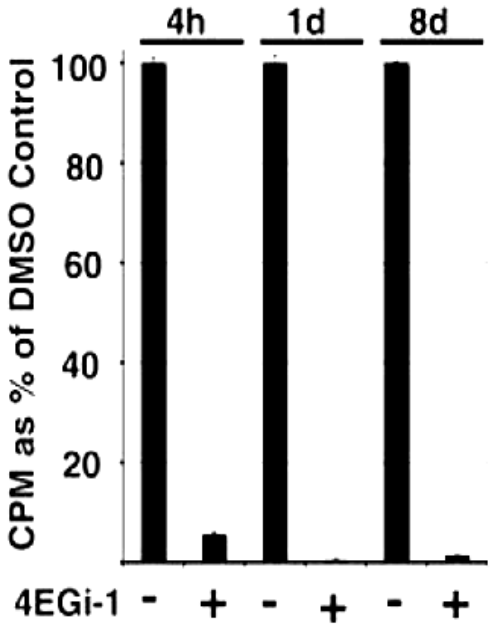

Figure 3.3.12 mRNA translation rates determined by TCA precipitation in NHDFs cultures treated were DMSO or 40μM 4EGi-1 for either 4 hours, 1 day or 8 days. 1 hour prior to the indicated time-points, cultures were metabolically-labelled and whole cell extracts prepared. For 8 day samples, drugs were replenished at day 3 and 7. [^{35}S] incorporation was quantified as counts per minute (CPM) as a percentage of control cultures treated with DMSO, arbitrarily set at 100%.

Figure 3.3.13

The effects of extended exposure to 4EGi-1 on patterns of translation and cell viability in NHDFs

Further characterisation of the effects of extended expose to 4EGi-1 was performed by metabolic labelling and cell viability assays to assess the levels of translation and what affects extended translational suppression had on cell viability.

(A) The degree of translational suppression at 8 days post-treatment with 4EGi-1 was evident on overexposed auto radiographs.

(B) It was found that NHDFs cultures treated with 4EGi-1 had no reduction in cell viability.

(A)

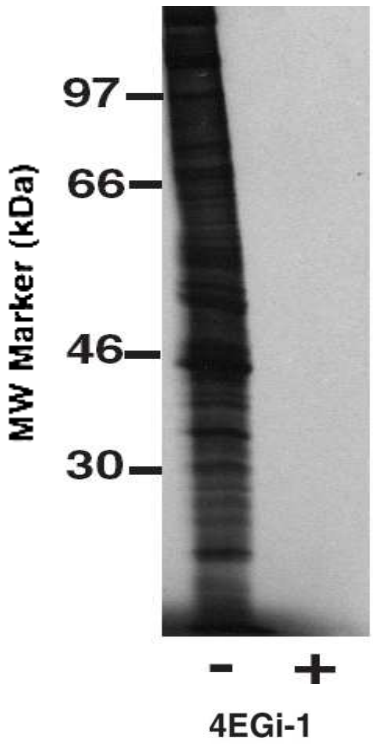

Figure 3.3.13 NHDFs were treated with DMSO or 40μM 4EGi-1 for 8 days replenishing at day 3 and 7 and then metabolically-labelled with [^{35}S]-Methionine/Cysteine for 1 hour. Whole cell extracts were resolved by SDS-PAGE and fixed dried gels were exposed to x-ray film. MW standards are indicated to the left of the panel.

(B)

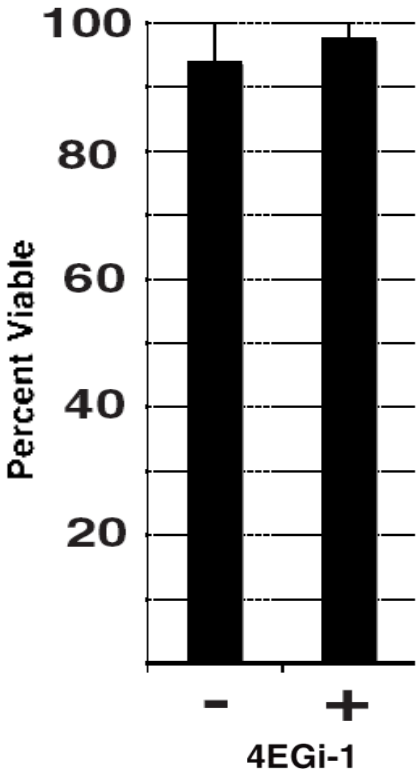

Figure 3.3.13 NHDFs were treated with DMSO or 40μM 4EGi-1 for 8 days then cells were trypsinized and incubated with trypan blue. Percentage viability represents the number of dye-excluding cells as a percentage of the total cell number.

Figure 3.3.14

Effects of prolonged 4EGi-1 exposure on protein abundance

To measure the effects of prolonged 4EGi-1 exposure on protein levels, whole cell extracts from NHDFs treated for 8 days with either DMSO or 40μM 4EGi-1 were analyzed by western blotting with the indicated antibodies. Phosphorylated forms of 4E-BP1 were assessed using 17.5% gels.

The abundance of cellular antigens was modestly reduced along with the activity of the mTOR signalling pathway as determined by a reduction of 4E-BP1 phosphorylation. The levels of the apoptotic indicators Caspase 3 or 7 were also reduced in 4EGi-1-treated cultures. However, this was apparently a result of a global decrease in abundance of proteins in these cultures, rather than apoptotic associated cleavage, as cleavage products were not evident in 4EGi-1 samples. The arrow (>) points to a low abundance Casp-7 cleavage product in DMSO-treated cultures.

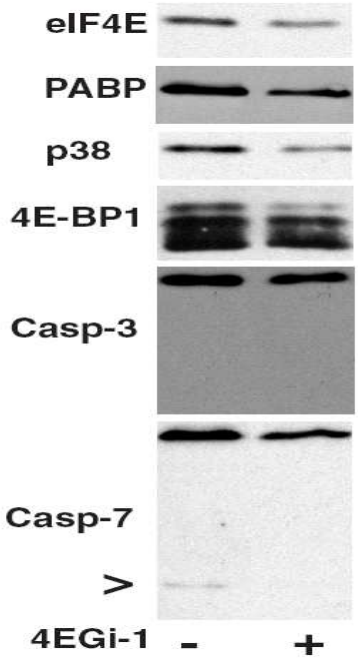

Figure 3.3.14 Western blot of protein abundance and cell signalling pathway activities in NHDFs that were treated for 8 days with DMSO or 40μM 4EGi-1. Whole-cell extracts were analysed with the indicated antibodies. Full length (FL) of Caspase 3 and 7 in cultures were also analysed to show if extended exposure caused apoptosis. The (>) represents the cleaved form of Caspase 7.

Figure 3.3.15

The effects of extended expose to 4EGi-1 on actin stabilization in NHDFs

To examine the effects of extended 4EGi-1 treatment on cytoskeletal integrity, NHDFs were treated with DMSO or 40μM 4EGi-1 for 8 days then washed in PBS and fixed in formaldehyde after which the cultures were permeablised and actin stained.

Although the intensity of actin staining decreased in 4EGi-1 treated cells, correlating with the global decrease in protein production, the cells treated with 4EGi-1 had actin morphologies similar to control cells suggesting cellular homeostasis was maintained over the extended period of 4EGi-1treatment.

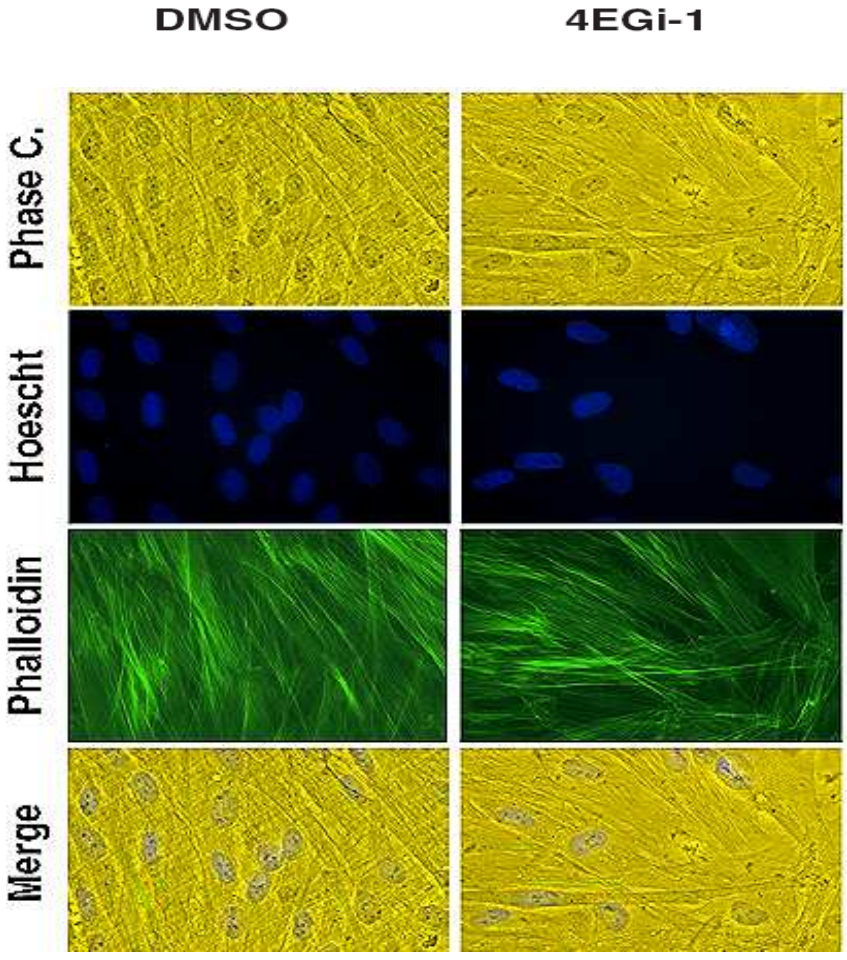

Figure 3.3.15 Fluorescent analysis of actin filaments in cultures treated with either DMSO or 4EGi-1 for 8 days and subsequently stained with FITC-conjugated phalloidin (actin; green). Nuclei were counterstained with Hoescht (DNA; blue). Phase contrast and fluorescent images were captured on a Leica DFC 500 microscope at 63x magnification.

Figure 3.3.16

4EGi-1-treated NHDFs remain tolerant of and responsive to proteasome inhibition

While cells remained viable when translation was inhibited to barely detectable levels over extended periods, it was necessary to understand the degree of sensitivity to stress within cultures exposed to 4EGi-1.

To address this question, NHDFs were treated with DMSO or 40μM 4EGi-1 for 7 days, replacing the drugs at day four and seven followed by treatment with DMSO or 10μM MG132, a broad-spectrum chemical inhibitor of proteasome and lysosome function, for 24 hours. Whole cell extracts were prepared and resolved by SDS-PAGE and blots were probed for Hsp27, Hsp70, and eIF4E as a control antigen. The MG132 drug concentration used were chosen from experiments as outlined in (Walsh, Mohr 2004).

In control samples that were not exposed to MG132, levels of each protein were again modestly reduced in 4EGi-1-treated cultures correlating with the global decrease in protein production. Cells exposed to MG132 had high levels of Hsp70 expression in both DMSO and 4EGi-1-treated cultures, illustrating that cultures were viable and stress-responsive.

Interestingly, 4EGi-1 reduced the accumulation of Hsp27 in response to MG132, demonstrating distinct mechanisms by which these small and large Hsps were induced. Finally, the pattern of eIF4E expression remained unaltered in cultures treated with MG132 treatment, only being modestly reduced in the presence of 4EGi-1.

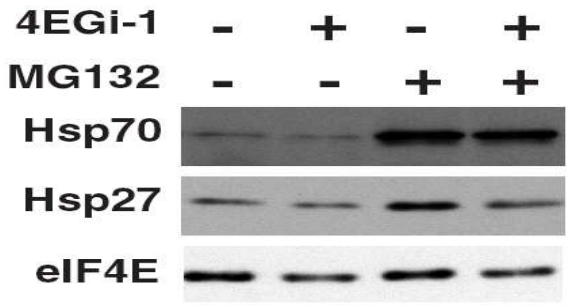

Figure 3.3.16 Western blot of Heat shock proteins (Hsp) 70 and 27 in NHDF cultures that were with either DMSO or 40μM 4EGi-1 for 7 days and subsequently treated with 10μM MG132 for 24 hours in addition DMSO or 40μM 4EGi-1.Whole-cell extracts were resolved by SDS-page and eIF4E was probed for as a loading control.

Figure 3.3.17

4EGi-1-treated NHDFs remain tolerant of and responsive to heat shock

To confirm that 4EGi-1 treated cells had a general capacity to tolerate stress and not a specific response with regard to proteasome inhibition, NHDFs were treated for 7 days as described in figure 3.3.16, followed by either continued incubation at 37°C or heat shock (H.S.) at 41°C for 24 hours. Whole cell extracts were prepared and resolved by SDS-PAGE and blots were probed for Hsp27, Hsp70, and PABP as a control antigen.

It was discovered that control cultures that were not exposed to heat shock had a modest reduction in protein levels when treated with 4EGi-1 for 8 days, again correlating with the global decrease in protein production.

The levels of PABP expression remained unaltered in cells subjected to heat shock.

In contrast, cells exposed to heat shock had high levels of Hsp70 expression in both DMSO and 4EGi-1-treated cultures, proving that cultures remain capable of mounting a stress response. Finally, in agreement with the MG132 experiment, the presence of 4EGi-1 reduced the accumulation of Hsp27 relative to the DMSO control in response to heat shock.

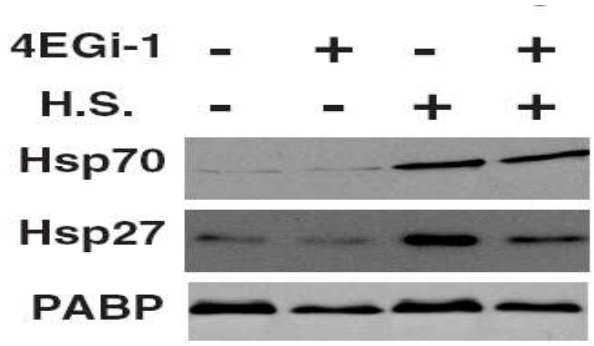

Figure 3.3.17 Western blot of Heat shock proteins (Hsp) 70 and 27 in NHDF cultures that were treated with either DMSO or 40μM 4EGi-1 for 7 days and subsequently heat shocked at 41°C for 24 hours in the presence of either DMSO or 40μM 4EGi-1. Whole-cell extracts were resolved by SDS-page and PABP was probed for as a loading control.

Figure 3.3.18

Effects of prolonged exposure to 4EGi-1 and heat shock on actin structures

The effects of heat shock on cells that had been exposed to 4EGi-1 for 7 days was further assessed by fluorescence microscopy. NHDFs were treated for 7 days as described in Figure 3.3.16 then heat-shocked at 41°C for a further 24 hours.

Although the intensity of actin staining decreased in 4EGi-1 treated cells, the Phase-contrast and fluorescent imaging illustrated that the morphology of cells together with the integrity of the actin cytoskeleton in both DMSO and 4EGi-1-treated cultures remained intact under heat-shock conditions

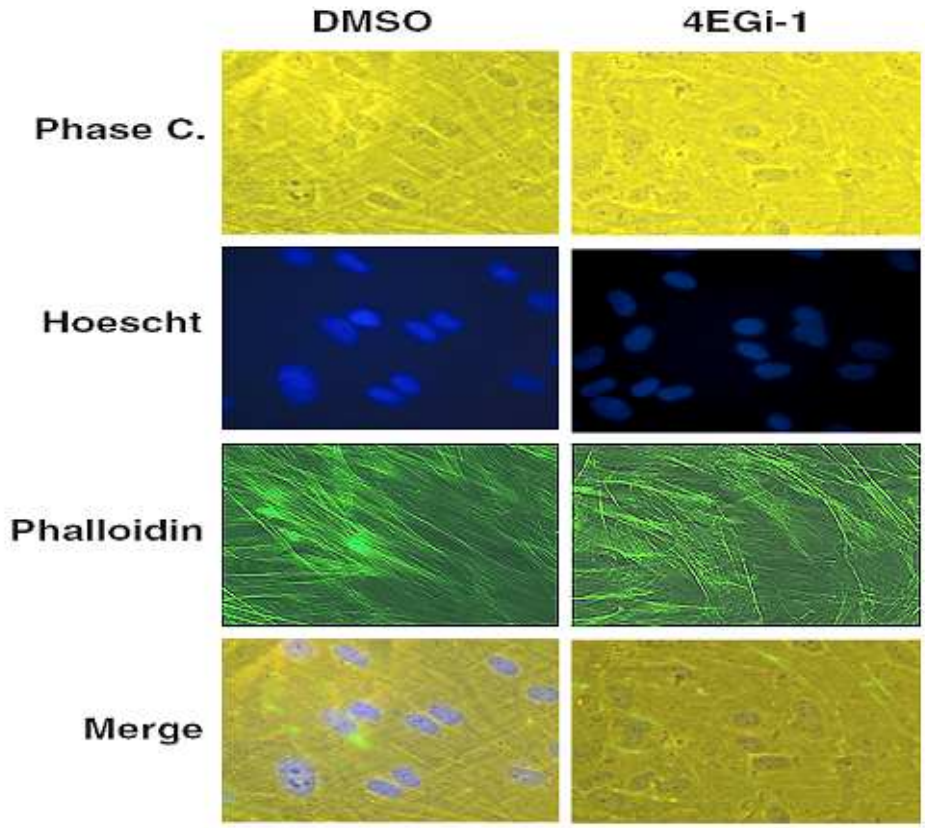

Figure 3.3.18 Fluorescent analysis of actin filaments in cultures treated with either DMSO or 4EGi-1 for 7 days and subsequently heat shocked at 41°C for 24 hours in the presence of either DMSO or 40μM 4EGi-1. Cultures were stained with FITC-conjugated phalloidin (actin; green). Nuclei were counterstained with Hoescht (DNA; blue). Phase contrast and fluorescent images were captured on a Leica DFC 500 microscope at 63x magnification.

Figure 3.3.19

Effects of prolonged exposure to 4EGi-1 and heat shock on apoptotic markers

To assess if an apoptotic profile of caspase activation was present in cells exposed to 4EGi-1 and heat shock, NHDFs were treated for 7 days as described in figure 3.3.15, followed by continued incubation and heat shock, (H.S.) at 41°C for 24 hours. Whole cell extracts were prepared and resolved by SDS-PAGE, then blots were probed with an antibody against PARP-1, which detects both full length (FL) and cleaved (C) forms of the protein, or an antibody against the cleaved form of PARP-1, NHDFs were also treated with 1μM of Staurosporin for 4 hours as a control for detection of apoptosis.

The processing of PARP-1 indicative of apoptosis was not detected in either DMSO or 4EGi-1-treated cultures which had been heat shocked, unlike cells treated with staurosporin which showed a large an increase in cleaved PARP and a reduction of full length PARP.

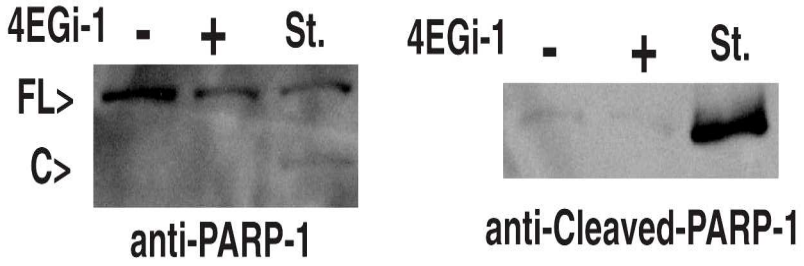

Figure 3.3.19 Western blot of anti-Parp-1 and anti-cleaved Parp-1 in cultures of NHDFs treated with DMSO or 40μM 4EGi-1 for 7 days followed by continued incubation and heat shock, (H.S.) at 41°C for 24 hours in the presence of either drug. NHDFs were also treated with 1μM of Staurosporin for 4 hours as a control for detection of apoptosis.

Figure 3.3.20

4EGi-1 inhibition of HSV-1 reactivation from quiescence

The discovery that primary human cells were extremely tolerant of 4EGi-1-mediated translational suppression led us to examine whether this inhibitor could be used to inhibit viral replication. To investigate what effects 4EGi-1 would have on HSV-1 reactivation from quiescence, NHDFs were mock-infected (M) or infected with HSV-1 day at elevated temperature to establish a quiescent infection, which was maintained for 6 days.

(A) Cultures were then returned to 37°C and quiescent virus was allowed to spontaneously reactivate in the presence of DMSO or 40μM 4EGi-1 for 5 days. Whole cell extracts were prepared in 1x Lemmli lysis buffer and resolved by SDS-PAGE. Blots were probed with the indicated antibodies.

(B) NHDFs were quiescently-infected with HSV-1 for 6 days then returned to 37°C. Cultures were mock transduced with medium (Q) or transduced with adenovirus encoding HSV-1 ICP0 (Reactivated) in the presence of DMSO or 40μM 4EGi-1 for 48 hours. Whole cell extracts were prepared by laemmli lysis and resolved by SDS-PAGE, then blots were probed with the indicated antibodies.

While the accumulation of viral proteins remains unencumbered during spontaneous and controlled reactivation in the presence of DMSO, the treatment of cells with 40μM 4EGi-1 prevented production of the immediate early gene ICP4, leaky late gene ICP5 and the late gene Us11 during both spontaneous and controlled reactivation. Notably, 4EGi-1 also prevented ICP0 production during controlled reactivation.

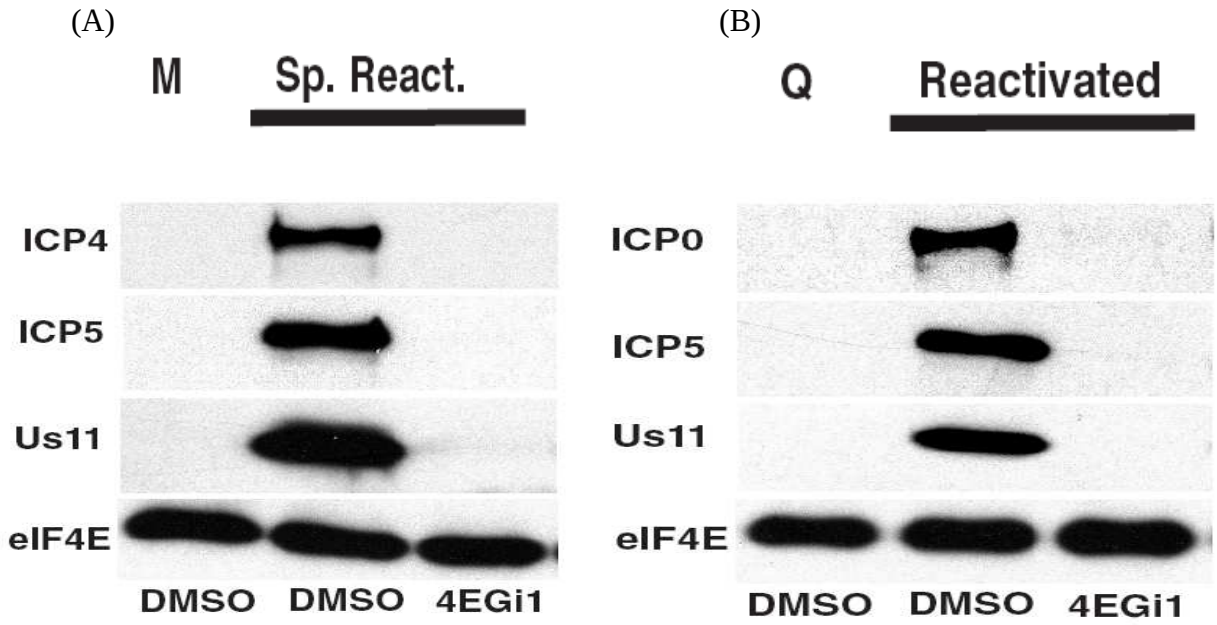

Figure 3.3.20 (A) Western blot analysis for production of HSV-1 viral proteins: ICP4, ICP5, Us11 and load control protein eIF4E in mock infected or quiescently-infected NHDFs that were spontaneously reactivated in the presence of equal volumes of DMSO, or 40μM 4EGi-1. Whole cell extracts were prepared at 5 days post reactivation. (B) Western blot analysis for production of HSV-1 viral proteins: ICP0, ICP5, Us11 and load control protein eIF4E in mock infected and quiescently-infected NHDFs that were transduced with adenovirus encoding HSV-1 ICP0 (Reactivated) in the presence of DMSO or 40μM 4EGi-1 for 48 hours.

Figure 3.3.21

4EGi-1 inhibition of infectious HSV-1 productiom during reactivation from quiescence

To confirm that 4EGi-1 prevented the production of viable infectious virus.

NHDFs were quiescently infected for 6 days at 41°C and then returned to 37°C prior to reactivation with an adenovirus encoding HSV-1 ICP0 in the presence of DMSO or 40μM 4EGi-1 for 48 hours. The levels of infectious virus in cultures was quantified by titration of freez thawed cell culture lysates on Vero cells and were represented as plaque-forming units per culture (Log p.f.u/culture).

The presence of 4EGi-1 during reactivation completely prevented production of infectious virus, in agreement with the inhibition of viral antigen accumulation (Figure 3.3.20)

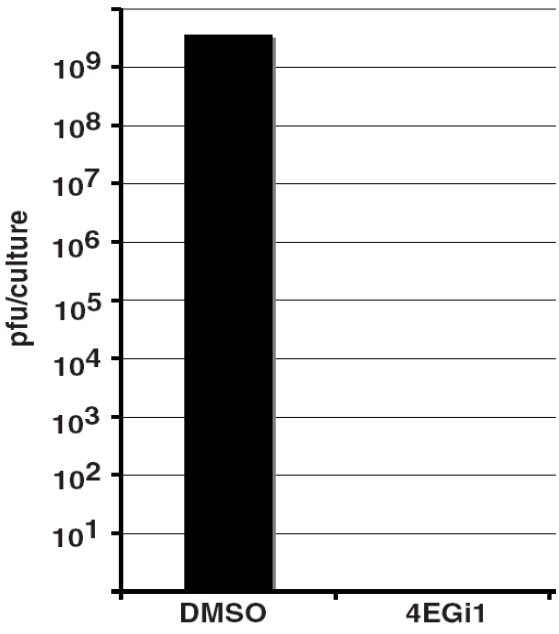

Figure 3.3.21 Viral titers of HSV-1 produced during a Ad-0 controlled reactivation in the presence of either DMSO or 40μM 4EGi-1 over a 48 hour period. Titers are represented as Log/p.f.u/culture in supernatants taken from cultures which had been freez thawed and are the average of three independent experiments.

Figure 3.3.22

4EGi-1 inhibition of translation during lytic HSV-1 replication

As 4EGi-1 was found to inhibit reactivation from quiescence, we therefore assessed whether 4EGi-1 was effective against primary lytic infection. To address this question, NHDFs were mock-infected (M) or infected with HSV-1 of m.o.i. 5 in the presence of increasing μM concentrations of 4EGi-1. 10 hours post-infection cultures were metabolically labelled for 1 hour and whole cell extracts were resolved by SDS-PAGE. Fixed dried gels were exposed to x-ray film. MW standards are indicated to the left panel.

At concentrations above 30 μM, 4EGi-1 caused potent inhibition of viral protein synthesis.

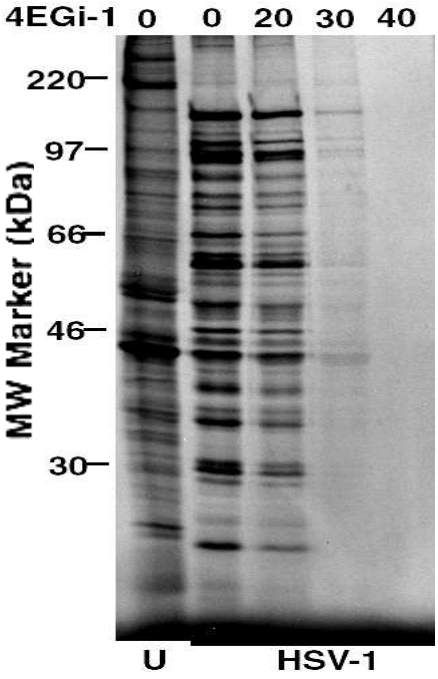

Figure 3.3.22 Metabolic labelling of NHDF cultures treated with either DMSO or increasing concentrations of 4EGi-1 in uninfected (U) or infected with HSV-1 at m.o.i 5 for 11 hours. At 1 hour prior to sampling, cultures were incubated with [^{35}S]-Methionine/Cysteine in the presence of the drugs. Total protein was harvested by lysing cells in Laemmli buffer. Samples were resolved by SDS-PAGE where 50μl of sample was loaded into each well. Molecular weight standards (in kDa) are shown to the left of the panel.

Figure 3.3.23

Rates of protein synthesis in HSV-1 infected cultures treated with 4EGi-1

To quantify the rates of translation in 4EGi-1 treated cells infected with HSV-1. NHDFs were mock-infected (M) or infected with HSV-1 at m.o.i. 5 in the presence of increasing μM concentrations of 4EGi-1, 10 hours post-infection cultures were metabolically labelled for 1 hour. Translation rates were quantified by TCA precipitation and represented as a percentage of DMSO control arbitrarily set at 100%.

It was found that 4EGi-1 at a concentration of 40μM reduced translation rates to 1.4% of control samples during lytic infection.

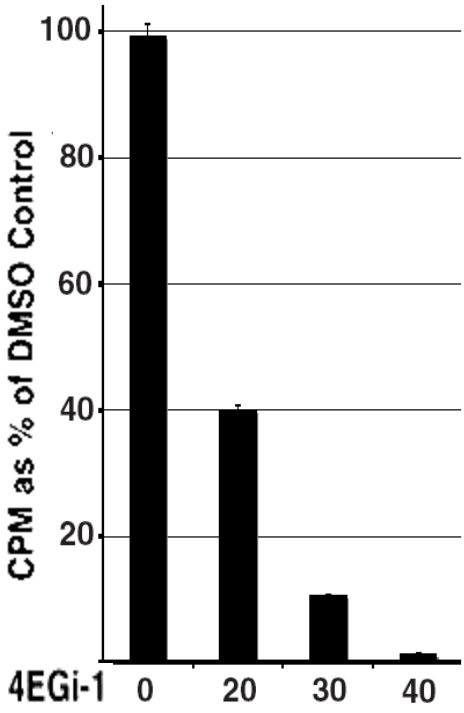

Figure 3.3.23 mRNA translation rates determined by TCA precipitation in NHDFs treated with DMSO or increasing μM concentrations of 4EGi-1 and infected with HSV-1 for 11 hours. At one hour prior to sampling cultures were [^{35}S]-Methionine/Cysteine metabolically-labelled and whole cell extracts prepared. [^{35}S] incorporation was quantified as counts per minute (CPM) as a percentage of control cultures treated with DMSO, arbitrarily set at 100%.

Figure 3.3.24

The affects of 4EGi-1 treatment on viral antigen accumulation during lytic replication

The ability of 4EGi-1 to reduce translation in HSV-1 infected cells led us to investigate what effects 4EGi-1 had on viral protein accumulation during lytic infection.

NHDFs were mock-infected (M) or infected with HSV-1 at m.o.i. 5 in the presence of increasing μM concentrations of 4EGi-1. 11 hours post-infection whole cell extracts were prepared. Samples were analyzed by western blotting using the indicated antibodies.

The abundance of viral antigens was found to decrease with increasing concentrations of 4EGi-1, with 40μM 4EGi-1 reducing the production of viral proteins examined to below detectable limits.

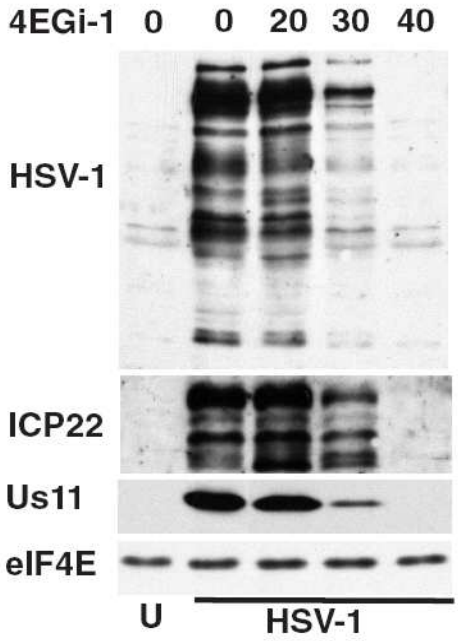

Figure 3.3.24 Western blot analysis for production of HSV-1 viral proteins: HSV-1, ICP22, Us11 in NHDFs cultures that were mock infected (U) or infected with HSV-1 at m.o.i 5 (HSV-1) in the presence of equal volumes of DMSO, or increasing μM concentrations of 4EGi-1. Whole cell extracts were prepared at 11 hours post infection. eIF4E was probed for as a load control.

Figure 3.3.25

Effects of 4EGi-1 treatment on infectious progeny production during lytic replication

Acknowledging the ability of 4EGi-1 to inhibit rates of translation and viral antigen accumulation, the amounts of infectious progeny being produced in cultures infected in the presence of 40μM 4EGi-1 were measured.

NHDFs were infected with HSV-1 at m.o.i. 5 in the presence of increasing μM concentrations of 4EGi-1 for 11 hours. Infectious virus production was determined by titration of freez thaw culture lysates on permissive Vero cells and represented as Log p.f.u/culture. Titration results are representative of three experiments.

Cultures treated with 40μM 4EGi-1 contained on average around 400 particles per culture, more than 10^6-fold lower than DMSO controls.

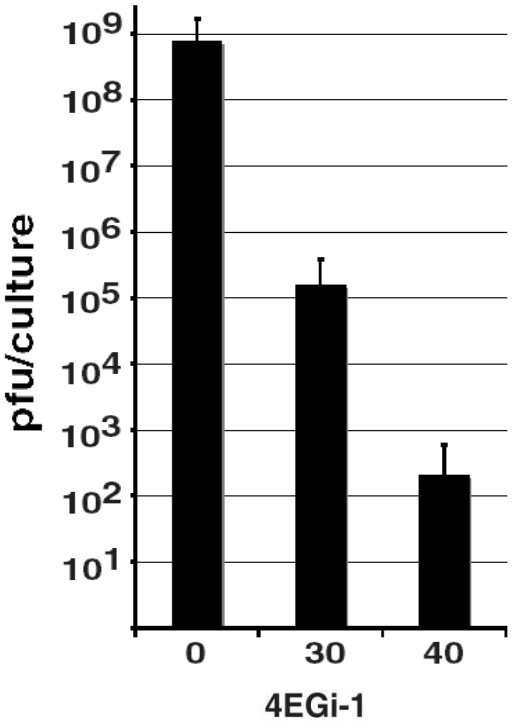

Figure 3.3.25 Viral titers of HSV-1 produced in NHDFs cultures that were infected with HSV-1 m.o.i 5 in the presence of equal volumes of DMSO, or increasing 30μM and 40μM concentrations of 4EGi-1 for 11 hours. Titers are represented as Log/p.f.u/culture supernatant and are the average of three independent experiments.

Figure 3.3.26

The effects of Torin1 on eIF4F complexes during HSV-1 infection

Considering that both Mnk and mTOR inhibition reduced reactivation from quiescence, it was deemed plausible that inhibition of eIF4F activity may be a viable therapeutic target for inhibition of HSV-1 lytic replication. Torin1 has been reported previously to prevent 4E-BP phosphorylation thus sequesting eIF4E and preventing eIF4G binding, therefore inhibiting eIF4F assembly (Thoreen et al. 2009).

To determine if the addition of Torin1 during HSV-1 infection caused inhibition of eIF4F complex formation, NHDFs were pretreated with 100nM Torin1 for 1 hour prior to infection. Cells were then infected at m.o.i 5 for 11 hours in the presence of Torin1 (100nM). Cell extracts were prepared in NP-40 lysis buffer, precleared with sepharose 4B and subsequently subjected to 7-Methyl-GTP Chromatography. Cap-bound and input samples were resolved by SDS-PAGE and membranes were probed with the indicated antibodies. 4E-BP1 was examined using 17.5% gels to resolve phosphorylated species.

Torin1 caused hypophosphorylation of 4E-BP1 and increased binding to eIF4E causing a significant reduction of eIF4G binding, demonstrating that during HSV-1 infection Torin1 effectively disrupted eIF4F formation.

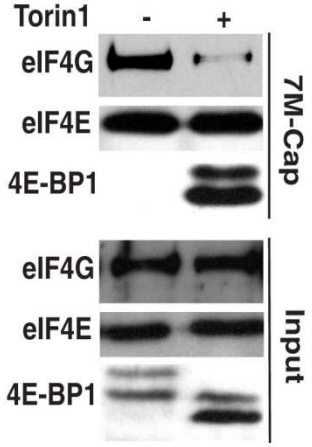

Figure 3.3.26 Western blot of translation factor abundance and 4E-BP1 phosphorylation in NHDFs that were treated with DMSO or 100nM Torin1 and then infected at m.o.i 5 for 11 hours in the presence of either DMSO or Torin1 (100nM). Soluble extracts were subjected to 7-Methyl-GTP chromatography. 7M-Cap-bound and input samples resolved by SDS-PAGE. Membranes were probed with the indicated antibodies. 4E-BP1 was examined using 17.5% gels to resolve phosphorylated species.

Figure 3.3.27

The affects of Torin1 on translation rates during HSV-1 high multiplicity infection.

To investigate whether Torin1 could inhibit HSV-1 translation, NHDF cells were either mock infected or infected at m.o.i 5 for 11 hours in the presence of DMSO or 100Nm Torin1. Cells were then metabolically labelled with [^{35}S]-Methionine/Cysteine for 1 hour in the presence of DMSO or 100nM Torin1. Whole cell extracts were prepared in 1x Laemmli and resolved by SDS-PAGE. The gels were then fixed dried and exposed to x-ray film. Migration of molecular weight (MW) standards is indicated to the left of the panel.

The metabolic labelling of mock infected cells treated with either DMSO or Torin1 illustrated that Torin1 reduces the translation rate modestly compared to DMSO treatment. During infection Torin1 also modestly represses translation.

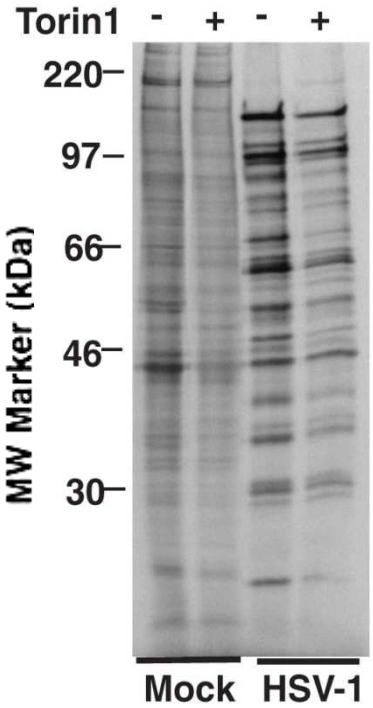

Figure 3.3.27 Metabolic labelling of NHDF cultures treated with either DMSO or 100nM Torin1 and mock infected (Mock) or infected (HSV-1) for 11 hours with HSV-1 at m.o.i 5. At 1 hour prior to sampling, cultures were incubated with [^{35}S]-Methionine/Cysteine in the presence of the drugs, and total protein was harvested by lysing cells in Laemmli buffer. Samples were resolved by SDS-PAGE where 50µl of sample was loaded into each well. Molecular weight standards (in kDa) are shown to the left of the panel.

Figure 3.3.28

A comparison of the effects of 4EGi-1 and Torin1 on viral protein production.
To observe what effects 4EGi-1 and Torin1 have on the production of viral proteins and downstream targets of mTOR in either uninfected or infected cells, NHDF cultures were mock infected of infected at a m.o.i 5 for 12 hours in either the presence of DMSO, 100nM Torin or 40μM 4EGi-1. Whole cell extracts were prepared and analyzed by western blotting and probed with antisera against p70S6K, HSV-1, ICP22, ICP0, US11 and eIF4E as a loading control.

As expected Torin1 caused the appearance of dephosphorylaed forms of p70S6K in both the uninfected and infected cultures. 4EGi-1 had no affect on the phosphorylation of p70S6K relative to the DMSO control. The DMSO treated infected culture showed an increase in p70S6K which was blocked by 4EGi-1 due to the inhibition of infection, while Torin1 caused hypophosphorylation of p70S6K due to mTOR inhibition.

In the infected cells treated with DMSO HSV-1 replicated efficiently as evident by the accumulation of HSV-1, ICP22, ICP0 and US11. Torin1 slightly reduced the accumulation of all viral antigens tested whereas 4EGi-1 completely inhibited their accumulation.

Figure 3.3.28

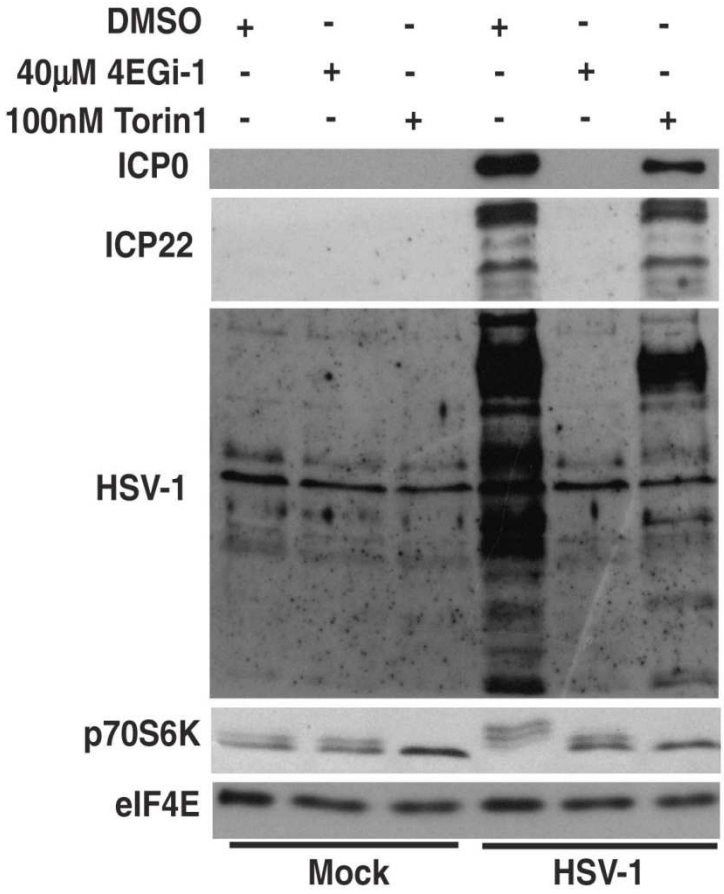

Figure 3.3.28 Western blot of HSV-1 viral proteins: ICP0, ICP22 and HSV-1 in addition to cellular protein p70S6K in NHDFs cultures that were mock infected (Mock) or infected with HSV-1 m.o.i 5 in the presence of equal volumes of DMSO, 40μM 4EGi-1 or 100nM Torin1. Whole cell extracts were prepared at 11 hours post infection. eIF4E was probed for as a load control. All drugs used were disolved in DMSO.

Figure 3.3.29

4EGI-1 is capable of suppressing translation mid way through a HSV-1 lytic infection.

To determine whether 4EGI-1 was capable of suppressing translation mid way through a HSV-1 lytic infection, NHDFs were mock-infected (M) or infected with HSV-1 at m.o.i. 5. 4 hours into the infection the infection the cells were treated with either DMSO or 40μM 4EGi-1. 8 hours post-infection cultures were metabolically labelled for 1 hour and whole cell extracts were resolved by SDS-PAGE. Fixed dried gels were exposed to x-ray film. MW standards are indicated to the left of the panel. Samples were analyzed by western blotting using the indicated antibodies.

4EGi-1 added to cultures at mid stages of infection retained the capacity to inhibit ongoing viral protein synthesis as illustrated by metabolic labelling and the suppression of the accumulation of late protein Us11.

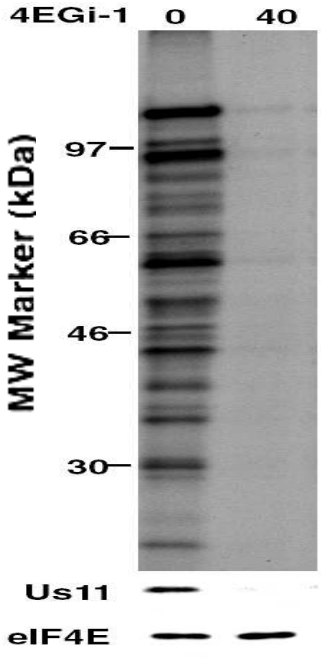

Figure 3.3.29 Metabolic labelling of NHDF cultures that were infected (HSV-1) for 9 hours with HSV-1 at m.o.i 5. 4 hours into the infection the cultures were treated with either DMSO (0) or 40 μM 4EGi-1. At 1 hour prior to sampling, cultures were incubated with [^{35}S]-Methionine/Cysteine in the presence of the drugs. Total protein was harvested by lysing cells in Laemmli buffer. Samples were resolved by SDS-PAGE where 50μl of sample was loaded into each well. Samples were also subjected to western blot and probed for Us11 and eIF4E. Molecular weight standards (in kDa) are shown to the left of the panel.

Figure 3.3.30

4EGi-1 translational suppression during late stage HSV-1 infection.

As 4EGi-1 inhibited translation when added prior to and during infection of cells, it was decided to test whether 4EGi-1 could suppress translation when added at very late stages during infection. NHDF cultures were infected with HSV-1 m.o.i 5 for 12 hours and subsequently treated with either DMSO or 4EGi-1 for 4 hours and labelled with ^{35}S-Methioine/Cysteine for 1 hour in the presence of drugs.

It was discovered that when 4EGi-1 is added to cells very late in HSV-1 infection still potently suppressed translation, although some viral protein synthesis was detectable.

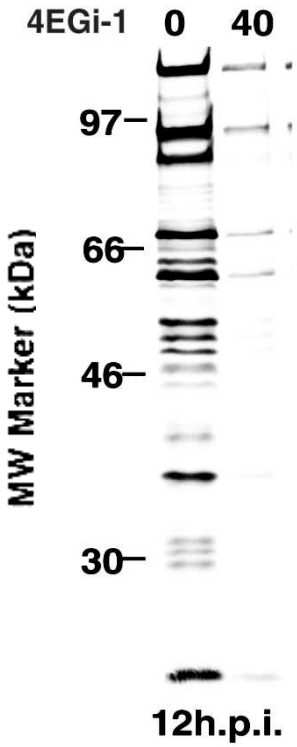

Figure 3.3.30 Metabolic labelling of NHDF cultures that were infected (HSV-1) for 16 hours with HSV-1 at m.o.i 5. 12 hours into the infection the cultures were treated with either DMSO or 40 µM 4EGi-1 for 4 hours. At 1 hour prior to sampling, cultures were incubated with [^{35}S]-Methionine/Cysteine in the presence the drugs. Total protein was harvested by lysing cells in Laemmli buffer. Samples were resolved by SDS-PAGE where 50µl of sample was loaded into each well. Molecular weight standards (in kDa) are shown to the left of the panel.

Figure 3.3.31

Suppression of poxvirus protein synthesis by 4EGi-1

To determine whether 4EGI-1 was capable of suppressing the replication of viruses other than HSV-1 we tested 4EGi-1 effects on Vaccinia Virus (VacV), the laboratory prototype for poxvirus infection. NHDFs were mock-infected (M) or infected with VacV at m.o.i. 10 for 15 hours in the presence of increasing concentrations of 4EGi-1 then metabolically labelled for 1 hour. Whole cell extracts were resolved on SDS-PAGE gels that were then dried and exposed to x-ray film.

Poxvirus infection of DMSO-treated NHDFs resulted in the characteristic shut-off of host translation and robust synthesis of poxvirus polypeptides. While lower concentrations of 4EGi-1 had no significant effect on the pattern or rates of viral protein synthesis, modest inhibition of translation was evident at 10μM while concentrations above 20μM resulted in dramatic suppression of translation.

Figure 3.3.22

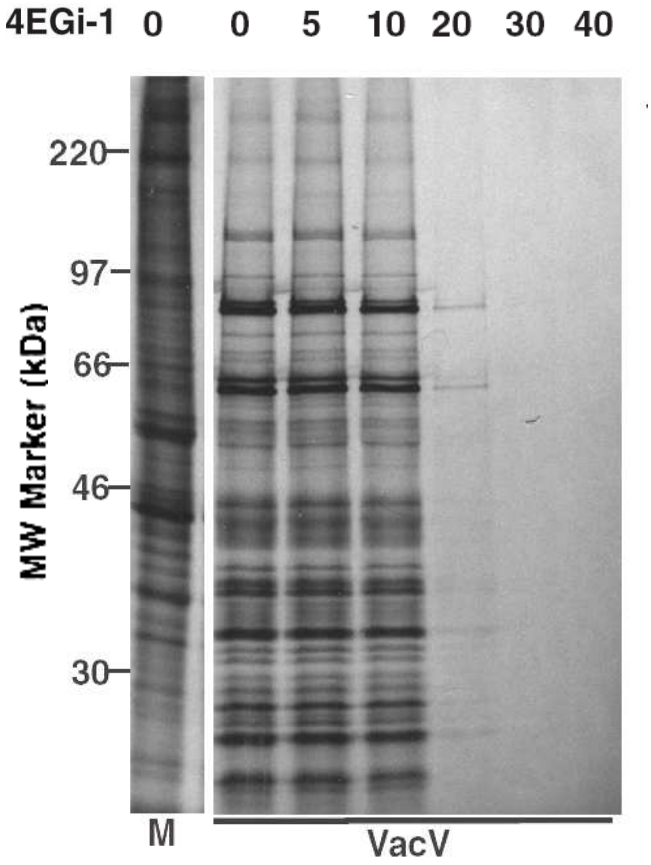

Figure 3.3.22 Metabolic labelling of NHDF cultures infected with Vaccinia virus at a m.o.i of 10 for 16 hours in the presence of either DMSO (0) or increasing μM concentrations of 4EGi-1. At 1 hour prior to sampling, cultures were incubated with [^{35}S]-Methionine/Cysteine in the presence of the drugs. Total protein was harvested by lysing cells in Laemmli buffer. Samples were resolved by SDS-PAGE where 50μl of sample was loaded into each well. Molecular weight standards (in kDa) are shown to the left of the panel.

Figure 3.3.32

Rates of protein synthesis in Vaccinia infected cultures treated with 4EGi-1

To determine the rates of translation in VacV samples described in figure 3.3.31, TCA precipitation was performed and presented as a percentage of DMSO controls.

TCA precipitation and quantification showed that 30μM and 40μM 4EGi-1 reduced translation rates in infected cultures to 2.4% and 2.3% respectively, of those in DMSO-treated cultures

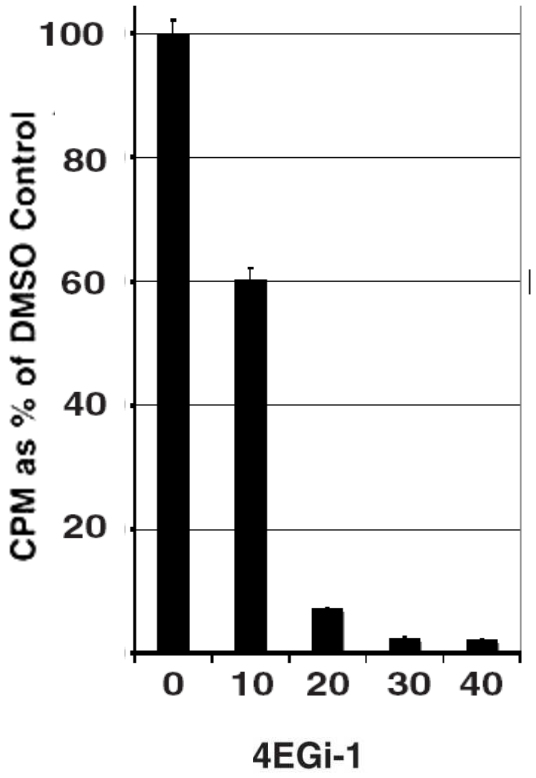

Figure 3.3.23 mRNA translation rates determined by TCA precipitation in NHDFs treated with DMSO or increasing concentrations of 4EGi-1 and infected with Vaccinia virus m.o.i 10 for 16 hours. At one hour prior to sampling cultures were [^{35}S]-Methionine/Cysteine metabolically-labelled and whole cell extracts prepared. [^{35}S] incorporation was quantified as counts per minute (CPM) as a percentage of control cultures treated with DMSO, arbitrarily set at 100%.

Figure 3.3.33

Effects of 4EGi-1 treatment on infectious progeny production during Vaccinia virus replication.

To test the effects of this translational suppression on production of infectious virus, NHDFs were treated with DMSO or 30μM 4EGi-1 and infected at a multiplicity of either 1 or 10 pfu per cell to determine whether the effects of this inhibitor were also influenced by the amount of incoming virus particles. At 16 hours post infection the samples were harvested by freeze-thaw and infectious virus determined by titration on permissive BSC40 cells.

(A) In both instances 4EGi-1 reduced virus replication to approximately 3% of control DMSO-treated cultures, demonstrating that 4EGi-1 suppressed virus replication regardless of the amount of incoming virus and that its effects on virus replication closely mirrored the degree to which it suppressed rates of viral protein synthesis (Figure 3.3.32). However, when represented on a logarithmic scale (B) although 4EGi-1 potently suppressed VacV replication its effects on infectious virus production were much lower than that observed with HSV-1.

Figure 3.3.33

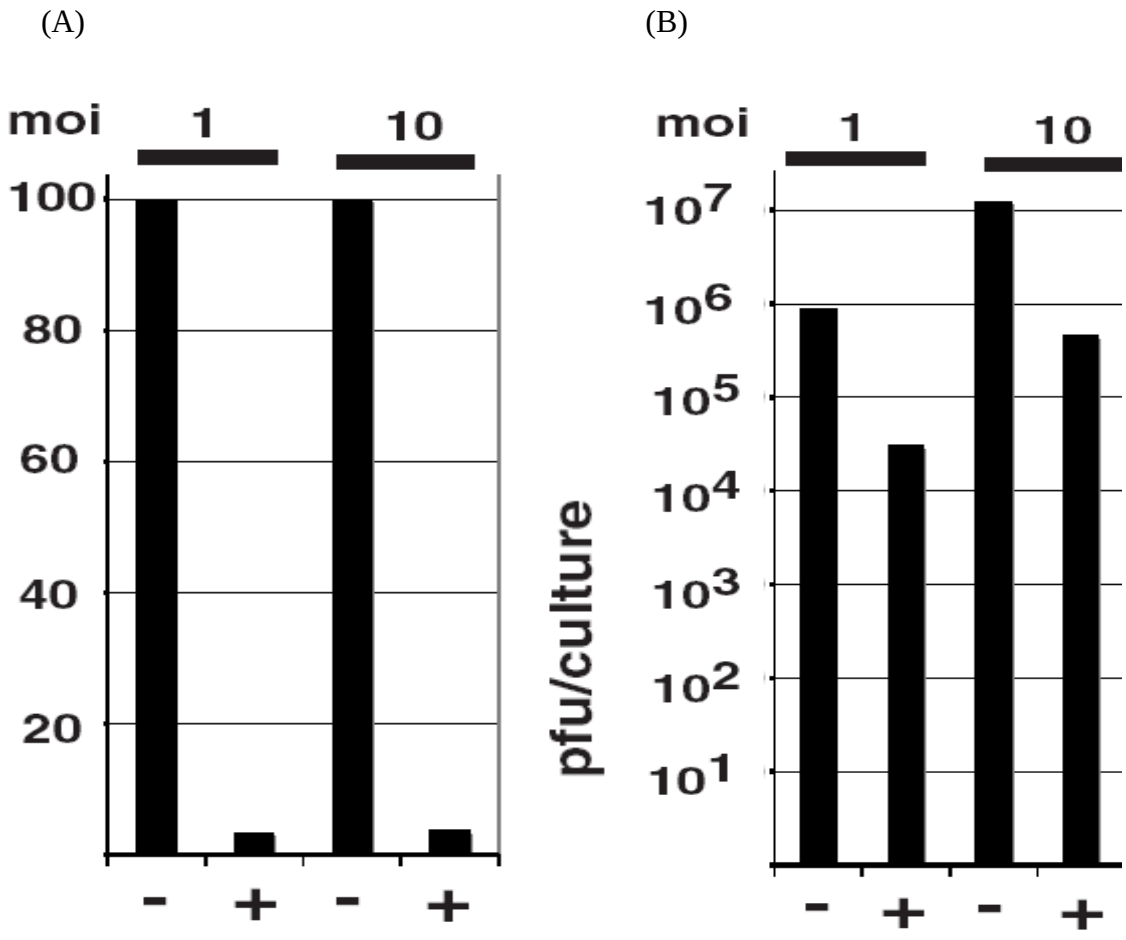

Figure 3.3.33 Viral titers of Vaccinia Virus produced in NHDFs cultures that were infected with Vaccinia Virus at m.o.i 1 and 10 in the presence of equal volumes of DMSO or 30μM of 4EGi-1 for 16 hours. Titers are represented as Log/p.f.u/culture supernatant and are the average of three independent experiments. (A) Represents the amounts of viral progeny production in cultures treated with 4EGi-1 as expressed as a percentage relative to a DMSO infection. (B) Represents the amounts of viral progeny production as expressed on the logarithmic scale.

Figure 3.3.34

The effects of 4EGi-1 on Vaccinia virus antigen accumulation

To determine the effects of 4EGi-1 on Vaccinia antigen accumulation during infection, NHDFs were mock-infected (M) or infected with VacV at m.o.i. 10 in the presence of increasing μM concentrations of 4EGi-1 and whole cell extracts prepared after 16 hours. Samples were analyzed by western blotting using anti-VacV antibody.

In contrast to HSV-1 antigens (Figure.3.3.24) overexposure of western blots against VacV antigens demonstrated that the accumulation of viral proteins was still detectable in 30μM 4EGi-1-treated samples. As such, while 4EGi-1 affected translation rates in both cases it had distinct effects on the overall fate of Poxvirus and Herpes virus infection.

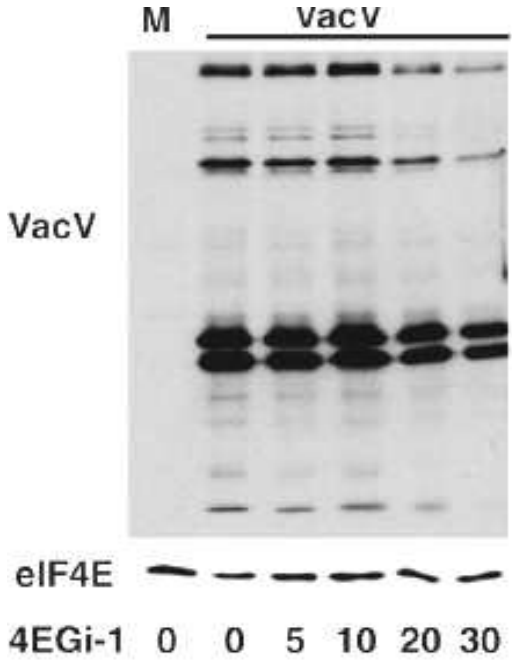

Figure 3.3.24 Western blot analysis for production of Vaccinia viral protein: VacV in NHDF cultures that were infected with Vaccinia Virus at m.o.i of 10 for 16 hours in the presence of equal volumes of DMSO increasing μM concentrations of 4EGi-1. eIF4E was probed for as a loading control.

Figure 3.3.35

4EGi-1 translational suppression during late stage Vaccinia infection

As 4EGi-1 inhibited translation when added prior to and during HSV-1 infection, it was decided to test whether 4EGi-1 could suppress translation when added at late stages of VacV infection. NHDF cultures were infected with Vaccinia virus m.o.i 10 for 12 hours and subsequently treated with either DMSO or 4EGi-1 for 4 hours and labelled with ^{35}S-Methioine/Cysteine for 1 hour in the presence of drugs.

It was discovered that when 4EGi-1 was added to cells late in Vaccinia virus infection 4EGi-1 retained the capacity to potently suppress translation.

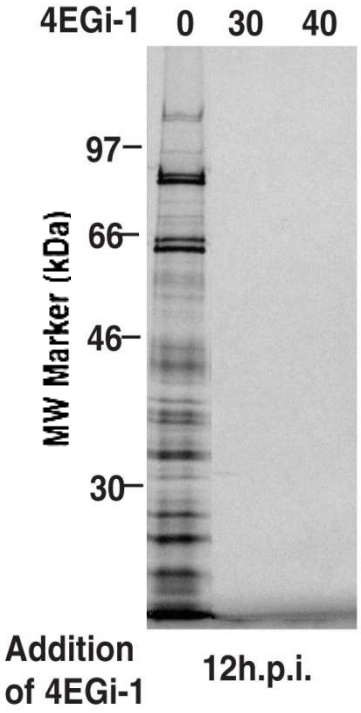

Figure 3.3.35 Metabolic labelling of NHDF cultures that were infected with Vaccina at m.o.i 10 for 16 hours. 12 hours into the infection the cultures were treated with either DMSO or 30µM and 40 µM 4EGi-1 for 4 hours. At 1 hour prior to sampling, cultures were incubated with [^{35}S]-Methionine/Cysteine in the presence of the drugs. Total protein was harvested by lysing cells in Laemmli buffer. Samples were resolved by SDS-PAGE where 50µl of sample was loaded into each well. Molecular weight standards (in kDa) are shown to the left of the panel.

Section 4: Discussion

4.0 Discussion:

In contrast to our relatively detailed understanding of HSV-1 lytic replication, latent infection and reactivation remain very poorly understood. This project was aimed at understanding the roles cell of signalling and translational regulation during the establishment and reactivation from HSV-1 quiescence. In an attempt to achieve our goals we endeavoured to create an in vitro model to study these phenomena. Our decision to create such a model was due to the fact that latent infection has proven to be difficult to model in the past. Consequently in order to study cellular changes caused by reactivation events an efficient model was required.

4.1 Developing a cell culture model of HSV-1 latency.

In the past many attempts in studying latency have employed the use of both in vitro and in vivo models. Of the in vivo models, mice and rabbit models are used most frequently for the study of latency. The use of mice during studies has been considered advantageous due to the low cost relative to other animal models and some aspects of mouse HSV-1 latency mimic aspects of human HSV-1 latency, and led to identifying the neuron as the site of latency and the expression of LATS during latent infection. However, the limitations associated with the use of mice to model human latency include the unusual routes by which infection is accomplished, such as infection of the cornea or through the footpad which do not represent a typical route of infection normally taken by an infecting virus. The main disadvantage postulated in using mice for the study of HSV-1 latency is that they are not human and mouse models lack the characteristic of spontaneous reactivation, a phenomena routinely seen in humans. Spontaneous reactivation along with LAT expression occurs in rabbits that are latently infected but their use has been limited as they are quite expensive and cumbersome to work with. Although spontaneous reactivation is seen in latently infected rabbits a fact which has led some to suggest that HSV-1 latency in rabbits mimic human infection more closely than mice, questions over the relevancy of these models have not eluded experts in the field. An example of this is that in mice latently infected with a LAT promoter deletion mutant, HSV-1 genomes are less associated with repressive histone dimethyl H3 K9 than with the wild type strains. Whereas HSV-1 LAT-negative genomes in infected rabbits do not become less

enriched in repressive histone marks relative to the wild type, therefore indicating that the LAT does not seem to exert a repressive effect on the chromatin state of latent genomes in rabbits. These findings highlight the opposing characteristics of infection in different species which raises questions on the relevancy of animal models to human infection by human pathogens (Wang *et al*., 2005; Giordani *et al*., 2008).

It should also be outlined that many clinical isolates of HSV-1 vary with regard to their neurovirulance and pathology in different species of animal. Therefore the amount of genotypes, serotypes and phenotypes available for study is limited in comparison to the plethora of naturally occurring ones. The study of HSV-1 mutants is also encumbered by the fact that specific characteristics of virus infection, latency and reactivation are often strain specific, so even though mutants are available for study in tissue culture they may not be viable for in vivo study. For example the 17syn+ strain is a standard strain used in mutational analysis but is much more virulent in mice than the Kos (M) strain (Thompson *et al*., 1986). Additionally, strain specific difference in viral glycoproteins as well as other proteins may drastically influence the capacity of the virus to spread in the nervous system and hypothetically alter the parameters of latency (Izumi, Stevens 1990; Yuhasz, Stevens 1993; Bloom, Stevens 1994; Mitchell, Stevens 1996).

The difficulties in creating in vitro models which can facilitate detailed mechanistic studies of HSV-1 latency has also largely contributed to our relatively poor understanding of latency. In the past, attempts have been made to create models which approximate human infection as accurately as possible but many of the models developed for such studies have used neuronal cells of non-human origin and transformed cell lines while employing the use of chemical inhibitors to suppress the initial production of immediate early genes. Alternatively HSV-1 mutants which lack the required genes to initiate viral replication have also been used. These strategies to suppress viral replication have attempted to increase the amount of virus that could infect and be coerced into a non replicating state as infecting cultures with wild type virus at high multiplicity without inhibitors is not viable and invariably results in the lytic replication and death of the culture. The main disadvantages of these models are the poor efficiency of both spontaneous and controllable reactivation and the fact that

they use animal cells, which again raises questions of the results gathered using these models with regard to their relevancy to human infection.

Due to Ethical and accessibility issues associated with the development of human neuronal systems using primary cell cultures, research groups have developed models to study quiescence using Human fibroblasts. This is mainly due to the low metabolic state of fibroblasts which is thought to resemble that of neurons more closely than transformed cell lines which do not support quiescent infection. It is also thought that fibroblasts express factors which restrict viral replication (Hancock, Corcoran & Smiley 2006).

Although primary fibroblasts may resemble neurons metabolically, difficulties still remain in forming efficient quiescent infection. In the past, just as in animal neuronal models, the inability to infect at high multiplicity has led research groups to create mutant stains of HSV-1 which are either lacking in the immediate early gene ICP0 or lacking ICP0 in combination with other essential genes such as ICP4. While these mutant strains have been useful in the study of HSV-1 quiescence, doubt still remains about whether quiescence established using IE mutants follows an identical infection paradigm to wild type infection. The caveats of these models raise questions in relation to the viruses natural characteristics during the formation of latency. For example, does immediate early protein production occur during establishment of quiescence, and if so, does that mean when certain genes are deleted from a genome, are there alterations in the virus host balance during initial establishment of quiescence, as many of the genes initially produced have regulatory functions which control ordered cascades that may determine the overall fate of the genome as it resides in either a strongly repressed or weakly suppressed state during its residency within the nucleus.

To begin to ask questions about these issues and the mechanisms of virus reactivation from a dormant state, we developed a model to study HSV-1 quiescence requiring only simple culture conditions to support the establishment of quiescence in human cells without having to employ mutant forms of the virus or chemical inhibitors of viral replication. In considering the parameters to be tested to establish quiescence in primary NHDF's our choices were influenced by the observation that old models used cycling cells which would presumably provide a more favourable environment for

lytic replication to proceed. Indeed previous studies could only achieve a m.o.i of 0.003. As a result the first selective pressure employed for the establishment of the quiescence model was serum starvation which synchronizes confluent cultures into the g0 quiescent phase, where the cells have left the cycle and stopped dividing thus limiting kinase pathway and metabolic activity to basal levels creating a less favourable environment for efficient viral replication. This non dividing state may resemble the low metabolic state of neurons which are the primary environment for the establishment of latency in humans. When experiments were conducted to answer whether serum starvation was required for the establishment of quiescence, the selective pressure was removed from the quiescent model protocol and cells were infected at elevated temperature in medium containing 5% normal serum, upon which it was discovered that although replication was suppressed, some Us11 was observed at 48 hours post infection and low level viral replication was observed at 72 hours postinfection, illustrated by small areas of CPE. This suggested that a mix of quiescent and replicating viruses were present, likely reflecting the mixture of dividing and nondividing cells present in unstarved cultures and signifying that temperature elevation alone is not sufficient to suppress lytic replication and that a restricted metabolic environment is required for maximal non productive infection (Figure 3.1.16, 3.1.15).

The second selective pressure employed to establish our quiescence model was temperature elevation. As mentioned previously, during latency of herpes simplex virus type 1, the latency associated transcript (LAT) 2-kb intron is expressed, this intron is excised from the larger (10kb) precursor primary transcript. During infection of neuronal cells with HSV that express LATs, it was observed that their presence increase the accumulation of the Hsp70 protein. This HSP has molecular chaperone activity and regulates processes involved in protein biogenesis e.g stabilization of unstable misfolded proteins, localisation of proteins to particular cellular compartments and control of the switch between active/inactive protein conformation (Whitley, Goldberg & Jordan 1999). It has therefore been hypothesised that Hsp overexpression during infection may be used by the virus to increase the viability of the infected cells during the establishment of a latent infection.

Considering that LAT expression is inefficient in quiescently infected rat neuronal and human fibroblast cultures, in addition to many neuronal cells in vivo, it is likely that the induction of Hsp expression by temperature elevation may allow for increased cell viability during infection with HSV-1. In our model the over expression of Hsp27 and Hsp70 was successfully achieved by temperature elevating NHDF cultures for 30 hours at 41°C despite the metabolic suppression of serum starvation (Figure 3.1.2). Western blot analysis showed Hsp27 and Hsp70 expression was up-regulated compared to mock infected or infected cells at 37°C and no increase in the expression of the control protein PABP was observed. Critically, the infection of cells at 41°C did not alter the degree of Hsp expression relative to mock infected cells. Whether Hsps are truly important for protecting cells during the establishment of quiescence could be tested in future experiments by siRNA-mediated knockdown of Hsps or the use of inhibitors of Hsp function such as Geldanamycin.

Although temperature elevation induces heat shock proteins, it also has the added benefit of suppressing HSV-1 replication. Temperature elevation had no inhibitory affect on entry into the cell or the expression of ICP4. However, unlike cells infected at 37°C, where virus replicated efficiently and subsequently spread to 100% of the culture by 24h postinfection, the percentage of cells expressing ICP4 at 41°C remained static (Figure 3.1.3, 3.1.4), which suggested that the virus was unable to replicate at elevated temperature. Measuring the amount of infectious virus in cultures at either temperature validated that virus was being actively synthesised in cells infected at 37°C while only minimal amounts of infectious virus were detectable in cultures at 41°C over the first 24 to 48 hours (an observation seen previously in neuronal cell systems (Su *et al*., 1999). This was also evident in the small amounts of Us11 produced at 41°C in the first 48 hours. This production of virus became undetectable by 72 hours postinfection (Figure 3.1.5) validating that as long as cultures were maintained at 41°C, the infection was maintained in this nonproductive state. This was further confirmed by metabolic labelling that monitored population-wide changes in protein production during entry into a nonproductive state. The experiment illustrated that at 12 hours postinfection, cells infected at 41°C differentially expressed a small number of proteins that due to their size and comigration with viral proteins in lytically infected cultures most likely represent viral polypeptides. By 24 hours postinfection, synthesis of these proteins had begun to

diminish, and by 48 hours postinfection they became undetectable relative to cultures infected at 37°C (Figure 3.1.6). Notably, many of the viral proteins normally characteristic with productive infection were not synthesized in cells infected at the elevated temperature, suggesting that they were either not produced or were made at levels below detection. In addition, at no point did infection at the elevated temperature alter host cell protein synthesis patterns or elicit the shutoff of host translation associated with lytic replication, a well known phenomena of HSV-1 infection which is facilitated by the viral proteins VHS protein and ICP27 (Feng, Everly & Read 2001; Taddeo, Zhang & Roizman 2010) These results suggest that as the quiescent infection progressed, the synthesis of viral proteins declined and production of progeny ceased, seemingly indicating that the virus was entering into a non productive state and agrees with previous reports showing that elevated temperature suppress lytic replication provided the infection has not proceed past the stage of genome replication stage (Crouch, Rapp 1972).

As mentioned previously the many clinical isolates of HSV-1 vary with regard to their neurovirulance and pathology. The question of whether alternate strains of HSV-1 could be coerced to a state of non productive infection using this system was answered in figure 3.17 and 3.18. When cultures which were treated as outlined in the models protocol were infected with the Patton strain of HSV-1 it was shown that although serum starvation and temperature elevation of cultures to 41°C repressed viral replication, some replication was observed. This phenomena was prevented by increasing the temperature to 42°C and it was found that virus was recoverable from quiescence as evident by tittering experiments performed (Figure 3.1.18). The exact reason for this requirement is unknown, but Patton is known to be a more temperature insensitive strain than Kos, which further supports the theory that temperature elevation suppresses HSV-1 replication and this degree of suppression can vary depending on the strain used. To illuminate the reasons for this occurrence, an analysis of the production of immediate early, early and late antigens produced by Patton at both 41°C and 42°C may indicate whether the expression of specific Patton strain proteins are less sensitive to temperature elevation thus making the lytic programme of the Patton more resistant to suppression relative to the Kos strain. Another possibility is that Kos ICP4 may be more sensitive to elevated temperature than the Patton strain, thus altering its transactivating capacity at a lower temperature.

This could be possibly tested by electrophoretic mobility shift and Chromatin immunoprecipitation assays.

4.1.1 Protein production and entry into quiescence.

The four bands initially visible at the 12 hour point post-infection at 41°C sample (Figure 3.1.6) were suspected to be viral antigens and western blotting with antiserum directed against an array of viral IE proteins confirmed that viral antigens were being robustly produced at 37°C whereas at 41°C the accumulation of viral antigens was greatly repressed relative to the 37°C infection (Figure 3.1.7). Although ICP4 was still being produced efficiently at 41°C, the production of the critical regulator of lytic replication ICP0 was highly suppressed relative to a normal lytic infection, whereas ICP22 was produced but not posttranslationally modified, which suggests that infection was impeded at an early stage of the viral life cycle. Additionally the Us11 protein which is an indicator of viral progeny production was only visible in minute quantities on the blots taken, signifying low level lytic replication, an observation confirmed by immunofluorescence analysis of another late antigen, ICP5 which showed at 24 hours post infection that cultures infected at 37°C expressed extremely high levels of ICP5 (100% of culture), whereas an average of only 15% of cells infected at 41°C faintly expressed ICP5 at very low levels. Whether these cells eventually died from productive infection or survived due to the level of lytic antigen production being below the threshold of cellular tolerance remains unknown, but as no large scale CPE or accumulation or cell debris was found in these cultures it was unlikely that these cells harbour robust lytic infection.

During lytic infection, the viral transcription factor VP16 binds to cellular transcription factors HCF and OCT-1 to initiate immediate early gene expression. In cells lacking HCF and OCT-1, HSV-1 infects poorly (Wysocka, Herr 2003; Nogueira *et al*., 2004). Interestingly during entry into quiescence, IE genes such as ICP4 were being produced abundantly throughout the first 48 hours (Figure 3.1.7) and when samples taken from a 6 day infection at 41°C were analysed it was found that residual ICP4 was present. The presence of ICP4 indicates that either ICP4 was continually synthesized at low quantities or was proteolytically stable in cells quiescently infected. This result agrees with previous reports showing that low levels of ICP4

transcript are detected in mouse ganglia latently infected with HSV-1 (Kramer, Coen 1995) and seems to indicate that Vp16 may be functionally active at elevated temperature. Taking this into consideration, an understanding of how these cells allow for the production of some immediate early genes while suppressing ICP0 production may provide some important mechanistic understanding into how certain host cells prevent HSV-1 from productively replicating. This may involve heat shock responses that regulate (NF)-kappa B which controls ICP0 expression (Amici et al. 2006).

Another mechanism by which lytic replication may be prevented is the fact that ICP22 is being produced but not processed. ICP22 is a protein 420-amino-acids in length and is encoded by the α22 gene and is required for regulation of early and late gene expression. The α22 gene is not required for replication in permissive Vero and HEp-2 cell lines, but $\alpha22^{-}$ mutant viruses replicate inefficiently in restrictive primary human fibroblasts (Post, Roizman 1981; Meignier *et al*., 1988). Interestingly, in the restrictive cells, the mutant HSV-1 strain R325 which lacks the carboxyl-terminal 220 codons of the α22 gene exhibits a reduction of ICP0 and of Us11 mRNA and proteins (Purves, Ogle & Roizman 1993). During the early stages of infection, ICP22 localizes in punctuate nuclear structures. Upon the onset of viral DNA synthesis, ICP22 colocalizes in infected nuclei with ICP4, viral DNA, RNA polymerase II, and the small cellular protein EAP. This localisation requires the presence of the functional HSV-1 protein kinase encoded by the U_L13 gene and is required for optimal late-gene expression (Purves, Ogle & Roizman 1993; Leopardi *et al*., 1997). During the procession of lytic infection ICP22 is extensively modified. These modifications include phosphorylation by the viral protein kinases Us3 and U_L13 (Post, Roizman 1981; Purves, Ogle & Roizman 1993) and nucleotidylylation by casein kinase II (Blaho, Mitchell & Roizman 1993; Mitchell, Blaho & Roizman 1994; Mitchell *et al*., 1997). A recombinant virus carrying a deletion in the U_L13 gene was found to be similar to R325 with respect to several properties. Studies of the U_L13^{-} virus in restricted cells led to the conclusion that the phosphorylation of ICP22 is necessary for the functions described above. At least one of the sequences required for posttranslational modification of ICP22 maps in the carboxyl-terminal domain of ICP22. Therefore it is interesting to note that the lack of ICP22 posttranslational modification observed in cultures infected at elevated temperature may also play a role in the repression of ICP0 synthesis. Indeed, ICP22 has been shown to

downregulate ICP0 expression in cotransfection assays where increasing amounts of the ICP22 expression vector, and unprocessed ICP22 protein, resulted in loss of ICP0 expression (Bowman *et al*., 2009).

4.1.2 Controllable and spontaneous reactivation of quiescent infection.

Of the characteristics exhibited by the virus during infection at elevated temperature, the failure to efficiently process ICP22 and the robust inhibition of ICP0 production (Figure 3.1.7) were likely the primary events hindering the establishment of productive infection. During a normal productive infection the IE protein ICP0 disrupts nuclear structures within the host cell known as ND10 or PML bodies. These PML bodies are then reorganised coinciding with formation of viral replication compartments (Chee *et al*., 2003; Everett, Maul 1994; Everett, Murray 2005; Everett *et al*., 2006; Ishov, Maul 1996; Gerd G. Maul 1998).

In neuronal cell lines, infection with mutant HSV-1 that lacks ICP0 results in enlarged PML bodies and the formation of a quiescent infection (Hsu, Everett 2001; Hsu, Everett 2001). PML structures are also known to become enlarged in human fibroblast lines when infected with HSV-1 mutants that lack IE gene products including ICP0. Interestingly, during infection at elevated temperature PML structures were also observed to be enlarged (Figure 3.1.24).

Although the capacity of the repressive culture conditions to prevent lytic replication was clear, there was a concern that the virus might be inactived. ICP0 is a critical regulator of lytic replication and reactivation from quiescence, whereby it acts as a promiscuous transactivator that functions in the nucleus to enhance the expression of genes by degrading and dispersing repressive ND10 bodies (Everett *et al*., 2006; Everett, Chelbi-Alix 2007). Its low level production at 41°C likely contributes to the failure to lytically replicate. Demonstrating that the virus was not inactivated and could indeed be coerced back into productive infection even after 6 days of quiescence was recovery by transduction of cells with an Adeno viral vector encoding ICP0 (Figure 3.1.13). This was evident in robust production of Us11 at levels comparable to a 37°C lytic infection of identical multiplicity. In addition, viral titering experiments showing that the yields of infectious virus from ICP0-transduced cultures that had been quiescently infected for either 3 or 6 days were equivalent to those from

cells infected at 37°C and harvested at 48 hours postinfection. Phase contrast imaging also showed extensive CPE in cultures that had been quiescently infected for 6 days and reactivated with ICP0, demonstrating that the virus could be robustly reactivated in a controlled manner upon exogenous addition of ICP0 (Figure 3.1.14).

Whereas exogenous expression of ICP0 caused reactivation of virus, exogenous expression of ICP4 could not reactivate quiescent virus above spontaneous levels in our system as evident by metabolic labelling experiments which showed a primarily cellular pattern of protein synthesis (Figure 3.1.19). This result was further supported by phase contrast imaging of quiescent cultures treated with either ICP0 or ICP4 vectors which illustrated that the extensive CPE in ICP0 reactivated cultures was absent in quiescent cultures treated with ICP4 (Figure 3.1.20). Additionally western blot analysis of Us11 from various quiescent cultures treated with either ICP0, ICP4 or medium alone showed that in cells harbouring quiescent virus that were transduced with ICP4 vector, the levels of Us11 expression were similar to quiescent cultures treated with medium alone (Figure 3.1.22). This was as expected as ICP4 was already being expressed in quiescent cells (Figure 3.1.12). In agreement with these results are previous reports describing the inability of exogenous expression of ICP4 to reactivate quiescent HSV in human cell line models (Harris *et al*., 1989; Zhu *et al.*, 1990; Harris, Preston 1991; Arthur *et al*., 2001).

In the past, the degree of viral genome repression has been observed to vary depending on the cell type used. Reports have shown that although ICP0 is the most efficient at causing reactivation, both ICP4 and VP16 also have the capacity to reactivate virus in neuronal lines (Halford *et al*., 2001). Also, it has been shown that various stress-inducing agents can reactivate virus in certain neuronal models. Conversely, human fibroblast models are resistant to most reactivation stimuli with the exception of ICP0. This information has led to the suggestion that viral genomes can be suppressed to varying degrees, as it seems that human fibroblasts maintain HSV-1 in a more repressed state than non human neuronal cell lines. To date our findings are in line with these previous reports.

Although exogenous expression of ICP0 caused the recovery of virus, spontaneous reactivation of virus could be also initiated by removal of the cells from the selective pressures of serum starvation and temperature elevation. Interestingly, low level

spontaneous reactivation has also been reported previously in quiescent systems. Additionally, low levels of viral production in rabbit ganglia causes shedding of virus which is contained by host immunity and asymtomatic shedding routinely occurs in infected humans (Feldman *et al*., 2002; Margolis *et al*., 2007). Therefore our results with regard to low level reactivation in cultures removed from their selective pressures likely reflect the natural dynamic state of HSV-1 in-vivo where the virus is spontaneously produced at low levels, but is continually prevented from causing a large scale productive infection by the hosts immunity. In agreement with this hypothesis is the fact that when quiescently infected cultures were removed from elevated temperature and maintained in 5% human serum that contains neutralizing antibodies, spread of virus from this small population undergoing spontaneous reactivation was prevented (Figure 3.1.23).

The main motivation in designing a quiescence model was to characterize what bearing HSV-1 quiescence and reactivation has on cellular processes such as cell signalling and translation. For our model to be able to detect cellular changes caused by the virus at various stages of its life cycle, the majority of the culture needed to be harbouring quiescent virus which could be viably reactivated. Through the use of Human serum during reactivation it was possible to reactivate virus and drastically reduce secondary spread of the virus to uninfected cells. This approach allowed for a more accurate quantification of cells harbouring reactivating virus. From results illustrated in figure 3.1.22 it was shown that when virus was reactivated in the presence of Human serum there was a reduction in the amount of Us11 accumulation relative to cultures reactivated in normal serum. This along with metabolic labelling showing that cultures reactivated in human serum had an amalgam of cellular and viral proteins being expressed simultaneously suggested that secondary spread was indeed being suppressed. This allowed us by indirect immunfluorescence to quantify that 40- 60% of the culture was harbouring reactivating virus. Therefore this model provides an amenable tool to study wild type HSV-1 quiescence in human cells, whereby the relatively simple selective pressures of serum starvation of primary normal human diploid fibroblasts and temperature elevation results in inhibition of productive infection and the establishment of quiescence. Importantly, the model can use different strains of HSV-1 with the proviso that the correct elevated temperature must first be elucidated to inhibit replication. In addition, as the majority of the

culture can be quiescently infected without causing cellular stress, this allows for population-wide scale occurrences to be readily detectable by experimentation. Furthermore, the use of human serum allows for the reduction of secondary spread of virus post reactivation. Consequently, this feature of the system therefore confers the capacity to differentiate between events associated with reactivation from those related to secondary spread of the virus. As such, this approach can be used to illuminate the mechanistics controlling the various stages in the life cycle of HSV-1.

4.3 The role of host signalling in reactivation:

The control of cell signalling pathways during viral replication is of paramount importance to the resulting fate of viruses within the host cell. During HSV-1 lytic infection, the activation of the p38 mitogen-activated protein kinase (MAPK) signalling pathway is onserved. This MAPK pathway is known as a stress-activated protein kinase (SAPKs) due its involvement in controlling cellular responses to various types of stress. Studies have discovered that p38 becomes activated as early as 3 hours postinfection during the initial stages of HSV-1 lytic infection and peaks at about 6 to 8 hours postinfection (McLean, Bachenheimer 1999). The activation of this SAPK signalling pathway appears to be important for normal viral replication since the presence of the pharmacological inhibitor p38 leads to a large drop in viral yields wheb cells are infected at low multiplicity but has little impact when large input doses of virus are used (Walsh, Mohr 2004). The timing of the p38 activation suggested that an IE gene product might be responsible for initial p38 stimulation as IE proteins are present at high levels at 3 hours postinfection. Consistent with this are studies which have shown that ICP27 is required in the context of viral infection for activation of the SAPK pathways (Hargett, McLean & Bachenheimer 2005). While host p38 signalling pathways exploited by Herpes Simplex Virus type 1 during lytic replication are relatively well characterized, little is known about kinase pathway activation during reactivation from the non-productive state of quiescence. Considering that viruses manipulate protein kinase signalling networks within host cells for the primary purpose of hijacking critical cellular functions to facilitate their replication, it was decided to characterize the activities the ERK and P38 kinase pathways during HSV-1 quiescence and reactivation from quiescence using the model described in figure 4.1.

4.2.1 The role of ERK and p38 in HSV-1 reactivation

To characterize the activities of these pathways during reactivation it was required to pinpoint the moment of viral reactivation subsequent to ICP0 transduction. A kinetic analysis of reactivation was illustrated by metabolic labeling and showed that while at 34 hours post transduction cells were producing primarily cellular proteins, there were four bands visible which migrated to areas of the gel where viral proteins are observed in cultures supporting complete reactivation at 48 hours post infection (Figure 3.2.1).

Upon analysis of viral antigen expression at various timepoints post reactivation, it was found that by 24 hours, ICP0 was not being produced in either the quiescent or reactivated cultures and levels of ICP4 in the reactivated cultures were similar to those observed in quiescently infected cultures (Figure 3.2.2), indicating that the virus had not yet reactivated. The fact that ICP4 was expressed during quiescence correlated with the results garnered in (Figure 3.1.12). At 34 hours post reactivation, there was robust ICP0 accumulation which coincided with the expression of immediate early ICP4 and processed ICP22. This, along with expression of the leaky late protein ICP5 suggested that the viral genomic replication was progressing, and when Us11 was analyzed it was found that small amounts of these proteins were already being produced. By 48 hours post reactivation all viral antigens analyzed were being robustly expressed. These results suggested that the initial processes of reactivation occurred upon ICP0 production somewhere between 24 and 34 hours post reactivation (Figure 3.2.2). It was likely that the population of quiescent virus reactivated in a relatively synchronous manner as immunofluorescence analysis of reactivating cultures showed no viral antigen staining at 24 hour post reactivation whereas by 30 hours post transduction approximately 40% of the cultures were expressing both ICP4 and ICP5 (Figure 3.2.3).

With at least 40% of the culture reactivating by 30 hours post infection, it was possible to observe changes in signalling activities for the duration of the initial stages of reactivation. Western blot characterization of p38 and ERK activity in quiescent cultures showed that although p38 was unaffected, surprisingly ERK activity was suppressed relative to mock infected cultures suggesting that quiescence has a suppressive effect on this pathway. Conversely, in reactivated cultures it was found that MEK-ERK signalling was modestly stimulated during reactivation from a non-productive state at the 34 hour time point. As the reactivation progressed ERK activity was suppressed and p38 stimulated most likely due to the production of ICP27 (Gillis, Okagaki & Rice 2009; Hargett, McLean & Bachenheimer 2005). The maintenance of ERK activity at early stages of infection was surprising given it has no role in HSV-1 lytic replication, as NHDFs that had been subjected to a mock quiescent infection i.e serum starvation and temperature elevation prior to a lytic infection at 37°C showed that ERK activity was not required and was in fact repressed during lytic replication (Figure 3.2.5). This result was in agreement with previous reports which have shown

that the activity of host MEK-ERK signalling pathways are stifled during lytic infection within a number of cell lines and ERK inhibitors do not suppress lytic replication (McLean, Bachenheimer 1999; Walsh, Mohr 2004; Sloan *et al*., 2006; Santamaría *et al*., 2009).

These differences in MEK-ERK signalling activities during lytic replication, quiescence or reactivation from quiescence suggested that distinct contextual requirements exist for ERK depending on the stage of HSV-1 infection. This was confirmed when quiescent virus was controllably reactivated by Ad-0 transduction in the presence of either MEK-ERK or p38 pathway inhibitors to see what affects these drugs would have on viral antigen accumulation.

The presence of U0126 caused a reduction of ICP5, ICP22 and Us11 at both 34 hours and 48 hours post transduction whereas SB203580 had no effect on antigen accumulation (Figure 3.2.7). This reduction of antigen accumulation by ERK inhibition was also seen in cells reactivated in the presence of human serum containing neutralizing antibodies, indicating that the reduction observed was not caused by a inhibition of secondary spread to uninfected cells within the culture (Figure 3.2.10).

The capacity of U0126 to suppress viral reactivation was also confirmed by immunofluorescence which showed that the amount of cells staining positive for both ICP5 and ICP4 was reduced in cultures reactivated in the presence of U0126 relative to cells reactivated in the presence of DMSO (Figure 3.2.8). Additionally, it was discovered that when viral progeny production was quantified from Ad-0 reactivated cultures, in contrast to SB203580, U0126 caused a 20 fold reduction of virus production compared to DMSO treated samples (Figure 3.2.9).

Whereas controllable reactivation is facilitated by transduction with adeno viral vectors coding for ICP0, a key feature of our quiescence model was that it also allowed the study of spontaneous reactivation, which most likely reflects more accurately the processes of reactivation in-vivo. This aspect of the model also allowed us to discount that the suppression observed was not due to a defect in Ad-0 transduction caused by UO126. Consequently, the affect of ERK inhibition on viral accumulation was investigated and was found to have an even more robust effect on spontaneous reactivation than controllable reactivation (Figure 3.2.12). Spontaneous

reactivation of HSV-1 in cells treated with either inhibitor showed that expression of both ICP0 and Us11 was reduced by both U0126 and SB503580. Furthermore it was found that U0126 significantly reduced spontaneous reactivation relative to DMSO with a 1200 fold reduction of infectious progeny and SB508230 had a 1000 fold reduction (Figure 3.2.13). In addition, immunofluorescence analysis illustrated that ICP5 expression was not evident in cultures reactivated in the presence of U0126 or SB203580 (Figure 3.2.14).

Although HSV-2 encodes ICP10, a ribonucleotide reductase (R1) protein that blocks apoptosis in cultured hippocampal neurons by activating the extracellular signal-regulated kinase (ERK) survival pathway (Perkins *et al.*, 2002) no reports have shown that its homologe in HSV-1 (ICP6) has a similar effect on ERK signalling. Whether an as-yet unidentified viral protein can activate ERK specifically during the reactivation process is unclear, but it is likely that the ERK phosphorylation observed at 34 hours post reactivation reflects metabolic changes in the host cell during reactivation of the dormant viral genomes. Interestingly, microarray studies have shown that the onset of HSV-1 reactivation elicits a wide range of responses in the host cell, identifying MAPKs, including ERKs as being rapidly induced upon reactivation in ganglia explanted from latently-infected animals (Singer *et al*., 1998; Tsavachidou *et al.*, 2001; Hill *et al*., 2001; Kent, Fraser 2005; Schang, Bantly & Schaffer 2002). These reports would seem to suggest that cellular responses are exploited by the virus to facilitate early events in the reactivation of the repressed genome. Indeed, during reactivation of HSV-1 within explanted ganglia both Cdk1/2 have been shown to be induced (Schang, Bantly & Schaffer 2002). Interestingly, Cdk1 has recently been shown to activate ERK and studies have also reported ERK dependent phosphorylation of Cdk2, suggesting that complicated feedback loops exist between Cdk activity and ERK signalling (Lents *et al*., 2002; Borysov, Guadagno 2008). Also it has been reported that the cyclin-dependent kinase inhibitor roscovitine inhibits the transactivating activity and alters the posttranslational modification of ICP0 (Davido, Leib & Schaffer 2002). Additionally, ERK activity has been shown to be involved in the reactivation of other herpes viruses (Chang *et al*., 2006; Ford *et al.*, 2006; Fahmi *et al*., 2000; Fukuda *et al*., 2002), and a number of chemical treatments understood to reactivate HSV-1 both in-vitro and in vivo are known to activate ERK signalling (Smith *et al*., 1992; Frost *et al*., 1994; Bartoli *et al*., 2003; Danaher *et al*.,

2005; Terry-Allison, Smith & DeLuca 2007; Wang *et al*., 2008). These findings illustrate that although the exact contributive mechanisms for HSV-1 reactivation remain unclear, they may be analogous to the role played by TPA mediated reactivation of KSHV (Cohen, Brodie & Sarid 2006). While it remains to be elucidated how ERK is activated, our results demonstrate its functional role in the initial stages of HSV-1 reactivation.

The requirement for activated p38 during the initial stages of lytic infection has been demonstrated during previous studies (Zachos, Clements & Conner 1999; Hargett, McLean & Bachenheimer 2005; Hargett, McLean & Bachenheimer 2005). During the characterization of HSV-1 reactivation in this study it was found that p38 was activated at 34 hours post transduction with Ad-0 and this activation of p38 coincided with the expression of early and mid phase proteins. Although this pathway is not essentially required during viral replication during high multiplicity infection, it does play a role during low level lytic replication and spread (Walsh, Mohr 2004). In agreement with these previous findings, our results demonstrated that p38 did not play a significant role in the reactivation process under conditions of efficient reactivation when the culture was transduced with Ad-0. However, when spontaneous reactivation was allowed to occur, a process resulting in asynchronous disparate reactivation, where low levels of reactivation and secondary spread occurs randomly and inefficiently throughout the culture, it was observed that p38 inactivation resulted in the reduction of viral antigen accumulation and infectious virus production within the culture.

This disparity between the ability of HSV-1 to replicate during controlled reactivation and the inability to accumulate virus during spontaneous reactivation when in the presence of SB203580 likely reflects the need for p38 kinase activity during low multiplicity lytic spread but also may be due to direct effects on virus reactivation. As spontaneous reactivation is a highly inefficient process compared to that of Ad-0 mediated reactivation, p38 might play a subtle but important role in the biology of HSV-1 during sub optimal conditions. Indeed, p38 is activated by a variety of stimuli in vivo, with stress being the main contributory factor (Kumar, Boehm & Lee 2003). As spontaneous reactivation is a dynamic event, it is frequently occurring in vivo but contained by the hosts immune system thus preventing frequent symptomatic lytic

replication in mucosal epithelia (Feldman *et al*., 2002; Sacks *et al*., 2004; Gilbert 2006; Margolis *et al*., 2007). Consequently, under conditions of physiological stress it is thought that a reduction in immune function alongside an increase in the efficiency of spontaneous reactivation can result in a greater chance of opportunistic re-entry into the productive stage viral replication and p38 may play a subtle but important role in this process. It may also be possible that the processes governing spontaneous and controllable reactivation differ. Indeed, the pattern of gene expression observed during primary lytic infection has been reported to be at variance from that of reactivation from quiescence (Danaher, Jacob & Miller October 2006). Additionally it has been observed that chemical treatments and various HSV-1 viral proteins can trigger reactivation both in-vitro and in-vivo (Halford *et al*., 2001; Miller, Danaher & Jacob 2006; Preston 2007), suggesting that the virus can exploit many different environmental cues to initiate reactivation through different routes. Indeed, evidence that ICP0 is not required for reactivation in vivo has been reported previously (Thompson, Sawtell 2006). Therefore if spontaneous reactivation occurs through a process which is distinct from the processes involved in ICP0 controllable reactivation, the fact that U0126 suppresses both spontaneous and controlled reactivation adds emphasis to the central importance of MEK-ERK signalling during HSV-1 reactivation, and possible roles for p38 in distinct processes.

4.2.2 The role of Mnk and mTORC1 in HSV-1 reactivation.

As described previously, HSV-1 increases mTORC1 and p38 cell signal pathway activities during lytic infection (Walsh, Mohr 2004; Walsh, Mohr 2006). We had also demonstrated that both ERK and p38 were required during spontaneous reactivation from quiescence. Considering that these kinases both have a function in phosphorylating the Mnk1 kinase which is known to phosphorylate eIF4E and enhances translation (Bianchini *et al*., 2008), we decided to inhibit Mnk and mTORC1, a serine/threonine protein kinase whose substrates are p70-S6 Kinase 1 (S6K1) and 4E-BP1 during virus reactivation. By targeting both kinases we hoped clarify if the downstream targets of ERK, p38 and mTORC1 that regulate translation were required for reactivation from quiescence.

Upon reactivation in the presence of either the Mnk1 inhibitor, CGP57380, or the mTORC1 inhibitor, Rapamycin, it was discovered that both kinase inhibitors

suppressed controlled and spontaneous reactivation from quiescence (Figure 3.2.16, 3.2.17). Rapamycin proved to be the more potent inhibitor of the two, suggesting that the disruption of eIF4E-eIF4G by 4E-BP1 sequestration of eIF4E reduced accumulation of viral antigens and infectious progeny production during reactivation more so than the inhibition of eIF4E phosphorylation, and agrees with the commonly held understanding of eIF4E phosphorylation having a more subtle regulatory function compared to sequestration of eIF4E by 4E-BP. This suggested that targeting the formation of the eIF4F complex directly may be a viable therapeutic target in preventing HSV-1 viral reactivation.

4.3 The role of initiation factors in HSV-1 reactivation

The importance of translation as an essential mechanism in the conversion of genes to protein for either host cells or invading viruses cannot be understated. The convergence of dependence is evident by the competition for translation factors between host cells and virus during infection. Due to this dependence on translation for both cell viability and productive replication of virus, questions relating to the suitability of targeting translation therapeutically to inhibit viral replication have remained unclear. Over the past decades our understanding of the mechanistics of translation regulation, and consequently, the affects of its deregulation has improved greatly. The result of this understanding has been the development of small molecule inhibitors which suppress translation initiation. These inhibitors have included Rapamycin, which binds the cytosolic protein FK-binding protein 12 (FKBP12) and inhibits mTORC1 (Dowling *et al*., 2010), Torin1, which is a highly potent and selective ATP-competitive mTORC inhibitor that directly inhibits both mTORC1 and mTORC2 complexes and impairs cell growth and proliferation to a far greater degree than Rapamycin (Thoreen *et al*., 2009) and NSC119889, a compound that prevents the association of eIF2 with Met-tRNA$_i^{Met}$ (Robert *et al*., 2006).

As our understanding of the detailed mechanistics of protein synthesis has increased, it has become apparent that mRNAs may have distinct requirements for initiation factors to mediate their translation. Previous reports have shown that the degree of complexity in the 5' UTRs of mRNAs may play an important role in whether their translation is affected when eIF4F is modulated. Structurally complex mRNAs have been found to be dependant on high levels of functionally active eIF4F while abundant housekeeping mRNA with little 5' UTR complexity are relatively insensitive to modulations in the activity of this complex (Coldwell, Morley 2006; Ramírez-Valle *et al*., 2008). Additionally, investigations into the dispensability of components within eIF4F have shown that many interactions within the complex are not required to maintain significant rates of global protein synthesis, indicating that this complex could play more a regulatory rather than essential role in translation (Hinton *et al*., 2007). With these developments in understanding, the potential for suppressing tumor growth through inhibition of eIF4F in a safe manner has gained recognition and driven the search for small molecules that exclusively target this

complex. One of the most promising inhibitors found recently was 4EGi-1. 4EGi-1 was discovered by high-throughput in vitro screening for molecules that can bind to eIF4E, and has been identified as a drug that could potentially prevent the interaction between eIF4G and eIF4E (Moerke *et al*., 2007). As cancer cells have been shown to rely on high rates of translation, this small molecule inhibitor has been proposed as a potential anti cancer drug.

Given recent findings which showed that DNA viruses stimulate eIF4F we wanted to test the effects of 4EGi-1 on viral replication (Walsh, Mohr 2004; Walsh *et al*., 2008; Arias *et al*., 2009; Castella *et al*., 2009). However, the potential effects of 4EGi-1 with regard to ongoing translation in normal cells had not been investigated and therefore must be considered if it is to be used therapeutically. With this in mind we endeavoured to investigate the effects of translation inhibition with regard cell stress tolerance, cell viability and finally what effect this drug had on replication of both the HSV-1 and Vacinna virus.

During our initial characterisation of 4EGi-1, metabolic labelling experiments of cells which had been treated with increasing concentrations of 4EGi-1 showed that the effective concentration to suppress translation below detectable levels was between 30 and 50μM. This concentration was much lower than that used in previous reports which showed that concentrations between 50 and 100μM and even higher were needed to disrupt eIF4F complex formation (Moerke *et al*., 2007; Fan *et al*., 2010).
The characterisation of initiation factor abundance and of the activity of cell signalling pathways which impart translational control illustrated that 40μM 4EGi-1 does not alter the levels of eIF4E, PABP or eIF4G and both the Mnk regulating kinases: ERK and p38 pathways remain unaffected during translational suppression (Figure 3.3.2). Interestingly the mTOR substrate: p70S6K, was slightly stimulated and may represent a failsafe mechanism whereby when translation is suppressed to below a certain threshold a feedback loop involving mTOR is activated. In agreement with this explanation is a report showing that upon cycloheximide treatment mTOR1 signalling is activated and that the change in mTOR signalling was inversely proportional to alterations in the expression of the short lived mTORC1 repressor, REDD1 (Kimball *et al*., 2008). In addition to p70S6K activation, a small increase in the phosphorylation of 4E-BP1 was also observed. The fact that p70S6K was more

stimulated than 4E-BP1 could be due to the fact that 4EGi-1 was reported in the initial characterisation paper to cause an accumulation of 4E-BP1 on eIF4E. This tripartate interaction may prevent 4E-BP1 phosphorylation and thus explain why one substrate of mTOR1 is more phosphorylated than the other. In addition, the regulator of global translation initiation (eIF2α) was assessed and found to have a phosphorylation profile identical to DMSO treated samples. This along with the absence of p38 activation suggested that even though the cells were translationally suppressed, cellular stress was not occurring.

eIF4E phosphorylation was also analysed as an indicator of 4EGi-1's capacity to prevent eIF4E/ eIF4G binding and iso-electric focusing demonstrated that 4EGi-1 had no effect on eIF4E phosphorylation (Figure 3.3.2). This result indicated that eIF4E/ eIF4G binding was still occurring and during subsequent cap pull-down experiments in both NHDF and Hela cells it was shown that 4EGi-1 did not prevent eIF4E/ eIF4G binding to any large degree.

4EGi-1 at concentrations sufficient to inhibit global translation was found to modestly stabilize the interaction of 4E-BP1 with eIF4E but did not affect the binding of eIF4G to any significant degree (Figure 3.3.3). In line with these data are numerous studies which have shown that modest changes in 4E-BP1 binding to eIF4E are insufficient to elicit changes in eIF4F levels in many cell types, including NHDFs (Walsh, Mohr 2004). This is most likely due to the differences in the relative amounts of each of these proteins. In agreement with this was the fact that high 4EGi-1 concentrations were previously reported necessary to achieve notable eIF4F disruption (Moerke *et al*., 2007). During our initial experiments, disruption of eIF4F was evident in NHDFs at higher 4EGi-1 concentrations but was coincident with the onset of cytotoxicity. Therefore it is unknown whether the drug was disrupting eIF4F or if the disruption observed was due processes associated with cell death. Taken together our findings demonstrated that 4EGi-1 suppressed translation at concentrations below the concentrations needed to cause a disruption to eIF4F. .Indeed, a reduction of eIF4F levels does not necessarily confer a reduction of translation rates in cells, as during cap pull-down analysis of initiation complexes from cells which had been treated with Torin1, it was found that Torin1 treatment caused a robust dephosphorylation of 4E-

BP1 in input samples, which correlated with a large increase in the association of 4E-BP1 with eIF4E and an associated loss of eIF4E-eIF4G binding, resulting in a robust disruption of eIF4F. However, Torin1 only had a modest effect on translation rates relative to 4EGi-1 treated NHDF and Hela cells (Figure 3.3.6) and was consistent with recent reports which documented the effects of Torin1 on eIF4F and viral protein production in Herpesvirus infected cells (Moorman, Shenk 2010). These results seem to indicate that when 4EGi-1 is used at the lower concentrations it may confer small fluctuations in eIF4F at undetectable levels, which may have specific effects on the translation of structurally complex mRNAs (Moerke *et al*., 2007), but the powerful effects of this inhibitor on global translation appear to be largely caused by an alternative mechanism independent of the eIF4E-eIF4G interaction.

It was shown in previous reports that 4EGi-1 disrupted eIF4F complexes in vitro and in vivo, but at relatively high concentrations (Moerke *et al*., 2007; Fan *et al*., 2010). Although a number of properties of this compound were elucidated during its initial characterization, questions of whether 4EGi-1 may have auxiliary effects on the processes of translation arose due to the facts that it inhibited translation and reduced the growth of cell lines at concentrations lower than those required to disrupt eIF4F. Additionally, 4EGi-1 affected the translation of mRNAs containing reporter constructs which employ Internal Ribosome Entry Sites from Encephelomyocarditis Virus (EMCV) or Hepatitis C Virus (HCV) and therefore do not require eIF4F but did not affect the translation of a mRNA containing the Cricket Paralysis Virus (CrPV) IRES, which is distinct from both the EMCV and HCV IRES elements in that it drives ribosome recruitment and translation initiation in a manner that is independent of both eIF4F and eIF2 (Sarnow, Cevallos 2005; Wilson et al. 2000).

We therefore examined the levels of other components of the translation initiation complex that forms on eIF4F. It was found that 4EGi-1 had no effect on the abundance or association of eIF3A, a component of the functional core of the eIF3 complex (Masutani *et al*., 2007), but did increase the binding of the 40S ribosomal protein, RPS3. In addition to the aforementioned proteins, eIF2α was examined due to its central role in regulating global protein synthesis. Similar to whole cell extracts (Figure 3.3.2), input samples demonstrated that 4EGi-1 did not induce eIF2α phosphorylation (Figure 3.3.3). However, large amounts of phosphorylated eIF2α

were found in complexes isolated from 4EGi-1-treated cultures. Overall these findings suggested that this compound increased the association of ribosomal complexes containing phosphorylated inactive eIF2 with eIF4F.

A recent report demonstrated that 100μM 4EGi-1 inhibited translation in HeLa cells with only a small induction of cellular stress over an 8 hour period, but the levels of eIF4F complex formation were not investigated (Mokas *et al*., 2009). We therefore treated HeLa cells with 60μM 4EGi-1 and first confirmed that this concentration also suppressed translation. Subsequently it was found that the composition of initiation complexes in HeLa cells treated 60μM 4EGi-1 for 4 hours were similar to the those found in NHDFs, the levels of translation initiation factors and RPS3, as well as the phosphorylation of eIF2α in input samples remained unaffected in 4EGi-1-treated samples (Figure 3.3.4), whereas analysis of 7-Methyl GTP-bound complexes demonstrated that 4EGi-1 had no significant effect on the association of eIF4E with eIF4G, or with eIF3A, but did increase the association of RPS3 and phosphorylated eIF2α, suggesting that phosphorylated eIF2α accumulation was not cell-type specific. This effect was also observed in samples treated with 4EGi-1 purchased from an independent source (Figure 3.3.5), demonstrating that the effects were not limited to 4EGi-1 sourced from Calbiochem.

While the exact mechanisms behind the accumulation of phosphorylated eIF2α remain unclear, this occurrence may explain how 4EGi-1 inhibited the translation of mRNAs containing reporter constructs driven by Encephelomyocarditis Virus (EMCV) or Hepatitis C Virus while being unable to prevent translation of mRNA's containing the Cricket Paralysis Virus (CrPV) IRES. It also may explain why this inhibitor supresses translation and reduces growth in cells at relatively low concentrations. Indeed, a previous report has shown that 4EGi-1 potently inhibited global translation in HeLa cells and resulted in the accumulation of 80S ribosomes, which was not observed when eIF4E levels were experimentally reduced (Mokas *et al*., 2009). 4EGi-1 may cause defects in the dissociation and recyling of both eIF4 and eIF2 that normally occurs during ribosome assembly and translation initiation, or alternatively may increase the abundance of inactive eIF2-bound ribosomes. Potential effects on correct eIF2 translocation and release during the formation of the 80S ribosome and the onset of translation might also fit with the polysome profiles of

Mokas and colleagues (Mokas *et al*., 2009). While it remains to be determined how 4EGi-1 mediates this effect, it may be analogous to how 4EGi-1 stabilizes the eIF4E-4EBP-1 interaction. This finding offers an explanation why this inhibitor affects global translation and cell growth at concentrations below those needed to disrupt eIF4F. As such, this is an important functional consideration if 4EGi-1 is to be considered a as tool to study eIF4F as a therapeutic agent.

During the initial characterisation it was observed that extensive dialysis of 4EGi-1 reverses its interaction with eIF4E, but the reversibility of its effects on translation had not been examined (Moerke *et al*., 2007). During our investigations we discovered that translation was restored in NHDFs upon removal of 4EGi-1 as illustrated in figure 3.3.10, which shows the amount of [^{35}S]-Methionine/Cysteine incorporated into proteins increased with increasing labelling time and was potently reduced in the continuous presence of 4EGi-1. However, in cultures where the inhibitor had been washed out, only a very small reduction in [^{35}S]-Methionine/Cysteine incorporation was observed after 10 minutes labelling, while after 30 minutes no significant differences were observed (Figure 3.3.5),, illustrating that the effects of 4EGi-1 were very rapidly reversible in cultured cells. Given the reversibility of effects on translation upon 4EGI-1 removal we examined whether its effect on RPS3 and eIF2α association with cap complexes were also reversible and found that the increased association of RPS3 and phosphorylated eIF2α was also reversed when 4EGI-1 was omitted from buffers during the final wash stages of the assay after recovery of cap-bound initiation complexes. Overall, these findings demonstrate that the effects of 4EGi-1 on both cellular translation and the composition of initiation complexes are rapidly reversible upon drug removal.

As 4EGi-1 had no obvious effects on NHDF morphology after 1 day, we then examined more prolonged exposure to this inhibitor. Previous studies demonstrated that 4EGi-1 inhibited cell growth or induced apoptosis in different cell types over 3-7 day periods (Moerke *et al*., 2007; Tamburini *et al*., 2009), suggesting that this inhibitor is relatively stable in culture. In agreement with these reports, when low density cultures of NHDFs were treated with 40μM 4EGi-1 it was found that the drug completely inhibited cell growth at least 3 days (Figure 3.3.11). When samples were

metabolically labelled after 4 hours, 1 day and 8 days, TCA precipitation demonstrated that rates of translation were reduced to 5% of control samples by 3-4 hours post-treatment while continued exposure to 4EGi-1 further reduced rates to 1% of control samples at the 8 day time point. The degree and global nature of translational suppression at 8 days post-treatment was evident on autoradiographs of samples resolved by SDS-PAGE but despite this, cell viability, as determined by trypan blue exclusion remained unaffected after 8 days (Figure 3.3.13). In addition, the structural integrity of both the cell and the actin cytoskeleton in 4EGi-1 treated cultures were found to be similar after 8 days of treatment as the DMSO treated control cultures (Figure 3.3.15), although the intensity of staining in inhibitor-treated cells was modestly decreased, in line with general decreases in protein abundance in these cells (Figure 3.3.14). In addition, removal of 4EGi-1 from cultures resulted in a substantial restoration of translation rates within just 30 minutes, demonstrating that cells remained viable and the effects of this inhibitor remained reversible.

The cytotoxicity observed when cells are treated with higher concentrations of 4EGi-1 suggested that primary cells can exist with a low basal level of protein production but below that threshold the cell cannot sustain viability. Indeed, above this threshold it was found that cells not only remain viable but retained the ability to tolerate stress as evident by Hsp70 accumulation in 4EGi-1 cells exposed to distinct chemical and thermal stresses. 4EGi-1-treatment in the absence of stress had no effect on Hsp70 expression, further demonstrating that 4EGi-1 does not cause cellular stress at this low concentrations and that Hsp70 accumulation is not sensitive to inhibition of global translation. Therefore Hsp70 accumulation is likely to be controlled by additional mechanisms during stress responses. Interestingly, translation of Hsp27 and Hsp70 mRNAs have been shown to be enhanced rather than repressed when eIF4F components are depleted in HeLa cells (Joshi-Barve, De Benedetti & Rhoads 1992). As 4EGi-1 did impair Hsp27 accumulation (Figure 3.3.16, 3.3.17), this further suggests that 4EGi-1 affects Hsp27 accumulation in an eIF4F-independent manner. Interestingly the accumulation of Hsp70 that was seen in 4EGi-1 treated cells during stress could be explained by a previous report that demonstrated selective synthesis of Hsp70 occuring during stress conditions which is facilitated by sequences specifically found in the 5’ UTR of Hsp70 mRNA (Yueh, Schneider 2000). These sequences may confer an advantage in competing for low levels of active initiation complexes.

Alternatively, the accumulation of Heat shock proteins may be the result of alterations in protein stability, or conversely, increases in the expression or stability of Hsp70 mRNA. The latter would give Hsp70 mRNA an advantage over other transcripts to compete for initiation complexes available in the cell. In any case, these findings demonstrated that extensive exposure to 4EGi-1 did not adversely affect the cells overall capacity to tolerate diverse stress conditions but it did alter the accumulation of specific Hsps, this raised the possibility that the drug may be a viable therapeutic to suppress viral replication given their dependence on host translation.

Currently, the only antiviral treatments presently available to treat HSV-1 are based on conversion of nucleotide analogues to cytotoxic forms by the viral Thymidine Kinase (Elion 1993). Consequently, the selective pressure of widespread use has caused the emergence of drug-resistant strains. As HSV-1 replication depends on the host cells translational machinery for production of viral proteins, manipulation of mRNA translation may prove an attractive antiviral strategy. Indeed, the inhibitors of Mnk and mTOR which reduce the capacity of the cell to translate mRNAs were recently shown to reduce herpes virus lytic replication and viral reactivation from quiescence, which suggests the importance for eIF4F activity during both stages of viral lifecycles (Walsh, Mohr 2004; Walsh *et al*., 2005; Arias et al. 2009; Moorman, Shenk 2010).

Upon investigation of the antiviral potential of targeting translation it was discovered that 4EGi-1 robustly inhibits both spontaneous and controlled reactivation of HSV-1 from quiescence as illustrated by readily detectable levels of early (ICP4), mid (ICP5) and late (Us11) stages proteins in samples from DMSO-treated cultures while levels of these proteins remained completely undetectable in 4EGi-1-treated samples (Figure 3.3.20). It was also found that the levels of infectious progeny produced during controlled reactivation were undetectable in cultures treated with 4EGi-1 (Figure 3.3.21).

In contrast to the process of virus reactivation from a quiescent state within the cell, infecting viral particles that establish a productive lytic infection contain within the tegument layer a plethora of viral proteins whose various functions manipulate the host cell environment to ensure the rapid onset of efficient viral replication (Karupiah

2002). We therefore tested the efficacy of 4EGi-1 against the lytic phase of HSV-1 infection and discovered that while 20μM 4EGi-1 had only modest effects, 30μM and 40μM concentrations caused an increasingly effective suppression of viral protein synthesis. When the abundance of viral antigens was analysed in the same samples the accumulation of HSV-1 proteins was reduced in a dose-dependent manner, reaching undetectable levels at 40μM 4EGi-1 (Figure 3.3.24). The effects on antigen accumulation were reflected in the levels of virus production in inhibitor-treated cultures infected at m.o.i. 5 for 11 hours, with those in 40μM 4EGi-1-treated cultures declining to just 400 infectious particles per culture, a 1 million fold reduction compared to DMSO controls (Figure 3.3.25). Additionally, when 4EGi-1 was added to cultures mid-way through infection it potently suppressed protein synthesis and reduced the accumulation of late proteins which are dependent upon ongoing translation. This demonstrates that the inhibitor was capable of interfering with protein synthesis even in an established infection and that its effects were not due to defects in viral entry.

Although 4EGi-1 potently suppressed rates of protein synthesis, some viral protein synthesis was evident when 4EGi-1 was added at very late in infection. HSV-1 encodes numerous proteins that interact with eIF4G, eIF4A, PABP and ribosomal proteins in addition to encoding proteins capable of modulating both the activity of eIF2 kinases and directly influencing phosphate turnover on eIF2 (Diaz *et al.*, 1993; Feng, Everly & Read 2001; Fontaine-Rodriguez *et al.*, 2004; Larralde *et al.*, 2006; Walsh, Mohr 2006). Future work might aim to elucidate whether any of these proteins might partially lessen the effects of 4EGi-1 at late stages of infection.

To determine the contribution of eIF4F to HSV-1 protein synthesis and accumulation, we then examined the effects of Torin1 during infection. In agreement with previous reports (Walsh, Mohr 2004), HSV-1 activated mTOR and induced phosphorylation of 4E-BP1 in input samples and resulted in a loss of 4E-BP1 associated with eIF4E in cap-bound samples (Figure 3.3.26). In contrast, Torin1 treatment resulted in a robust dephosphorylation of 4E-BP1. This was accompanied by a robust decrease in eIF4G binding to eIF4E compared to the DMSO-treated control (Figure 3.3.26). Upon analysis of effects of Torin1 on rates of host and viral protein synthesis metabolic labelling illustrated that Torin1 reduced rates of translation in both mock and infected

samples to comparable degrees, suggesting that host and viral mRNAs have a similar requirement for eIF4F activity. However, the extent of repression was not as dramatic as that observed for 4EGi-1 (Figure. 3.3.28).

When a comparison analysis on the effects of both inhibitors was conducted in relation to the production of viral proteins, it was found that Torin1 caused a notable reduction in the accumulation of viral immediate early proteins and late proteins detected with anti- HSV-1 antibody. These results were in agreement with a recent report detailing that Torin1 disrupted eIF4F, where it was shown that Torin1 reduced HSV-1 replication by approximately 100-fold (Moorman, Shenk 2010). However, it should be outlined that those experiments were performed in mouse embryo fibroblast cells that were infected at m.o.i. 0.05 for a period of 3 days. Therefore the viral growth defects observed may have been amplified by inefficient secondary spread in Torin1-treated cultures. Nevertheless, our findings are in agreement with this report and suggest that although eIF4F is important for maximal rates of translation, as significant disruption of this complex does not adversely affect HSV-1 protein synthesis to a major degree. In contrast to Torin1, each of the viral antigens tested were undetectable in 4EGi-1-treated samples. HSV-1 infection resulted in mTOR activation and phosphorylation of p70S6K, which was not only inhibited but was reduced to below basal mock-infected levels in the presence of Torin1 (Figure 3.3.28). In addition p70S6K phosphorylation in cells infected with HSV-1 in the presence of 4EGi-1 remained the same as in DMSO or 4EGi-1-treated mock-infected samples, which was most probably the result of the failure of the virus to establish lytic infection and produce the proteins required to activate mTOR.

We also examined whether 4EGi-1 was potentially effective against other viruses. Vaccinia Virus (VacV) is a large, complex, double-stranded DNA enveloped virus belonging to the Poxvirus family and is the laboratory prototype for Poxvirus infection (Knipe *et al.*, 2007). Compared to other human DNA viruses, Poxviruses are unique with regard their life cycle as they replicate exclusively in the cytoplasm of infected cells. To confirm if 4EGi-1 can inhibit the replication of viruses other than HSV-1 we then tested its effects on VacV. Upon treatment of NHDFs with increasing concentrations of 4EGi-1 it was found that while Poxvirus infection of DMSO-treated NHDFs resulted in the characteristic shut-off of host translation and robust synthesis

of Poxvirus polypeptides, a modest inhibition of translation was evident when cells were treated with 10μM 4EGi-1 while concentrations above 20μM resulted in dramatic suppression of translation. TCA precipitation and quantification showed that 30μM and 40μM 4EGi-1 reduced translation rates in infected cultures to 2.4% and 2.3% respectively, of those in DMSO-treated cultures (Figure 3.3.32). Notably, when 4EGi-1 was added to cultures at 12 hours post infection 4EGi-1 inhibited ongoing viral protein synthesis (Figure 3.3.35), demonstrating that its effects were again not due to 4EGi-1 preventing cell entry.

When the effect of this translational suppression on poxvirus replication was assessed it was found that 4EGi-1 reduced virus replication to approximately 3% of control DMSO-treated cultures (Figure 3.3.33), this result correlated with the degree to which 4EGi-1 suppressed rates of viral protein synthesis and demonstrated that 4EGi-1 inhibited virus replication regardless of the multiplicity of infection used. However, although 4EGi-1 potently suppressed VacV replication the degree of suppression was much lower than that observed with HSV-1 (Figure 3.3.25). Indeed, in contrast to HSV-1 antigens, western blotting against VacV antigens demonstrated that the accumulation of viral proteins remained detectable in 4EGi-1- treated samples albeit with significant reductions (Figure 3.3.34).

As mentioned previously, the degree to which 4EGi-1 suppressed translation Poxvirus-infected cells directly correlated with the reduction in infectious virus produced in 4EGi-1-treated cultures. However, although 4EGi-1 reduced translation to similar levels in HSV-1 infected cells it had a disproportionately large effect on viral antigen accumulation and production of infectious virus during HSV-1 infection. While this effect may be caused by a series of unknown negative effects on other processes during HSV-1 infection it may also be due to the fact that Herpesviruses and Poxviruses have lifecycles that are inherently different. For example, poxviruses replicate in the cytoplasm whereas HSV-1 replicates in the nucleus but can also exist in a dormant repressed state mediated by suppressive cellular factors. Upon infection, if the critical regulators of lytic replication are not produced in sufficient quantities these suppressive cellular factors can coerce the virus to a non productive state. Therefore 4EGi-1 may cause inefficient production of these proteins to below a level required to initiate lytic replication. This in collaboration with the cells natural

suppressive mechanisms likely results in an amplification of the inhibitory effects of 4EGi-1 on the HSV-1 lifecycle. Similarly, 4EGi-1 inhibition of HSV-1 reactivation is likely due its ability to suppress de-novo production of ICP0 therefore resulting in a maintained repression of viral genomes which may be similar to the phenonema observed in our quiescence model where low levels of ICP0 produced fail to disperse PML resulting in an inability to establish productive infection. Therefore, 4EGi-1 may be a particularly effective treatment in the repression of infectious agents that depend upon efficient expression of viral proteins to overcome the hosts natural cellular restrictions.

Section 5.0: Conclusions

Conclusions:

The main focus of this thesis was to investigate the roles of host signalling pathways and translation initiation factors during HSV-1 reactivation from quiescence and lytic replication.

To achieve these goals a tissue culture model for studying HSV-1 quiescence was developed. The system utilizes selective pressures of serum starvation of primary normal human diploid fibroblasts and temperature elevation, both of which proved to play an important role in the inhibition of productive infection and establishment of a stable quiescent infection. Approximately 40-60% of the culture can be quiescently infected and reactivated efficiently, which allows for population-wide occurrences to be readily examined. The model can be readily adapted for use with different strains of HSV-1. Additionally, the use of human serum allows for the reduction of secondary spread of virus post reactivation and therefore permits differentiation between mechanistic events associated with reactivation and secondary spread of the virus.

While the reduced metabolic state caused by serum starvation likely creates a sub-optimal environment for efficient viral infection, the role of temperature elevation in this system will prove important for our understanding of cellular mechanisms that repress viral infection. Notably, ICP0, a viral factor critical for establishing productive infection, failed to be produced efficiently during entry into quiescence in our system. Interestingly, it has been reported that Herpes simplex virus 1 is able to hijack the host-cell IkappaB kinase (IKK)/NF-kappaB pathway causing a redirection and recruitment of NF-kappaB to the ICP0 promoter. Various laboratories have documented in vitro and in vivo interactions between the heat shock response (HSR) and NF-kappa B (Amici *et al*., 2006). Alternatively, ICP0 may be unstable at higher temperatures in certain cell types and the degree of sensitivity may be HSV-1 strain-specific. In addition, the failure to process ICP22 may negatively impact on ICP0 production. Therefore, further experimentation to monitor these phenomena using our model may help to uncover why a block in ICP0 production occurs in heat shocked primary human cells.

Additionally the role of heat shock proteins in reducing cellular stress during viral infection may be further examined using siRNA knock-down of specific hsps. In addition, small molecule inhibitors of Hsp function might be possible to use to further examine their role in this system.

The characterisation of signalling pathways required for efficient reactivation showed that ERK activity was moderately stimulated during reactivation relative to quiescently-infected cells and in contrast to previous studies of ERK suppression during lytic infection. Indeed, inhibition of ERK failed to affect lytic infection but resulted in a suppression of viral reactivation, suggesting a specific role for ERK in the reactivation process. Considering that there was a suppression in ERK activity during quiescence but modest stimulation observed during reactivation, which was in line with previous reports of elevated ERK during reactivation from explanted ganglia, this may represent a cellular response to the reactivation of quiescent virus. As our system allows direct comparison of distinct phases of the viral lifecycle in the same cell type, it would be interesting to conduct parallel microarray and proteomic studies in mock and quiescent NHDF cultures, alongside reactivating cultures in the presence or absence of ERK inhibitors, such as U0126. This may help to determine the role of ERK activity in responding to or altering cellular metabolism during both quiescence and reactivation, and its role in virus reactivation.

Additionally, the activity of the down stream substrate of ERK and p38, Mnk1, along with the mTORC1 substrate 4E-BP, both of which regulate mRNA translation initiation factor eIF4E, were shown to be required for efficient reactivation. These results correlate with studies showing a requirement for efficient translation during HSV-1 lytic replication and highlight its importance in both phases of the virus lifecycle (Walsh, Mohr 2004). In line with this, the viability of directly inhibiting eIF4F as a potential therapeutic target against viral infection was also assessed using the mTORC1/2 inhibitor, Torin1. Results with this inhibitor showed that both host and viral protein synthesis was only modestly affected by disruption of eIF4F, suggesting that viral mRNAs have a similar low-level requirement for eIF4F activity to cellular housekeeping mRNAs.

In contrast to Torin1, cells treated with 4EGi-1, a small molecule reported to inhibit eIF4F formation was also assessed. Experiments showed that translation rates could be reduced to 0.5-1% of normal rates with 4EGi-1 concentrations that did not prevent eIF4F formation. At these relatively low concentrations, increased association of inactive eIF2α with initiation complexes was observed and may be the primary mechanism by which 4EGi-1 functions. Robust translation inhibition did not initiate apoptotic events in normal human cells, which also retained their capacity to mount stress responses in reply to heat shock or proteasome inhibition, suggesting that prolonged 4EGi-1 mediated inhibition of translation but did not confer fragility to primary NHDFs.

Our findings that 4EGi-1 causes increased binding of ribosomal protein and inactive eIF2α to cap-bound complexes suggests that this inhibitor may cause defects in the dissociation and recyling of both eIF4 and eIF2 that normally occurs during ribosome assembly and translation initiation, or alternatively may increase the abundance of inactive eIF2-bound ribosomes. A recent report showed that 4EGi-1 potently inhibited global translation while increasing the accumulation of 80S ribosomes (Mokas 2009). Therefor polysome profiling in addition to immunoprecipitation of 40S and 60s ribsosomal subunits in 4EGi-1 treated cells may help to understand potential effects of this inhibitor on the proper formation and dissociation of factors involved in translation, which may be perturbed in numerous ways by 4EGi-1 to induce its effects.

Importantly, 4EGi-1 powerfully suppressed both lytic replication and reactivation from quiescence. It would be interesting to investigate whether the lack of viral protein synthesis during lytic infection allows for suppressive cellular mechanisms that exist in the nucleus to silence the virus and force it into quiescence. The reorganisation of PML structures that were seen in the establishment of the quiescence model may also be observed during lytic infection in the presence of 4EGi-1 by immunofluorescence. Additionally, as 4EGi-1 is reversible, it would be interesting to determine if HSV-1 replication resumes upon wash-out of inhibitor after infection. This may occur rapidly or gradually, similar to spontaneous reactivation.

Overall, our findings show that inhibition of cellular signalling pathways that regulate eIF4F function or disruption of eIF4F itself only modestly reduce virus reactivation or lytic replication. However, 4EGi-1 mediated potent translational suppression and inhibits infection by both Herpesviruses and Poxviruses. As such, this may represent a potent, non-cytotoxic antiviral approach.

Section 6.0: References

Ahmed, M., Lock, M., Miller, C.G. & Fraser, N.W. 2002, "Regions of the Herpes Simplex Virus Type 1 Latency-Associated Transcript That Protect Cells from Apoptosis In Vitro and Protect Neuronal Cells In Vivo", *The Journal of Virology,* vol. 76, no. 2, pp. 717-729.

Allaudeen, H.S., Kozarich, J.W., Bertino, J.R. & De Clercq, E. 1981, "On the mechanism of selective inhibition of herpesvirus replication by (E)-5-(2-bromovinyl)-2'-deoxyuridine", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 78, no. 5, pp. 2698-2702.

Amici, C., Rossi, A., Costanzo, A., Ciafrè, S., Marinari, B., Balsamo, M., Levrero, M. & Santoro, M.G. 2006, "Herpes Simplex Virus Disrupts NF-κB Regulation by Blocking Its Recruitment on the IκBα Promoter and Directing the Factor on Viral Genes", *Journal of Biological Chemistry,* vol. 281, no. 11, pp. 7110-7117.

Arias, C., Walsh, D., Harbell, J., Wilson, A.C. & Mohr, I. 2009, "Activation of Host Translational Control Pathways by a Viral Developmental Switch", *PLoS Pathog,* vol. 5, no. 3, pp. e1000334.

Arthur, J.L., Scarpini, C.G., Connor, V., Lachmann, R.H., Tolkovsky, A.M. & Efstathiou, S. 2001, "Herpes Simplex Virus Type 1 Promoter Activity during Latency Establishment, Maintenance, and Reactivation in Primary Dorsal Root Neurons In Vitro", *The Journal of Virology,* vol. 75, no. 8, pp. 3885-3895.

Asano, K., Krishnamoorthy, T., Phan, L., Pavitt, G.D. & Hinnebusch, A.G. 1999, "Conserved bipartite motifs in yeast eIF5 and eIF2B[epsi], GTPase-activating and GDP-GTP exchange factors in translation initiation, mediate binding to their common substrate eIF2", *The EMBO journal,* vol. 18, no. 6, pp. 1673-1688.

Atanasiu, D., Kent, J.R., Gartner, J.J. & Fraser, N.W. 2006, "The stable 2-kb LAT intron of herpes simplex stimulates the expression of heat shock proteins and protects cells from stress", *Virology,* vol. 350, no. 1, pp. 26-33.

Avruch, J., Khokhlatchev, A., Kyriakis, J.M., Luo, Z., Tzivion, G., Vavvas, D. & Zhang, X. 2001, "Ras Activation of the Raf Kinase: Tyrosine Kinase Recruitment of the MAP Kinase Cascade", *Recent progress in hormone research,* vol. 56, no. 1, pp. 127-156.

Azwa, A. & Barton, S.E. October 2009, "Aspects of herpes simplex virus: a clinical review", *Journal of Family Planning and Reproductive Health Care,* vol. 35, pp. 237-242(6).

Baim, S.B., Pietras, D.F., Eustice, D.C. & Sherman, F. 1985, "A mutation allowing an mRNA secondary structure diminishes translation of Saccharomyces cerevisiae iso-1-cytochrome c.", *Molecular and cellular biology,* vol. 5, no. 8, pp. 1839-1846.

Banerjee, A.K. 1980, "5'-terminal cap structure in eucaryotic messenger ribonucleic acids.", *Microbiology and Molecular Biology Reviews,* vol. 44, no. 2, pp. 175-205.

Barklie Clements, J., Watson, R.J. & Wilkie, N.M. 1977, "Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome" *Cell,* vol, 12, no 1, pp 275-285.

Bartoli, A., Fettucciari, K., Fetriconi, I., Rosati, E., Ianni, M.D., Tabilio, A., Delfino, D.V., Rossi, R. & Marconi, P. 2003, "Effect of trichostatin a and 5′-azacytidine on transgene reactivation in U937 transduced cells", *Pharmacological Research,* vol. 48, no. 1, pp. 111-118.

Bastian, T.W. & Rice, S.A. 2009, "Identification of Sequences in Herpes Simplex Virus Type 1 ICP22 That Influence RNA Polymerase II Modification and Viral Late Gene Expression", *The Journal of Virology,* vol. 83, no. 1, pp. 128-139.

Belsham, G. & Sonenberg, N. 1996, "RNA-protein interactions in regulation of picornavirus RNA translation", *Microbiology and Molecular Biology Reviews,* vol. 60, no. 3, pp. 499-511.

Bergmann, M., Garcia-Sastre, A., Carnero, E., Pehamberger, H., Wolff, K., Palese, P. & Muster, T. 2000, "Influenza Virus NS1 Protein Counteracts PKR-Mediated Inhibition of Replication", *The Journal of Virology,* vol. 74, no. 13, pp. 6203-6206.

Bianchini, A., Loiarro, M., Bielli, P., Busa, R., Paronetto, M.P., Loreni, F., Geremia, R. & Sette, C. 2008, "Phosphorylation of eIF4E by MNKs supports protein synthesis, cell cycle progression and proliferation in prostate cancer cells", *Carcinogenesis,* vol. 29, no. 12, pp. 2279-2288.

Biberman, Y. & Meyuhas, O. 1997, "Substitution of just five nucleotides at and around the transcription start site of rat β-actin promoter is sufficient to render the resulting transcript a subject for translational control", *FEBS letters,* vol. 405, no. 3, pp. 333-336.

Blaho, J., Mitchell, C. & Roizman, B. 1993, "Guanylylation and adenylylation of the alpha regulatory proteins of herpes simplex virus require a viral beta or gamma function", *J Virol,* vol. 67, no. 7, pp. 3891-3900.

Block, T., Barney, S., Masonis, J., Maggioncalda, J., Valyi-Nagy, T. & Fraser, N.W. 1994, "Long term herpes simplex virus type 1 infection of nerve growth factor-treated PC12 cells", *Journal of General Virology,* vol. 75, no. 9, pp. 2481-2487.

Bloom, D.C. & Stevens, J.G. 1994, "Neuron-specific restriction of a herpes simplex virus recombinant maps to the UL5 gene.", *The Journal of Virology,* vol. 68, no. 6, pp. 3761-3772.

Boal, T.R., Chiorini, J.A., Cohen, R.B., Miyamoto, S., Frederickson, R.M., Sonenberg, N. & Safer, B. 1993, "Regulation of eukaryotic translation initiation factor expression during T-cell activation", *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1176, no. 3, pp. 257-264.

Boesen, T., Mohammad, S.S., Pavitt, G.D. & Andersen, G.R. 2004, "Structure of the Catalytic Fragment of Translation Initiation Factor 2B and Identification of a Critically Important Catalytic Residue", *Journal of Biological Chemistry*, vol. 279, no. 11, pp. 10584-10592.

Borysov, S.I. & Guadagno, T.M. 2008, "A Novel Role for Cdk1/Cyclin B in Regulating B-Raf Activation at Mitosis", *Molecular biology of the cell*, vol. 19, no. 7, pp. 2907-2915.

Bowman, J.J., Orlando, J.S., Davido, D.J., Kushnir, A.S. & Schaffer, P.A. 2009, "Transient expression of Herpes Simplex Virus type 1 ICP22 represses viral promoter activity and complements the replication of an ICP22 null virus", *The Journal of Virology*, .

Brand, S.R., Kobayashi, R. & Mathews, M.B. 1997, "The Tat protein of human immunodeficiency virus type 1 is a substrate and inhibitor of the interferon-induced, virally activated protein kinase, PKR", *Journal of Biological Chemistry*, vol. 272, no. 13, pp. 8388-8395.

Brunn, G.J., Hudson, C.C., Sekulic, A., Williams, J.M., Hosoi, H., Houghton, P.J., Lawrence, J.C., Jr. & Abraham, R.T. 1997, "Phosphorylation of the Translational Repressor PHAS-I by the Mammalian Target of Rapamycin", *Science*, vol. 277, no. 5322, pp. 99-101.

Bu, X., Haas, D.W. & Hagedorn, C.H. 1993, "Novel phosphorylation sites of eukaryotic initiation factor-4F and evidence that phosphorylation stabilizes interactions of the p25 and p220 subunits.", *Journal of Biological Chemistry*, vol. 268, no. 7, pp. 4975-4978.

Burysek, L. & Pitha, P.M. 2001, "Latently Expressed Human Herpesvirus 8-Encoded Interferon Regulatory Factor 2 Inhibits Double-Stranded RNA-Activated Protein Kinase", *The Journal of Virology*, vol. 75, no. 5, pp. 2345-2352.

Cai, W. & Schaffer, P.A. 1992, "Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells", *J Virol*, vol. 66, pp. 2904-2915.

Carroll, K., Elroy-Stein, O., Moss, B. & Jagus, R. 1993, "Recombinant vaccinia virus K3L gene product prevents activation of double- stranded RNA-dependent, initiation factor 2*α*-specific protein kinase", *Journal of Biological Chemistry*, vol. 268, no. 17, pp. 12837-12842.

Cassady, K.A. & Gross, M. 2002, "The Herpes Simplex Virus Type 1 US11 Protein Interacts with Protein Kinase R in Infected Cells and Requires a 30-Amino-Acid Sequence Adjacent to a Kinase Substrate Domain", *The Journal of Virology,* vol. 76, no. 5, pp. 2029-2035.

CastellÃ³, A., Quintas, A., SÃ¡nchez, E.G., Sabina, P., Nogal, M., Carrasco, L. & Revilla, Y. 2009, "Regulation of Host Translational Machinery by African Swine Fever Virus", *PLoS Pathog,* vol. 5, no. 8, pp. e1000562.

Chan, S. 2004, "Targeting the mammalian target of rapamycin (mTOR): a new approach to treating cancer", *British journal of cancer,* vol. 91, no. 8, pp. 1420-1424.

Chan, T.O., Rittenhouse, S.E. & Tsichlis, P.N. 1999, "AKT/PKB AND OTHER D3 PHOSPHOINOSITIDE-REGULATED KINASES: Kinase Activation by Phosphoinositide-Dependent Phosphorylation", *Annual Review of Biochemistry,* vol. 68, no. 1, pp. 965-1014.

Chang, Y., Lee, H., Chen, Y., Lu, J., Wu, S., Chen, C., Takada, K. & Tsai, C. 2006, "Induction of the Early Growth Response 1 Gene by Epstein-Barr Virus Lytic Transactivator Zta", *The Journal of Virology,* vol. 80, no. 15, pp. 7748-7755.

Chee, A.V., Lopez, P., Pandolfi, P.P. & Roizman, B. 2003a, "Promyelocytic Leukemia Protein Mediates Interferon-Based Anti-Herpes Simplex Virus 1 Effects", *The Journal of Virology,* vol. 77, no. 12, pp. 7101-7105.

Chee, A.V., Lopez, P., Pandolfi, P.P. & Roizman, B. 2003b, "Promyelocytic Leukemia Protein Mediates Interferon-Based Anti-Herpes Simplex Virus 1 Effects", *The Journal of Virology,* vol. 77, no. 12, pp. 7101-7105.

Chen, J. & London, I.M. 1995, "Regulation of protein synthesis by heme-regulated eIF-2α kinase", *Trends in biochemical sciences,* vol. 20, no. 3, pp. 105-108.

Chen, S., Kramer, M., Schaffer, P. & Coen, D. 1997, "A viral function represses accumulation of transcripts from productive- cycle genes in mouse ganglia latently infected with herpes simplex virus", *The Journal of Virology,* vol. 71, no. 8, pp. 5878-5884.

Chen, Z., Gibson, T.B., Robinson, F., Silvestro, L., Pearson, G., Xu, B., Wright, A., Vanderbilt, C. & Cobb, M.H. 2001, "MAP Kinases", *Chemical reviews,* vol. 101, no. 8, pp. 2449-2476.

Choi, K.M., McMahon, L.P. & Lawrence, J.C. 2003, "Two Motifs in the Translational Repressor PHAS-I Required for Efficient Phosphorylation by Mammalian Target of Rapamycin and for Recognition by Raptor", *Journal of Biological Chemistry,* vol. 278, no. 22, pp. 19667-19673.

Cigan, A.M., Pabich, E.K. & Donahue, T.F. 1988, "Mutational analysis of the HIS4 translational initiator region in Saccharomyces cerevisiae.", *Molecular and cellular biology,* vol. 8, no. 7, pp. 2964-2975.

Clemens, M.J. 2006, "Epstein-Barr virus: Inhibition of apoptosis as a mechanism of cell transformation", *The international journal of biochemistry & cell biology,* vol. 38, no. 2, pp. 164-169.

Clements, G.B. & Stow, N.D. 1989, "A Herpes Simplex Virus Type 1 Mutant Containing a Deletion within Immediate Early Gene 1 Is Latency-competent in Mice", *Journal of General Virology,* vol. 70, no. 9, pp. 2501-2506.

Cohen, A., Brodie, C. & Sarid, R. 2006, "An essential role of ERK signalling in TPA-induced reactivation of Kaposi's sarcoma-associated herpesvirus", *Journal of General Virology,* vol. 87, no. 4, pp. 795-802.

Coldwell, M.J. & Morley, S.J. 2006, "Specific Isoforms of Translation Initiation Factor 4GI Show Differences in Translational Activity", *Molecular and cellular biology,* vol. 26, no. 22, pp. 8448-8460.

Cook, M.L., Bastone, V.B. & Stevens, J.G. 1974, "Evidence that Neurons Harbor Latent Herpes Simplex Virus", *Infection and immunity,* vol. 9, no. 5, pp. 946-951.

Cook, M.L. & Stevens, J.G. 1976, "Latent Herpetic Infections Following Experimental Viraemia", *Journal of General Virology,* vol. 31, no. 1, pp. 75-80.

Cougot, N., van Dijk, E., Babajko, S. & Séraphin, B. 2004, "'Cap-tabolism'", *Trends in biochemical sciences,* vol. 29, no. 8, pp. 436-444.

Courtney, K.D., Corcoran, R.B. & Engelman, J.A. 2010, "The PI3K Pathway As Drug Target in Human Cancer", *Journal of Clinical Oncology,* vol. 28, no. 6, pp. 1075-1083.

Crouch, N.A. & Rapp, F. 1972, "Cell-Dependent Differences in the Production of Infectious Herpes Simplex Virus at a Supraoptimal Temperature", *The Journal of Virology,* vol. 9, no. 2, pp. 223-230.

Cuend, A., Rousseaua, S.," p38 MAP-Kinases pathway regulation, function and role in human diseases", *Molecular Cell Research,* vol.1773, no 8, pp. 1358-1375.

Cuesta R., Xi Q., Schneider, R.J. 2000. Adeno virus specific translation by selective disassembly of cap-initiation complex. *EMBO J*: 19: 3465-3474

Danaher, R.J., Jacob, R.J., Chorak, M.D., Freeman, C.S. & Miller, C.S. 1999, "Heat stress activates production of herpes simplex virus type 1 from quiescently infected neurally differentiated PC12 cells", *Journal of neurovirology,* vol. 5, no. 4, pp. 374.

Danaher, R.J., Jacob, R.J. & Miller, C.S. 1999, "Establishment of a quiescent herpes simplex virus type 1 infection in neurally-differentiated PC12 cells", *Journal of neurovirology,* vol. 5, no. 3, pp. 258-267.

Danaher, R.J., Jacob, R.J., Steiner, M.R., Allen, W.R., Hill, J.M. & Miller, C.S. 2005, "Histone deacetylase inhibitors induce reactivation of herpes simplex virus type 1 in a latency-associated transcriptâ€“independent manner in neuronal cells", *Journal of neurovirology,* vol. 11, no. 3, pp. 306-317.

Danaher, R., Jacob, R. & Miller, C. October 2006, "Reactivation from quiescence does not coincide with a global induction of herpes simplex virus type 1 transactivators", *Virus genes,* vol. 33, pp. 163-167(5).

Das, S., Ghosh, R. & Maitra, U. 2001, "Eukaryotic Translation Initiation Factor 5 Functions as a GTPase-activating Protein", *Journal of Biological Chemistry,* vol. 276, no. 9, pp. 6720-6726.

Datta, S.R., Brunet, A. & Greenberg, M.E. 1999, "Cellular survival: a play in three Akts", *Genes & development,* vol. 13, no. 22, pp. 2905-2927.

David M. Knipe, Peter M. Howley, M., Diane E. Griffin, MD, PhD, Robert A. Lamb, PhD, ScD, Malcolm A. Martin, M., Bernard Roizman, S. & Stephen E. Straus, M. (eds) 2007, *Field's Virology*.

Davido, D.J., Leib, D.A. & Schaffer, P.A. 2002, "The Cyclin-Dependent Kinase Inhibitor Roscovitine Inhibits the Transactivating Activity and Alters the Posttranslational Modification of Herpes Simplex Virus Type 1 ICP0", *The Journal of Virology,* vol. 76, no. 3, pp. 1077-1088.

Davies, M.V., Elroy-Stein, O., Jagus, R., Moss, B. & Kaufman, R.J. 1992, "The vaccinia virus K3L gene product potentiates translation by inhibiting double-stranded-RNA-activated protein kinase and phosphorylation of the alpha subunit of eukaryotic initiation factor 2", *Journal of virology,* vol. 66, no. 4, pp. 1943-1950.

Davis, R.J. 1993, *The mitogen-activated protein kinase signal transduction pathway.*

Deatly, A.M., Spivack, J.G., Lavi, E. & Fraser, N.W. 1987, "RNA from an immediate early region of the type 1 herpes simplex virus genome is present in the trigeminal ganglia of latently infected mice", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 84, no. 10, pp. 3204-3208.

Deng, J., Harding, H.P., Raught, B., Gingras, A., Berlanga, J.J., Scheuner, D., Kaufman, R.J., Ron, D. & Sonenberg, N. 2002, "Activation of GCN2 in UV-Irradiated Cells Inhibits Translation", *Current Biology,* vol. 12, no. 15, pp. 1279-1286.

Diaz, J., Simonin, D., Masse, T., Deviller, P., Kindbeiter, K., Denoroy, L. & Madjar, J. 1993, "The herpes simplex virus type 1 US11 gene product is a phosphorylated protein found to be non-specifically associated with both ribosomal subunits", *Journal of General Virology,* vol. 74, no. 3, pp. 397-406.

Dos D. Sarbassov, Ali, S.M., Kim, D., Guertin, D.A., Latek, R.R., Erdjument-Bromage, H., Tempst, P. & Sabatini, D.M. 2004, "Rictor, a Novel Binding Partner of mTOR, Defines a Rapamycin-Insensitive and Raptor-Independent Pathway that Regulates the Cytoskeleton", *Current Biology,* vol. 14, no. 14, pp. 1296-1302.

Dowling, R.J.O., Topisirovic, I., Fonseca, B.D. & Sonenberg, N. 2010, "Dissecting the role of mTOR: Lessons from mTOR inhibitors", *Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics,* vol. 1804, no. 3, pp. 433-439.

Duncan, R.F., Peterson, H. & Sevanian, A. 2005, "Signal transduction pathways leading to increased eIF4E phosphorylation caused by oxidative stress", *Free Radical Biology and Medicine,* vol. 38, no. 5, pp. 631-643.

Eguchi, S., Tokunaga, C., Hidayat, S., Oshiro, N., Yoshino, K., Kikkawa, U. & Yonezawa, K. 2006, "Different roles for the TOS and RAIP motifs of the translational regulator protein 4E-BP1 in the association with raptor and phosphorylation by mTOR in the regulation of cell size", *Genes to Cells,* vol. 11, no. 7, pp. 757-766.

Elion, G.B. 1993, "Acyclovir: Discovery, mechanism of action, and selectivity", *Journal of medical virology,* vol. 41, no. S1, pp. 2-6.

Esteban, M., Garcia, M.A., Domingo-Gil, E., Arroyo, J., Nombela, C. & Rivas, C. 2003, "The latency protein LANA2 from Kaposi's sarcoma-associated herpesvirus inhibits apoptosis induced by dsRNA-activated protein kinase but not RNase L activation", *Journal of General Virology,* vol. 84, no. 6, pp. 1463-1470.

Everett, R.D. 2000, "ICP0, a regulator of herpes simplex virus during lytic and latent infection", *BioEssays,* vol. 22, no. 8, pp. 761-770.

Everett, R.D. & Maul, G.G. 1994, "HSV-1 IE protein Vmw110 causes redistribution of PML", *EMBO J,* vol. 13, pp. 5062-5069.

Everett, R.D., Boutell, C. & Orr, A. 2004, "Phenotype of a Herpes Simplex Virus Type 1 Mutant That Fails To Express Immediate-Early Regulatory Protein ICP0", *The Journal of Virology,* vol. 78, no. 4, pp. 1763-1774.

Everett, R.D. & Chelbi-Alix, M.K. 2007, "PML and PML nuclear bodies: Implications in antiviral defence", *Biochimie,* vol. 89, no. 6-7, pp. 819-830.

Everett, R.D. & Murray, J. 2005, "ND10 Components Relocate to Sites Associated with Herpes Simplex Virus Type 1 Nucleoprotein Complexes

during Virus Infection", *The Journal of Virology,* vol. 79, no. 8, pp. 5078-5089.

Everett, R.D., Rechter, S., Papior, P., Tavalai, N., Stamminger, T. & Orr, A. 2006, "PML Contributes to a Cellular Mechanism of Repression of Herpes Simplex Virus Type 1 Infection That Is Inactivated by ICP0", *The Journal of Virology,* vol. 80, no. 16, pp. 7995-8005.

Fahmi, H., Cochet, C., Hmama, Z., Opolon, P. & Joab, I. 2000, "Transforming Growth Factor Beta 1 Stimulates Expression of the Epstein-Barr Virus BZLF1 Immediate-Early Gene Product ZEBRA by an Indirect Mechanism Which Requires the MAPK Kinase Pathway", *The Journal of Virology,* vol. 74, no. 13, pp. 5810-5818.

Fan, S., Li, Y., Yue, P., Khuri, F.R. & Sun, S. 2010, "The eIF4E/eIF4G interaction inhibitor 4EGI-1 augments TRAIL-mediated apoptosis through c-FLIP Down-regulation and DR5 induction independent of inhibition of cap-dependent protein translation.", *Neoplasia,* vol. 12, no. 4, pp. 346-356.

Feigenblum, D. & Schneider, R. 1996, "Cap-binding protein (eukaryotic initiation factor 4E) and 4E- inactivating protein BP-1 independently regulate cap-dependent translation", *Molecular and cellular biology,* vol. 16, no. 10, pp. 5450-5457.

Feldman, L.T., Ellison, A.R., Voytek, C.C., Yang, L., Krause, P. & Margolis, T.P. 2002, "Spontaneous molecular reactivation of herpes simplex virus type 1 latency in mice", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 99, no. 2, pp. 978-983.

Feng, P., Everly, D.N., Jr. & Read, G.S. 2001, "mRNA Decay during Herpesvirus Infections: Interaction between a Putative Viral Nuclease and a Cellular Translation Factor", *The Journal of Virology,* vol. 75, no. 21, pp. 10272-10280.

Ferguson, G., Mothe-Satney, I. & Lawrence, J.C. 2003, "Ser-64 and Ser-111 in PHAS-I Are Dispensable for Insulin-stimulated Dissociation from eIF4E", *Journal of Biological Chemistry,* vol. 278, no. 48, pp. 47459-47465.

Flynn, A. & Proud, C.G. 1996, "Insulin-stimulated phosphorylation of initiation factor 4E is mediated by the MAP kinase pathway", *FEBS letters,* vol. 389, no. 2, pp. 162-166.

Fontaine-Rodriguez, E.C., Taylor, T.J., Olesky, M. & Knipe, D.M. 2004, "Proteomics of herpes simplex virus infected cell protein 27: association with translation initiation factors", *Virology,* vol. 330, no. 2, pp. 487-492.

Ford, P.W., Bryan, B.A., Dyson, O.F., Weidner, D.A., Chintalgattu, V. & Akula, S.M. 2006, "Raf/MEK/ERK signalling triggers reactivation of Kaposi's sarcoma-associated herpesvirus latency", *Journal of General Virology,* vol. 87, no. 5, pp. 1139-1144.

Frias, M.A., Thoreen, C.C., Jaffe, J.D., Schroder, W., Sculley, T., Carr, S.A. & Sabatini, D.M. 2006, "mSin1 Is Necessary for Akt/PKB Phosphorylation, and Its Isoforms Define Three Distinct mTORC2s", *Current Biology,* vol. 16, no. 18, pp. 1865-1870.

Frost, J.A., Geppert, T.D., Cobb, M.H. & Feramisco, J.R. 1994, "A requirement for extracellular signal-regulated kinase (ERK) function in the activation of AP-1 by Ha-Ras, phorbol 12-myristate 13-acetate, and serum", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 91, no. 9, pp. 3844-3848.

Fuke, H. & Ohno, M. 2008, "Role of poly (A) tail as an identity element for mRNA nuclear export", *Nucleic acids research,* vol. 36, no. 3, pp. 1037-1049.

Fukuda, M., Kurosaki, W., Yanagihara, K., Kuratsune, H. & Sairenji, T. 2002, "A Mechanism in Epstein–Barr Virus Oncogenesis: Inhibition of Transforming Growth Factor-β1-mediated Induction of MAPK/p21 by LMP1", *Virology,* vol. 302, no. 2, pp. 310-320.

Fukunaga, R. & Hunter, T. 1997, "MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates", *The EMBO journal,* vol. 16, no. 8, pp. 1921-1933.

Geballe, A.P. & Morris, D.R. 1994, "Initiation codons within 5′-leaders of mRNAs as regulators of translation", *Trends in biochemical sciences,* vol. 19, no. 4, pp. 159-164.

Gerd G. Maul 1998, "Nuclear domain 10, the site of DNA virus transcription and replication", *BioEssays,* vol. 20, pp. 660-667.

Gilbert, S.C. 2006, "Oral shedding of herpes simplex virus type 1 in immunocompetent persons", *Journal of Oral Pathology and Medicine,* vol. 35, no. 9, pp. 548-553.

Gillis, P.A., Okagaki, L.H. & Rice, S.A. 2009, "Herpes Simplex Virus Type 1 ICP27 Induces p38 Mitogen-Activated Protein Kinase Signalling and Apoptosis in HeLa Cells", *The Journal of Virology,* vol. 83, no. 4, pp. 1767-1777.

Gingras, A.C., Svitkin, Y., Belsham, G.J., Pause, A. & Sonenberg, N. 1996, "Activation of the translational suppressor 4E-BP1 following infection with encephalomyocarditis virus and poliovirus", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 93, no. 11, pp. 5578-5583.

Gingras, A., Gygi, S.P., Raught, B., Polakiewicz, R.D., Abraham, R.T., Hoekstra, M.F., Aebersold, R. & Sonenberg, N. 1999, "Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism", *Genes & development,* vol. 13, no. 11, pp. 1422-1437.

Gingras, A., Kennedy, S.G., O'Leary, M.A., Sonenberg, N. & Hay, N. 1998, "4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signalling pathway", *Genes & development,* vol. 12, no. 4, pp. 502-513.

Gingras, A., Raught, B., Gygi, S.P., Niedzwiecka, A., Miron, M., Burley, S.K., Polakiewicz, R.D., Wyslouch-Cieszynska, A., Aebersold, R. & Sonenberg, N. 2001, "Hierarchical phosphorylation of the translation inhibitor 4E-BP1", *Genes & development,* vol. 15, no. 21, pp. 2852-2864.

Gingras, A., Raught, B. & Sonenberg, N. 2001, "Regulation of translation initiation by FRAP/mTOR", *Genes & development,* vol. 15, no. 7, pp. 807-826.

Gingras, A., Raught, B. & Sonenberg, N. 1999, "eIF4 INITIATION FACTORS: Effectors of mRNA Recruitment to Ribosomes and Regulators of Translation", *Annual Review of Biochemistry,* vol. 68, no. 1, pp. 913-963.

Gingras, A. & Sonenberg, N. 1997, "Adenovirus Infection Inactivates the Translational Inhibitors 4E-BP1 and 4E-BP2", *Virology,* vol. 237, no. 1, pp. 182-186.

Giordani, N.V., Neumann, D.M., Kwiatkowski, D.L., Bhattacharjee, P.S., McAnany, P.K., Hill, J.M. & Bloom, D.C. 2008, "During Herpes Simplex Virus Type 1 Infection of Rabbits, the Ability To Express the Latency-Associated Transcript Increases Latent-Phase Transcription of Lytic Genes", *The Journal of Virology,* vol. 82, no. 12, pp. 6056-6060.

Gomez, E., Mohammad, S.S. & Pavitt, G.D. 2002, "Characterization of the minimal catalytic domain within eIF2B: the guanine-nucleotide exchange factor for translation initiation", *The EMBO journal,* vol. 21, no. 19, pp. 5292-5301.

Goodbourn, S., Didcock, L. & Randall, R.E. 2000, "Interferons: Cell signalling, immune modulation, antiviral responses and virus countermeasures", *Journal of General Virology,* vol. 81, no. 10, pp. 2341-2364.

Graves, L.M., Bornfeldt, K.E., Argast, G.M., Krebs, E.G., Kong, X., Lin, T.A. & Lawrence, J.C. 1995, "cAMP- and rapamycin-sensitive regulation of the association of eukaryotic initiation factor 4E and the translational regulator PHAS-I in aortic smooth muscle cells", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 92, no. 16, pp. 7222-7226.

Guhaniyogi, J. & Brewer, G. 2001, "Regulation of mRNA stability in mammalian cells", *Gene,* vol. 265, no. 1-2, pp. 11-23.

Gwinn, D.M., Shackelford, D.B., Egan, D.F., Mihaylova, M.M., Mery, A., Vasquez, D.S., Turk, B.E. & Shaw, R.J. 2008, "AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint", *Molecular cell,* vol. 30, no. 2, pp. 214-226.

Haar, E.V., Lee, S., Bandhakavi, S., Griffin, T.J. & Kim, D. 2007, "Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40", ***Nature cell biology,*** **vol. 9, no. 3, pp. 316-323.**

Halford, W.P., Kemp, C.D., Isler, J.A., Davido, D.J. & Schaffer, P.A. 2001a, "ICP0, ICP4, or VP16 expressed from adenovirus vectors induces reactivation of latent herpes simplex virus type 1 in primary cultures of latently infected trigeminal ganglion cells", ***J Virol,*** **vol. 75, pp. 6143-6153.**

Halford, W.P., Kemp, C.D., Isler, J.A., Davido, D.J. & Schaffer, P.A. 2001b, "ICP0, ICP4, or VP16 Expressed from Adenovirus Vectors Induces Reactivation of Latent Herpes Simplex Virus Type 1 in Primary Cultures of Latently Infected Trigeminal Ganglion Cells", ***The Journal of Virology,*** **vol. 75, no. 13, pp. 6143-6153.**

Han, A., Yu, C., Lu, L., Fujiwara, Y., Browne, C., Chin, G., Fleming, M., Leboulch, P., Orkin, S.H. & Chen, J. 2001, "Heme-regulated eIF2[alpha] kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency", ***The EMBO journal,*** **vol. 20, no. 23, pp. 6909-6918.**

Hancock, M.H., Corcoran, J.A. & Smiley, J.R. 2006, "Herpes simplex virus regulatory proteins VP16 and ICP0 counteract an innate intranuclear barrier to viral gene expression", ***Virology,*** **vol. 352, no. 1, pp. 237-252.**

Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J. & Yonezawa, K. 2002a, "Raptor, a Binding Partner of Target of Rapamycin (TOR), Mediates TOR Action", ***Cell,*** **vol. 110, no. 2, pp. 177-189.**

Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J. & Yonezawa, K. 2002b, "Raptor, a Binding Partner of Target of Rapamycin (TOR), Mediates TOR Action", ***Cell,*** **vol. 110, no. 2, pp. 177-189.**

Harding, H.P. & Ron, D. 2002, "Endoplasmic reticulum stress and the development of diabetes: A review", ***Diabetes,*** **vol. 51, no. SUPPL. 3.**

Hargett, D., McLean, T. & Bachenheimer, S.L. 2005, "Herpes Simplex Virus ICP27 Activation of Stress Kinases JNK and p38", ***The Journal of Virology,*** **vol. 79, no. 13, pp. 8348-8360.**

Harris, R.A., Everett, R.D., Zhu, X.X., Silverstein, S. & Preston, C.M. 1989, "Herpes simplex virus type 1 immediate-early protein Vmw110 reactivates latent herpes simplex virus type 2 in an in vitro latency system.", ***The Journal of Virology,*** **vol. 63, no. 8, pp. 3513-3515.**

Harris, R.A. & Preston, C.M. 1991, "Establishment of latency in vitro by the herpes simplex virus type 1 mutant in1814", ***J Gen Virol,*** **vol. 72, pp. 907-913.**

Hatada, E., Saito, S. & Fukuda, R. 1999, "Mutant influenza viruses with a defective NS1 protein cannot block the activation of PKR in infected cells", *Journal of virology,* vol. 73, no. 3, pp. 2425-2433.

He, B., Gross, M. & Roizman, B. 1998, "The γ134.5 Protein of Herpes Simplex Virus 1 Has the Structural and Functional Attributes of a Protein Phosphatase 1 Regulatory Subunit and Is Present in a High Molecular Weight Complex with the Enzyme in Infected Cells", *Journal of Biological Chemistry,* vol. 273, no. 33, pp. 20737-20743.

Helliwell, S., Wagner, P., Kunz, J., Deuter-Reinhard, M., Henriquez, R. & Hall, M. 1994, "TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast", *Molecular biology of the cell,* vol. 5, no. 1, pp. 105-118.

Henderson, G., Peng, W., Jin, L., Perng, G., Nesburn, A.B., Wechsler, S.L. & Jones, C. 2002, "Regulation of Caspase 8- and Caspase 9-Induced Apoptosis by the Herpes Simplex Virus Type 1 Latency-Associated Transcript", *Journal of neurovirology,* vol. 8, no. 2 supp 1, pp. 103.

Hentze, M.W. & Kühn, L.C. "Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 93, no. 16, pp. 8175-8182.

Hill, J.M., Dudley, J.B., Shimomura, Y. & Kaufman, H.E. 1986, "Quantitation and kinetics of induced HSV-1 ocular shedding", *Current eye research,* vol. 5, no. 3, pp. 241-246.

Hill, J.M., Lukiw, W.J., Gebhardt, B.M., Higaki, S., Loutsch, J.M., Myles, M.E., Thompson, H.W., Kwon, B.S., Bazan, N.G. & Kaufman, H.E. 2001, *Gene Expression Analyzed by Microarrays in HSV-1 Latent Mouse Trigeminal Ganglion Following Heat Stress*.

Hinton, T.M., Coldwell, M.J., Carpenter, G.A., Morley, S.J. & Pain, V.M. 2007, "Functional Analysis of Individual Binding Activities of the Scaffold Protein eIF4G", *Journal of Biological Chemistry,* vol. 282, no. 3, pp. 1695-1708.

Hobbs, W.E., Brough, D.E., Kovesdi, I. & DeLuca, N.A. 2001, "Efficient Activation of Viral Genomes by Levels of Herpes Simplex Virus ICP0 Insufficient To Affect Cellular Gene Expression or Cell Survival", *The Journal of Virology,* vol. 75, no. 7, pp. 3391-3403.

Hovanessian, A.G. 2007, "On the discovery of interferon-inducible, double-stranded RNA activated enzymes: The 2′–5′oligoadenylate synthetases and the protein kinase PKR", *Cytokine & growth factor reviews,* vol. 18, no. 5-6, pp. 351-361.

Hsu, W. & Everett, R.D. 2001, "Human Neuron-Committed Teratocarcinoma NT2 Cell Line Has Abnormal ND10 Structures and Is Poorly Infected by

Herpes Simplex Virus Type 1", *The Journal of Virology,* vol. 75, no. 8, pp. 3819-3831.

Huang, J. & Schneider, R.J. 1991, "Adenovirus inhibition of cellular protein synthesis involves inactivation of cap-binding protein", *Cell,* vol. 65, no. 2, pp. 271-280.

Huez, I., Creancier, L., Audigier, S., Gensac, M., Prats, A. & Prats, H. 1998, "Two Independent Internal Ribosome Entry Sites Are Involved in Translation Initiation of Vascular Endothelial Growth Factor mRNA", *Molecular and cellular biology,* vol. 18, no. 11, pp. 6178-6190.

Inoki, K., Li, Y., Xu, T. & Guan, K. 2003, "Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling", *Genes & development,* vol. 17, no. 15, pp. 1829-1834.

Inoki, K., Zhu, T. & Guan, K. 2003, "TSC2 Mediates Cellular Energy Response to Control Cell Growth and Survival", *Cell,* vol. 115, no. 5, pp. 577-590.

Ishov, A.M. & Maul, G.G. 1996, "The periphery of nuclear domain 10 (ND10) as site of DNA virus deposition", *J Cell Biol,* vol. 134, no. 4, pp. 815-826.

Izumi, K.M. & Stevens, J.G. 1990, "Molecular and biological characterization of a herpes simplex virus type 1 (HSV-1) neuroinvasiveness gene.", *The Journal of experimental medicine,* vol. 172, no. 2, pp. 487-496.

Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S.Y., Huang, Q., Qin, J. & Su, B. 2006, "SIN1/MIP1 Maintains rictor-mTOR Complex Integrity and Regulates Akt Phosphorylation and Substrate Specificity", *Cell,* vol. 127, no. 1, pp. 125-137.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M.A., Hall, A. & Hall, M.N. 2004, "Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive", *Nature cell biology,* vol. 6, no. 11, pp. 1122-1128.

Jamieson, D.R.S., Robinson, L.H., Daksis, J.I., Nicholl, M.J. & Preston, C.M. 1995, "Quiescent viral genomes in human fibroblasts after infection with herpes simplex virus type 1 Vmw65 mutants", *Journal of General Virology,* vol. 76, no. 6, pp. 1417-1431.

Jang, S.K., Krausslich, H.G., Nicklin, M.J., Duke, G.M., Palmenberg, A.C. & Wimmer, E. 1988, "A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation.", *The Journal of Virology,* vol. 62, no. 8, pp. 2636-2643.

Jin, L., Peng, W., Perng, G., Brick, D.J., Nesburn, A.B., Jones, C. & Wechsler, S.L. 2003, "Identification of Herpes Simplex Virus Type 1 Latency-Associated Transcript Sequences That both Inhibit Apoptosis and Enhance the Spontaneous Reactivation Phenotype", *The Journal of Virology,* vol. 77, no. 11, pp. 6556-6561.

Johnson, P.A., Wang, M.J. & Friedmann, T. 1994, "Improved cell survival by the reduction of immediate-early gene expression in replication-defective mutants of herpes simplex virus type 1 but not by mutation of the virion host shutoff function.", *The Journal of Virology,* vol. 68, no. 10, pp. 6347-6362.

Jones, R., Branda, J., Johnston, K., Polymenis, M., Gadd, M., Rustgi, A., Callanan, L. & Schmidt, E. 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", *Molecular and cellular biology,* vol. 16, no. 9, pp. 4754-4764.

Joshi-Barve, S., De Benedetti, A. & Rhoads, R.E. 1992, "Preferential translation of heat shock mRNAs in HeLa cells deficient in protein synthesis initiation factors eIF-4E and eIF-4 gamma.", *Journal of Biological Chemistry,* vol. 267, no. 29, pp. 21038-21043.

Kapoor, A. & Sanyal, A.J. 2009, "Endoplasmic Reticulum Stress and the Unfolded Protein Response", *Clinics in liver disease,* vol. 13, no. 4, pp. 581-590.

Karupiah, G. 2002, "Fields Virology, 4TH Edition", *Immunology and cell biology,* vol. 80, no. 3, pp. 314-315.

Kaspar, R.L., Kakegawa, T., Cranston, H., Morris, D.R. & White, M.W. 1992, "A regulatory cis element and a specific binding factor involved in the mitogenic control of murine ribosomal protein L32 translation", *Journal of Biological Chemistry,* vol. 267, no. 1, pp. 508-514.

Keith, C.T. & Schreiber, S.L. 1995, "PIK-Related Kinases: DNA Repair, Recombination, and Cell Cycle Checkpoints", *Science,* vol. 270, no. 5233, pp. 50.

Kennedy, P.G.E., Al-Saadi, S.A. & Clements, G.B. 1983, "Reactivation of Latent Herpes Simplex Virus from Dissociated Identified Dorsal Root Ganglion Cells in Culture", *Journal of General Virology,* vol. 64, no. 7, pp. 1629-1635.

Kent, J.R. & Fraser, N.W. 2005, "The cellular response to herpes simplex virus type 1 (HSV-1) during latency and reactivation", *Journal of neurovirology,* vol. 11, no. 4, pp. 376.

Kim, D., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P. & Sabatini, D.M. 2002, "mTOR Interacts with Raptor to Form a Nutrient-Sensitive Complex that Signals to the Cell Growth Machinery", *Cell,* vol. 110, no. 2, pp. 163-175.

Kim, D., Sarbassov, D.D., Ali, S.M., Latek, R.R., Guntur, K.V.P., Erdjument-Bromage, H., Tempst, P. & Sabatini, D.M. 2003, "GβL, a Positive Regulator of the Rapamycin-Sensitive Pathway Required for the Nutrient-Sensitive Interaction between Raptor and mTOR", *Molecular cell,* vol. 11, no. 4, pp. 895-904.

Kimball, S.R. 1999, "Eukaryotic initiation factor eIF2", *The international journal of biochemistry & cell biology,* vol. 31, no. 1, pp. 25-29.

Kimball, S.R., Do, A.N.D., Kutzler, L., Cavener, D.R. & Jefferson, L.S. 2008, "Rapid Turnover of the mTOR Complex 1 (mTORC1) Repressor REDD1 and Activation of mTORC1 Signaling following Inhibition of Protein Synthesis", *Journal of Biological Chemistry,* vol. 283, no. 6, pp. 3465-3475.

Knauf, U., Tschopp, C. & Gram, H. 2001, "Negative Regulation of Protein Translation by Mitogen-Activated Protein Kinase-Interacting Kinases 1 and 2", *Molecular and cellular biology,* vol. 21, no. 16, pp. 5500-5511.

Knickelbein, J.E., Khanna, K.M., Yee, M.B., Baty, C.J., Kinchington, P.R. & Hendricks, R.L. 2008, "Noncytotoxic Lytic Granule-Mediated CD8+ T Cell Inhibition of HSV-1 Reactivation from Neuronal Latency", *Science,* vol. 322, no. 5899, pp. 268-271.

Kotwal, G. 1997, "Microorganisms and their interaction with the immune system", *Journal of leukocyte biology,* vol. 62, no. 4, pp. 415-429.

Kozak, M. "The scanning model for translation: an update.", *The Journal of cell biology,* vol. 108, no. 2, pp. 229-241.

Kozak, M. 1987, "An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs", *Nucleic acids research,* vol. 15, no. 20, pp. 8125-8148.

Kramer, M. & Coen, D. 1995, "Quantification of transcripts from the ICP4 and thymidine kinase genes in mouse ganglia latently infected with herpes simplex virus", *The Journal of Virology,* vol. 69, no. 3, pp. 1389-1399.

Krishnamoorthy, T., Pavitt, G.D., Zhang, F., Dever, T.E. & Hinnebusch, A.G. 2001, "Tight Binding of the Phosphorylated {alpha} Subunit of Initiation Factor 2 (eIF2{alpha}) to the Regulatory Subunits of Guanine Nucleotide Exchange Factor eIF2B Is Required for Inhibition of Translation Initiation", *Molecular and cellular biology,* vol. 21, no. 15, pp. 5018-5030.

Kristie, T.M., Vogel, J.L. & Sears, A.E. 1999, "Nuclear Localization of the C1 Factor (Host Cell Factor) in Sensory Neurons Correlates with Reactivation of Herpes Simplex Virus from Latency", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 96, no. 4, pp. 1229-1233.

Kumar, S., Boehm, J. & Lee, J.C. 2003, "p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases", *Nat Rev Drug Discov,* vol. 2, no. 9, pp. 717-726.

Kyriakis, J. 2003, "At the crossroads: AMP-activated kinase and the LKB1 tumor suppressor link cell proliferation to metabolic regulation", *Journal of Biology,* vol. 2, no. 4, pp. 26.

Larralde, O., Smith, R.W.P., Wilkie, G.S., Malik, P., Gray, N.K. & Clements, J.B. 2006, "Direct Stimulation of Translation by the Multifunctional Herpesvirus ICP27 Protein", *The Journal of Virology*, vol. 80, no. 3, pp. 1588-1591.

Laycock, K., Lee, S., Brady, R. & Pepose, J. 1991, "Characterization of a murine model of recurrent herpes simplex viral keratitis induced by ultraviolet B radiation", *Investigative Ophthalmology Visual Science*, vol. 32, no. 10, pp. 2741-2746.

Le, S. & Maizel, J.,Jr 1997, "A common RNA structural motif involved in the internal initiation of translation of cellular mRNAs", *Nucleic acids research*, vol. 25, no. 2, pp. 362-369.

Lents, N.H., Keenan, S.M., Bellone, C. & Baldassare, J.J. 2002, "Stimulation of the Raf/MEK/ERK Cascade Is Necessary and Sufficient for Activation and Thr-160 Phosphorylation of a Nuclear-targeted CDK2", *Journal of Biological Chemistry*, vol. 277, no. 49, pp. 47469-47475.

Leopardi, R., Ward, P., Ogle, W. & Roizman, B. 1997, "Association of herpes simplex virus regulatory protein ICP22 with transcriptional complexes containing EAP, ICP4, RNA polymerase II, and viral DNA requires posttranslational modification by the U(L)13 proteinkinase", *J Virol*, vol. 71, no. 2, pp. 1133-1139.

Lewis, J.D. & Izaurflde, E. 1997, "The Role of the Cap Structure in RNA Processing and Nuclear Export", *European Journal of Biochemistry*, vol. 247, no. 2, pp. 461-469.

Li, S., Min, J., Krug, R.M. & Sen, G.C. 2006, "Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA", *Virology*, vol. 349, no. 1, pp. 13-21.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J.L., Bonenfant, D., Oppliger, W., Jenoe, P. & Hall, M.N. 2002, "Two TOR Complexes, Only One of which Is Rapamycin Sensitive, Have Distinct Roles in Cell Growth Control", *Molecular cell*, vol. 10, no. 3, pp. 457-468.

Loret, S., Guay, G. & Lippe, R. 2008, "Comprehensive Characterization of Extracellular Herpes Simplex Virus Type 1 Virions", *The Journal of Virology*, vol. 82, no. 17, pp. 8605-8618.

Lu, L., Han, A. & Chen, J. 2001, "Translation Initiation Control by Heme-Regulated Eukaryotic Initiation Factor 2alpha Kinase in Erythroid Cells under Cytoplasmic Stresses", *Molecular and cellular biology*, vol. 21, no. 23, pp. 7971-7980.

Lu, Y., Wambach, M., Katze, M.G. & Krug, R.M. 1995, "Binding of the Influenza Virus NS1 Protein to Double-Stranded RNA Inhibits the Activation of the Protein Kinase That Phosphorylates the eIF-2 Translation Initiation Factor", ***Virology,*** **vol. 214, no. 1, pp. 222-228.**

Macejak, D.G. & Sarnow, P. 1991, "Internal initiation of translation mediated by the 5[prime] leader of a cellular mRNA", ***Nature,*** **vol. 353, no. 6339, pp. 90-94.**

Mader, S., Lee, H., Pause, A. & Sonenberg, N. 1995, "The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins", ***Molecular and cellular biology,*** **vol. 15, no. 9, pp. 4990-4997.**

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J. & Cantley, L.C. 2002, "Identification of the Tuberous Sclerosis Complex-2 Tumor Suppressor Gene Product Tuberin as a Target of the Phosphoinositide 3-Kinase/Akt Pathway", ***Molecular cell,*** **vol. 10, no. 1, pp. 151-162.**

Mao, X., Green, J.M., Safer, B., Lindsten, T., Frederickson, R.M., Miyamoto, S., Sonenberg, N. & Thompson, C.B. 1992, "Regulation of translation initiation factor gene expression during human T cell activation.", ***Journal of Biological Chemistry,*** **vol. 267, no. 28, pp. 20444-20450.**

Marcotrigiano, J., Gingras, A., Sonenberg, N. & Burley, S.K. 1999, "Cap-Dependent Translation Initiation in Eukaryotes Is Regulated by a Molecular Mimic of eIF4G", ***Molecular cell,*** **vol. 3, no. 6, pp. 707-716.**

Marcotrigiano, J., Gingras, A., Sonenberg, N. & Burley, S.K. 1997, "Cocrystal Structure of the Messenger RNA 5′ Cap-Binding Protein (eIF4E) Bound to 7-methyl-GDP", ***Cell,*** **vol. 89, no. 6, pp. 951-961.**

Margolis, T.P., Elfman, F.L., Leib, D., Pakpour, N., Apakupakul, K., Imai, Y. & Voytek, C. 2007, "Spontaneous Reactivation of Herpes Simplex Virus Type 1 in Latently Infected Murine Sensory Ganglia", ***The Journal of Virology,*** **vol. 81, no. 20, pp. 11069-11074.**

Marignani, P.A. 2005, "LKB1, the multitasking tumour suppressor kinase", ***Journal of clinical pathology,*** **vol. 58, no. 1, pp. 15-19.**

Marshall, C.J. 1994, "MAP kinase kinase kinase, MAP kinase kinase and MAP kinase", ***Current opinion in genetics & development,*** **vol. 4, no. 1, pp. 82-89.**

Masutani, M., Sonenberg, N., Yokoyama, S. & Imataka, H. 2007, "Reconstitution reveals the functional core of mammalian eIF3", ***The EMBO journal,*** **vol. 26, no. 14, pp. 3373-3383.**

Maul, G.G., Guldner, H.H. & Spivack, J.G. 1993, "Modification of discrete nuclear domains induced by herpes simplex virus type 1 immediate early

gene 1 product (ICP0)", *Journal of General Virology,* vol. 74, no. 12, pp. 2679-2690.

Maul, G.G. & Everett, R.D. 1994, "The nuclear location of PML, a cellular member of the C3HC4 zinc-binding domain protein family, is rearranged during herpes simplex virus infection by the C3HC4 viral protein ICP0", *Journal of General Virology,* vol. 75, no. 6, pp. 1223-1233.

McKendrick, L., Morley, S.J., Pain, V.M., Jagus, R. & Joshi, B. 2001, "Phosphorylation of eukaryotic initiation factor 4E (eIF4E) at Ser209 is not required for protein synthesis in vitro and in vivo", *European Journal of Biochemistry,* vol. 268, no. 20, pp. 5375-5385.

McLean, T.I. & Bachenheimer, S.L. 1999, "Activation of cJUN N-Terminal Kinase by Herpes Simplex Virus Type 1 Enhances Viral Replication", *The Journal of Virology,* vol. 73, no. 10, pp. 8415-8426.

Meignier, B., Longnecker, R., Mavromara-Nazos, P., Sears, A. & Roizman, B. 1988, "Virulence of and establishment of latency by genetically engineered deletion mutants of herpes simplex virus 1", *Virology,* vol. 162, no. 1, pp. 251-254.

Miller, C.S., Danaher, R.J. & Jacob, R.J. 2006, "ICP0 Is Not Required for Efficient Stress-Induced Reactivation of Herpes Simplex Virus Type 1 from Cultured Quiescently Infected Neuronal Cells", *The Journal of Virology,* vol. 80, no. 7, pp. 3360-3368.

Minich, W.B., Balasta, M.L., Goss, D.J. & 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", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 91, no. 16, pp. 7668-7672.

Mitchell, B. & Stevens, J. 1996, "Neuroinvasive properties of herpes simplex virus type 1 glycoprotein variants are controlled by the immune response", *The Journal of Immunology,* vol. 156, no. 1, pp. 246-255.

Mitchell, C., Blaho, J., McCormick, A. & Roizman, B. 1997, "The nucleotidylylation of herpes simplex virus 1 regulatory protein alpha22 by human casein kinase II", *J Biol Chem,* vol. 272, no. 40, pp. 25394-25400.

Mitchell, C., Blaho, J. & Roizman, B. 1994, "Casein kinase II specifically nucleotidylylates in vitro the amino acid sequence of the protein encoded by the alpha 22 gene of herpes simplex virus 1", *Proc Natl Acad Sci U S A,* vol. 91, no. 25, pp. 11864-11868.

Moerke, N.J., Aktas, H., Chen, H., Cantel, S., Reibarkh, M.Y., Fahmy, A., Gross, J., Degterev, A., Yuan, J., Chorev, M., Halperin, J.A. & Wagner, G. 2007,

"Small-Molecule Inhibition of the Interaction between the Translation Initiation Factors eIF4E and eIF4G", *Cell,* vol. 128, no. 2, pp. 257-267.

Mohr, I. 2006, "Phosphorylation and dephosphorylation events that regulate viral mRNA translation", *Virus research,* vol. 119, no. 1, pp. 89-99.

Mokas, S., Mills, J.R., Garreau, C., Fournier, M., Robert, F., Arya, P., Kaufman, R.J., Pelletier, J. & Mazroui, R. 2009, "Uncoupling Stress Granule Assembly and Translation Initiation Inhibition", *Molecular biology of the cell,* vol. 20, no. 11, pp. 2673-2683.

Moorman, N.J. & Shenk, T. 2010, "Rapamycin-Resistant mTORC1 Activity is Required for Herpesvirus Replication", *The Journal of Virology, .*

Morley, S.J. & 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 4E in NIH 3T3 Cells.*

Morley, S.J. & 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", *Journal of Biological Chemistry,* vol. 277, no. 36, pp. 32855-32859.

Mothe-Satney, I., Yang, D., Fadden, P., Haystead, T.A.J. & Lawrence, J.C., Jr. 2000, "Multiple Mechanisms Control Phosphorylation of PHAS-I in Five (S/T)P Sites That Govern Translational Repression", *Molecular and cellular biology,* vol. 20, no. 10, pp. 3558-3567.

Nanbru, C., Lafon, I., Audigier, S., Gensac, M., Vagner, S., Huez, G. & Prats, A. "Alternative Translation of the Proto-oncogene c-mycby an Internal Ribosome Entry Site", *Journal of Biological Chemistry,* vol. 272, no. 51, pp. 32061-32066.

Nesburn, A.B., Elliott, J.H. & Leibowitz, H.M. 1967, "Spontaneous Reactivation of Experimental Herpes Simplex Keratitis in Rabbits", *Archives of Ophthalmology,* vol. 78, no. 4, pp. 523-529.

Newcomb, W.W., Homa, F.L., Thomsen, D.R., Ye, Z. & Brown, J.C. 1994, "Cell-free assembly of the herpes simplex virus capsid.", *The Journal of Virology,* vol. 68, no. 9, pp. 6059-6063.

Niedzwiecka, A., Marcotrigiano, J., Stepinski, J., Jankowska-Anyszka, M., Wyslouch-Cieszynska, A., Dadlez, M., Gingras, A., Mak, P., Darzynkiewicz, E., Sonenberg, N., Burley, S.K. & 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", *Journal of Molecular Biology,* vol. 319, no. 3, pp. 615-635.

Nobukuni, T., Joaquin, M., Roccio, M., Dann, S.G., Kim, S.Y., Gulati, P., Byfield, M.P., Backer, J.M., Natt, F., Bos, J.L., Zwartkruis, F.J.T. &

Thomas, G. 2005, "Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 102, no. 40, pp. 14238-14243.

Nogueira, M.L., Wang, V.E.H., Tantin, D., Sharp, P.A. & Kristie, T.M. 2004, "Herpes simplex virus infections are arrested in Oct-1-deficient cells", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 101, no. 6, pp. 1473-1478.

Nojima, H., Tokunaga, C., Eguchi, S., Oshiro, N., Hidayat, S., Yoshino, K., Hara, K., Tanaka, N., Avruch, J. & Yonezawa, K. 2003, "The Mammalian Target of Rapamycin (mTOR) Partner, Raptor, Binds the mTOR Substrates p70 S6 Kinase and 4E-BP1 through Their TOR Signaling (TOS) Motif", *Journal of Biological Chemistry,* vol. 278, no. 18, pp. 15461-15464.

Oliver, L., Wald, A., Kim, M., Zeh, J., Selke, S., Ashley, R. & Corey, L. 1995, "Seroprevalence of Herpes Simplex Virus Infections in a Family Medicine Clinic", *Archives of Family Medicine,* vol. 4, no. 3, pp. 228-232.

Orr, M.T., Mathis, M.A., Lagunoff, M., Sacks, J.A. & Wilson, C.B. 2007, "CD8 T Cell Control of HSV Reactivation from Latency Is Abrogated by Viral Inhibition of MHC Class I", Cell Host & Microbe, vol. 2, no. 3, pp. 172-180.

Pain, V.M. 1996, "Initiation of Protein Synthesis in Eukaryotic Cells", *European Journal of Biochemistry,* vol. 236, no. 3, pp. 747-771.

Pause, A., Belsham, G.J., Gingras, A., Donze, O., Lin, T., Lawrence, J.C., & Sonenberg, N. 1994, "Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function", *Nature,* vol. 371, no. 6500, pp. 762-767.

Pavitt, G.D., Yang, W. & Hinnebusch, A.G. 1997, "Homologous segments in three subunits of the guanine nucleotide exchange factor eIf2B mediate translational regulation by phosphorylation of eIF2", *Molecular and cellular biology,* vol. 17, no. 3, pp. 1298-1313.

Pelletier, J. & Sonenberg, N. 1988, "Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA", *Nature,* vol. 334, no. 6180, pp. 320-325.

Pelletier, J. & Sonenberg, N. 1985, "Insertion mutagenesis to increase secondary structure within the 5′ noncoding region of a eukaryotic mRNA reduces translational efficiency", *Cell,* vol. 40, no. 3, pp. 515-526.

Peng, W., Jin, L., Henderson, G., Perng, G.C., Brick, D.J., Nesburn, A.B., Wechsler, S.L. & Jones, C. 2004, "Mapping herpes simplex virus type 1 latency-associated transcript sequences that protect from apoptosis mediated by a plasmid expressing caspase-8", *Journal of neurovirology,* vol. 10, no. 4, pp. 260-265.

Perng, G., Jones, C., Ciacci-Zanella, J., Stone, M., Henderson, G., Yukht, A., Slanina, S.M., Hofman, F.M., Ghiasi, H., Nesburn, A.B. & Wechsler, S.L. 2000, "Virus-Induced Neuronal Apoptosis Blocked by the Herpes Simplex Virus Latency-Associated Transcript", *Science,* vol. 287, no. 5457, pp. 1500-1503.

Peters, G.A., Khoo, D., Mohr, I. & Sen, G.C. 2002, "Inhibition of PACT-Mediated Activation of PKR by the Herpes Simplex Virus Type 1 Us11 Protein", *The Journal of Virology,* vol. 76, no. 21, pp. 11054-11064.

Peterson, T.R., Laplante, M., Thoreen, C.C., Sancak, Y., Kang, S.A., Kuehl, W.M., Gray, N.S. & Sabatini, D.M. 2009, "DEPTOR Is an mTOR Inhibitor Frequently Overexpressed in Multiple Myeloma Cells and Required for Their Survival", *Cell,* vol. 137, no. 5, pp. 873-886.

Poppers, J., Mulvey, M., Perez, C., Khoo, D. & Mohr, I. 2003, "Identification of a Lytic-Cycle Epstein-Barr Virus Gene Product That Can Regulate PKR Activation", *The Journal of Virology,* vol. 77, no. 1, pp. 228-236.

Post, L. & Roizman, B. 1981, "A generalized technique for deletion of specific genes in large genomes: alpha gene 22 of herpes simplex virus 1 is not essential for growth", *Cell,* vol. 25, no. 1, pp. 227-232.

Poulin, F., Gingras, A., Olsen, H., Chevalier, S. & Sonenberg, N. 1998, "4E-BP3, a New Member of the Eukaryotic Initiation Factor 4E-binding Protein Family", *Journal of Biological Chemistry,* vol. 273, no. 22, pp. 14002-14007.

Preiss, T., Muckenhaler, M. & Hentze, M.W. 1998, "Poly(A)-tail-promoted translation in yeast: Implications for translational control", *RNA,* vol. 4, no. 11, pp. 1321-1331.

Preston, C.M. 1979, "Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant tsK.", *The Journal of Virology,* vol. 29, no. 1, pp. 275-284.

Preston, C.M. 2007, "Reactivation of Expression from Quiescent Herpes Simplex Virus Type 1 Genomes in the Absence of Immediate-Early Protein ICP0", *The Journal of Virology,* vol. 81, no. 21, pp. 11781-11789.

Preston, C. & Nicholl, M. 1997, "Repression of gene expression upon infection of cells with herpes simplex virus type 1 mutants impaired for immediate-early protein synthesis", *The Journal of Virology,* vol. 71, no. 10, pp. 7807-7813.

Proud, C.G. 2002, "Regulation of mammalian translation factors by nutrients", *European Journal of Biochemistry,* vol. 269, no. 22, pp. 5338-5349.

Purves, F., Ogle, W. & Roizman, B. 1993, "Processing of the herpes simplex virus regulatory protein alpha 22 mediated by the UL13 protein kinase determines the accumulation of a subset of alpha and gamma mRNAs and

proteins in infected cells", *Proc Natl Acad Sci U S A,* vol. 90, no. 14, pp. 6701-6705.

Pyronnet, S., Imataka, H., Gingras, A., Fukunaga, R., Hunter, T. & Sonenberg, N. 1999, "Human eukaryotic translation initiation factor 4G (eIF4G) recruits Mnk1 to phosphorylate eIF4E", *The EMBO journal,* vol. 18, no. 1, pp. 270-279.

Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D.B., Perera, S., Roberts, T.M. & Sellers, W.R. 1999, "Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 96, no. 5, pp. 2110-2115.

Ramírez-Valle, F., Braunstein, S., Zavadil, J., Formenti, S.C. & Schneider, R.J. 2008, "eIF4GI Links Nutrient Sensing by mTOR to Cell Proliferation and Inhibition of Autophagy", *The Journal of cell biology,* vol. 181, no. 2, pp. 293-307.

Rao, G.N., Griendling, K.K., Frederickson, R.M., Sonenberg, N. & Alexander, R.W. 1994, "Angiotensin II induces phosphorylation of eukaryotic protein synthesis initiation factor 4E in vascular smooth muscle cells.", *Journal of Biological Chemistry,* vol. 269, no. 10, pp. 7180-7184.

Raught, B. & Gingras, A. 1999, "eIF4E activity is regulated at multiple levels", *The international journal of biochemistry & cell biology,* vol. 31, no. 1, pp. 43-57.

Rivas, C., Gil, J., Melková, Z., Esteban, M. & Díaz-Guerra, M. 1998, "Vaccinia Virus E3L Protein Is an Inhibitor of the Interferon (IFN)-Induced 2-5A Synthetase Enzyme", *Virology,* vol. 243, no. 2, pp. 406-414.

Rivas, C., Thlick, A., Parravicini, C., Moore, P.S. & Chang, Y. 2001, "Kaposi's Sarcoma-Associated Herpesvirus LANA2 Is a B-Cell-Specific Latent Viral Protein That Inhibits p53", *The Journal of Virology,* vol. 75, no. 1, pp. 429-438.

Robert, F., Kapp, L.D., Khan, S.N., Acker, M.G., Kolitz, S., Kazemi, S., Kaufman, R.J., Merrick, W.C., Koromilas, A.E., Lorsch, J.R. & Pelletier, J. 2006, "Initiation of Protein Synthesis by Hepatitis C Virus Is Refractory to Reduced eIF2 {middle dot} GTP {middle dot} Met-tRNAiMet Ternary Complex Availability", *Molecular biology of the cell,* vol. 17, no. 11, pp. 4632-4644.

Rodriguez-Viciana, P., Oses-Prieto, J., Burlingame, A., Fried, M. & McCormick, F. 2006, "A Phosphatase Holoenzyme Comprised of Shoc2/Sur8 and the Catalytic Subunit of PP1 Functions as an M-Ras Effector to Modulate Raf Activity", *Molecular cell,* vol. 22, no. 2, pp. 217-230.

Romano, P.R., Zhang, F., Tan, S.-., Garcia-Barrio, M.T., Katze, M.G., Dever, T.E. & Hinnebusch, A.G. 1998, "Inhibition of double-stranded RNA-dependent protein kinase PKR by vaccinia virus E3: Role of complex formation and the E3 N-terminal domain", *Molecular and cellular biology,* vol. 18, no. 12, pp. 7304-7316.

Rosenwald, I.B., Rhoads, D.B., Callanan, L.D., Isselbacher, K.J. & 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", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 90, no. 13, pp. 6175-6178.

Roux, P.P. & Blenis, J. 2004, "ERK and p38 MAPK-Activated Protein Kinases: a Family of Protein Kinases with Diverse Biological Functions", *Microbiology and Molecular Biology Reviews,* vol. 68, no. 2, pp. 320-344.

Ruf, I.K., Lackey, K.A., Warudkar, S. & Sample, J.T. 2005, "Protection from Interferon-Induced Apoptosis by Epstein-Barr Virus Small RNAs Is Not Mediated by Inhibition of PKR", *The Journal of Virology,* vol. 79, no. 23, pp. 14562-14569.

Russell, J., Stow, N.D., Stow, E.C. & Preston, C.M. 1987, "Herpes Simplex Virus Genes Involved in Latency in vitro", *Journal of General Virology,* vol. 68, no. 12, pp. 3009-3018.

Sacks, S.L., Griffiths, P.D., Corey, L., Cohen, C., Cunningham, A., Dusheiko, G.M., Self, S., Spruance, S., Stanberry, L.R., Wald, A. & Whitley, R.J. 2004a, "HSV shedding", *Antiviral Research,* vol. 63, no. Supplement 1, pp. S19-S26.

Sacks, S.L., Griffiths, P.D., Corey, L., Cohen, C., Cunningham, A., Dusheiko, G.M., Self, S., Spruance, S., Stanberry, L.R., Wald, A. & Whitley, R.J. 2004b, "HSV shedding", *Antiviral Research,* vol. 63, no. Supplement 1, pp. S19-S26.

Samaniego, L.A., Neiderhiser, L. & DeLuca, N.A. 1998, "Persistence and Expression of the Herpes Simplex Virus Genome in the Absence of Immediate-Early Proteins", *The Journal of Virology,* vol. 72, no. 4, pp. 3307-3320.

Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R.A., Kang, S.A., Spooner, E., Carr, S.A. & Sabatini, D.M. 2007, "PRAS40 Is an Insulin-Regulated Inhibitor of the mTORC1 Protein Kinase", *Molecular cell,* vol. 25, no. 6, pp. 903-915.

Santamaría, E., Mora, M.I., Potel, C., Fernández-Irigoyen, J., Carro-Roldán, E., Hernández-Alcoceba, R., Prieto, J., Epstein, A.L. & Corrales, F.J. 2009, "Identification of Replication-competent HSV-1 Cgal+ Strain Signaling Targets in Human Hepatoma Cells by Functional Organelle Proteomics", *Molecular & Cellular Proteomics,* vol. 8, no. 4, pp. 805-815.

Sarbassov, D.D., Guertin, D.A., Ali, S.M. & Sabatini, D.M. 2005, "Phosphorylation and Regulation of Akt/PKB by the Rictor-mTOR Complex", *Science,* vol. 307, no. 5712, pp. 1098-1101.

Sarnow, P., Cevallos, R.C., & Jan, E. 2005, " Takeover of host ribosomes by divergent IRES elements". Biochem Soc Trans. 33:1479 -1482

Schalm, S.S., Fingar, D.C., Sabatini, D.M. & Blenis, J. 2003, "TOS Motif-Mediated Raptor Binding Regulates 4E-BP1 Multisite Phosphorylation and Function", *Current Biology,* vol. 13, no. 10, pp. 797-806.

Schang, L.M., Bantly, A. & Schaffer, P.A. 2002, "Explant-Induced Reactivation of Herpes Simplex Virus Occurs in Neurons Expressing Nuclear cdk2 and cdk4", *The Journal of Virology,* vol. 76, no. 15, pp. 7724-7735.

Scheper, G.C., Morrice, N.A., Kleijn, M. & Proud, C.G. 2001, "The Mitogen-Activated Protein Kinase Signal-Integrating Kinase Mnk2 Is a Eukaryotic Initiation Factor 4E Kinase with High Levels of Basal Activity in Mammalian Cells", *Molecular and cellular biology,* vol. 21, no. 3, pp. 743-754.

Scheper, G.C., Mulder, J., Kleijn, M., Voorma, H.O., Thomas, A.A.M. & van Wijk, R. 1997, "Inactivation of eIF2B and Phosphorylation of PHAS-I in Heat-shocked Rat Hepatoma Cells", *Journal of Biological Chemistry,* vol. 272, no. 43, pp. 26850-26856.

Scheper, G.C., van Kollenburg, B., Hu, J., Luo, Y., Goss, D.J. & Proud, C.G. 2002, "Phosphorylation of Eukaryotic Initiation Factor 4E Markedly Reduces Its Affinity for Capped mRNA", *Journal of Biological Chemistry,* vol. 277, no. 5, pp. 3303-3309.

Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonner-Weir, S. & Kaufman, R.J. 2001, "Translational Control Is Required for the Unfolded Protein Response and In Vivo Glucose Homeostasis", *Molecular cell,* vol. 7, no. 6, pp. 1165-1176.

Schneider, A., Younis, R.H. & Gutkind, J.S. 2008. "Hypoxia-Induced Energy Stress Inhibits the mTOR Pathway by Activating an AMPK/REDD1 Signaling Axis in Head and Neck Squamous Cell Carcinoma1", *Neoplasia,* vol 10, no. 11, pp. 1295–1302.

Schneider, R.J. & Mohr, I. 2003, "Translation initiation and viral tricks", *Trends in biochemical sciences,* vol. 28, no. 3, pp. 130-136.

Sharp, T.V., Moonan, F., Romashko, A., Joshi, B., Barber, G.N. & Jagus, R. 1998, "The Vaccinia Virus E3L Gene Product Interacts with both the Regulatory and the Substrate Binding Regions of PKR: Implications for PKR Autoregulation", *Virology,* vol. 250, no. 2, pp. 302-315.

Sharp, T.V., Schwemmle, M., Jeffrey, I., Laing, K., Mellor, H., Proud, C.G., Hilse, K. & Clemens, M.J. 1993, "Comparative analysis of the regulation of the interferon-inducible protein kinase PKR by Epstein-Barr virus RNAs EBER-1 and EBER-2 and adenovirus VA(I) RNA", *Nucleic acids research,* vol. 21, no. 19, pp. 4483-4490.

Shenk, T.E. & Stinski, M.F. 2004, *Human Cytomegalovirus,* .

Sheridan, B.S., Knickelbein, J.E. & Hendricks, R.L. 2007, "CD8 T cells and latent herpes simplex virus type 1: keeping the peace in sensory ganglia", Expert Opinion on Biological Therapy, vol. 7, no. 9, pp. 1323-1331.

Shimeld, C., Hill, T.J., Blyth, W.A. & Easty, D.L. 1990, "Reactivation of latent infection and induction of recurrent herpetic eye disease in mice", *Journal of General Virology,* vol. 71, no. 2, pp. 397-404.

Shors, T., Kibler, K.V., Perkins, K.B., Seidler-Wulff, R., Banaszak, M.P. & Jacobs, B.L. 1997, "Complementation of Vaccinia Virus Deleted of the E3L Gene by Mutants of E3L", *Virology,* vol. 239, no. 2, pp. 269-276.

Sloan, D.D., Han, J., Sandifer, T.K., Stewart, M., Hinz, A.J., Yoon, M., Johnson, D.C., Spear, P.G. & Jerome, K.R. 2006, "Inhibition of TCR Signaling by Herpes Simplex Virus", *The Journal of Immunology,* vol. 176, no. 3, pp. 1825-1833.

Smith, J. & Robinson, N. . 2002, "Age‐Specific Prevalence of Infection with Herpes Simplex Virus Types 2 and 1: A Global Review", *The Journal of infectious diseases,* vol. 186, pp. S3-S28.

Smith, R.L., Pizer, L.I., Johnson Jr., E.M. & Wilcox, C.L. 1992, "Activation of second-messenger pathways reactivates latent herpes simplex virus in neuronal cultures", *Virology,* vol. 188, no. 1, pp. 311-318.

Sonenberg, N. & Gingras, A. 1998, "The mRNA 5′ cap-binding protein eIF4E and control of cell growth", *Current opinion in cell biology,* vol. 10, no. 2, pp. 268-275.

Spivack, J.G. & Fraser, N.W. 1987, "Detection of herpes simplex virus type 1 transcripts during latent infection in mice.", *The Journal of Virology,* vol. 61, no. 12, pp. 3841-3847.

Stevens, J.G. & Cook, M.L. 1971, "Latent Herpes Simplex Virus in Spinal Ganglia of Mice", *Science,* vol. 173, no. 3999, pp. 843-845.

Stevens, J., Wagner, E., Devi-Rao, G., Cook, M. & Feldman, L. 1987, "RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons", *Science,* vol. 235, no. 4792, pp. 1056-1059.

Stow, E.C. & Stow, N.D. 1989, "Complementation of a Herpes Simplex Virus Type 1 Vmw110 Deletion Mutant by Human Cytomegalovirus", *Journal of General Virology,* vol. 70, no. 3, pp. 695-704.

Su, Y., Meegalla, R.L., Chowhan, R., Cubitt, C., Oakes, J.E., Lausch, R.N., Fraser, N.W. & Block, T.M. 1999, "Human Corneal Cells and Other Fibroblasts Can Stimulate the Appearance of Herpes Simplex Virus from Quiescently Infected PC12 Cells", *The Journal of Virology,* vol. 73, no. 5, pp. 4171-4180.

Subkhankulova, T., Mitchell, S.A. & Willis, A.E. 2001, " Internal ribosome entry segment-mediated initiation of c-Myc protein synthesis following genotoxic stress". Biochem J. 2001 October 1; 359 (Pt 1): 183-192

Subramanian, R.P. & Geraghty, R.J. 2007, "Herpes simplex virus type 1 mediates fusion through a hemifusion intermediate by sequential activity of glycoproteins D, H, L, and B", *Proceedings of the National Academy of Sciences,* vol. 104, no. 8, pp. 2903-2908.

Taddeo, B., Zhang, W. & Roizman, B. 2010, "The role of herpes simplex virus ICP27 in the degradation of mRNA by the virion host shutoff (VHS) RNase", *The Journal of Virology* .

Tal-Singer, R., Podrzucki, W., Lasner, T.M., Skokotas, A., Leary, J.J., Fraser, N.W. & Berger, S.L. 1998, "Use of Differential Display Reverse Transcription-PCR To Reveal Cellular Changes during Stimuli That Result in Herpes Simplex Virus Type 1 Reactivation from Latency: Upregulation of Immediate-Early Cellular Response Genes TIS7, Interferon, and Interferon Regulatory Factor-1", *The Journal of Virology,* vol. 72, no. 2, pp. 1252-1261.

Tamburini, J., Green, A.S., Bardet, V., Chapuis, N., Park, S., Willems, L., Uzunov, M., Ifrah, N., Dreyfus, F., Lacombe, C., Mayeux, P. & Bouscary, D. 2009a, "Protein synthesis is resistant to rapamycin and constitutes a promising therapeutic target in acute myeloid leukemia", *Blood,* vol. 114, no. 8, pp. 1618-1627.

Tamburini, J., Green, A.S., Bardet, V., Chapuis, N., Park, S., Willems, L., Uzunov, M., Ifrah, N., Dreyfus, F., Lacombe, C., Mayeux, P. & Bouscary, D. 2009b, "Protein synthesis is resistant to rapamycin and constitutes a promising therapeutic target in acute myeloid leukemia", *Blood,* vol. 114, no. 8, pp. 1618-1627.

Teleman, A.A., Chen, Y. & Cohen, S.M. 2005, "4E-BP functions as a metabolic brake used under stress conditions but not during normal growth", *Genes & development,* vol. 19, no. 16, pp. 1844-1848.

Terry-Allison, T., Smith, C.A. & DeLuca, N.A. 2007, "Relaxed Repression of Herpes Simplex Virus Type 1 Genomes in Murine Trigeminal Neurons", *The Journal of Virology,* vol. 81, no. 22, pp. 12394-12405.

Thompson, R.L., Cook, M.L., Devi-Rao, G.B., Wagner, E.K. & Stevens, J.G. 1986, "Functional and molecular analyses of the avirulent wild-type herpes simplex virus type 1 strain KOS.", *The Journal of Virology,* vol. 58, no. 1, pp. 203-211.

Thompson, R.L. & Sawtell, N.M. 2006, "Evidence that the Herpes Simplex Virus Type 1 ICP0 Protein Does Not Initiate Reactivation from Latency In Vivo", *The Journal of Virology,* vol. 80, no. 22, pp. 10919-10930.

Thoreen, C.C., Kang, S.A., Chang, J.W., Liu, Q., Zhang, J., Gao, Y., Reichling, L.J., Sim, T., Sabatini, D.M. & Gray, N.S. 2009, "An ATP-competitive Mammalian Target of Rapamycin Inhibitor Reveals Rapamycin-resistant Functions of mTORC1", *Journal of Biological Chemistry,* vol. 284, no. 12, pp. 8023-8032.

Tomoo, K., Shen, X., Okabe, K., Nozoe, Y., Fukuhara, S., Morino, S., Sasaki, M., Taniguchi, T., Miyagawa, H., Kitamura, K., Miura, K. & Ishida, T. 2003, "Structural Features of Human Initiation Factor 4E, Studied by X-ray Crystal Analyses and Molecular Dynamics Simulations", *Journal of Molecular Biology,* vol. 328, no. 2, pp. 365-383.

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. & Miura, K. 2002, Crystal structures of 7-methylguanosine 5'-triphosphate (m(7)GTP)- and P(1)-7-methylguanosine-P(3)-adenosine-5',5'-triphosphate (m(7)GpppA)-bound human full-length eukaryotic initiation factor 4E: biological importance of the C-terminal flexible region. *Biochem J* . vol. 362, pp 539-544

Tsavachidou, D., Podrzucki, W., Seykora, J. & Berger, S.L. 2001, "Gene Array Analysis Reveals Changes in Peripheral Nervous System Gene Expression following Stimuli That Result in Reactivation of Latent Herpes Simplex Virus Type 1: Induction of Transcription Factor Bcl-3", *The Journal of Virology,* vol. 75, no. 20, pp. 9909-9917.

Tullo, A.B., Shimeld, C., Blyth, W.A., Hill, T.J. & Easty, D.L. 1982, "Spread of Virus and Distribution of Latent Infection Following Ocular Herpes Simplex in the Non-immune and Immune Mouse", *Journal of General Virology,* vol. 63, no. 1, pp. 95-101.

Vagner, S., Gensac, M., Maret, A., Bayard, F., Amalric, F., Prats, H. & Prats, A. 1995, "Alternative translation of human fibroblast growth factor 2 mRNA occurs by internal entry of ribosomes", *Molecular and cellular biology,* vol. 15, no. 1, pp. 35-44.

Vanhaesebroeck, B., Leevers, S.J., Panayotou, G. & Waterfield, M.D. 1997, "Phosphoinositide 3-kinases: A conserved family of signal transducers", *Trends in biochemical sciences,* vol. 22, no. 7, pp. 267-272.

Varnum, S.M., Streblow, D.N., Monroe, M.E., Smith, P., Auberry, K.J., Pasa-Tolic, L., Wang, D., Camp, D.G.,II, Rodland, K., Wiley, S., Britt, W., Shenk, T., Smith, R.D. & Nelson, J.A. 2004, "Identification of Proteins in Human Cytomegalovirus (HCMV) Particles: the HCMV Proteome", *The Journal of Virology,* vol. 78, no. 20, pp. 10960-10966.

Volpon, L., Osborne, M.J., Topisirovic, I., Siddiqui, N. & Borden, K.L.B. 2006, "Cap-free structure of eIF4E suggests a basis for conformational regulation by its ligands", *The EMBO journal,* vol. 25, no. 21, pp. 5138-5149.

Von Manteuffel, S.R., Gingras, A.C., Ming, X.F., Sonenberg, N. & Thomas, G. 1996, "4E-BP1 phosphorylation is mediated by the FRAP-p70s6k pathway and is independent of mitogen-activated protein kinase", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 93, no. 9, pp. 4076-4080.

Walsh, D., Arias, C., Perez, C., Halladin, D., Escandon, M., Ueda, T., Watanabe-Fukunaga, R., Fukunaga, R. & Mohr, I. 2008, "Eukaryotic Translation Initiation Factor 4F Architectural Alterations Accompany Translation Initiation Factor Redistribution in Poxvirus-Infected Cells", *Molecular and cellular biology,* vol. 28, no. 8, pp. 2648-2658.

Walsh, D. & Mohr, I. 2006, "Assembly of an active translation initiation factor complex by a viral protein", *Genes & development,* vol. 20, no. 4, pp. 461-472.

Walsh, D. & Mohr, I. 2004, "Phosphorylation of eIF4E by Mnk-1 enhances HSV-1 translation and replication in quiescent cells", *Genes & development,* vol. 18, no. 6, pp. 660-672.

Walsh, D., Perez, C., Notary, J. & Mohr, I. 2005, "Regulation of the Translation Initiation Factor eIF4F by Multiple Mechanisms in Human Cytomegalovirus-Infected Cells", *The Journal of Virology,* vol. 79, no. 13, pp. 8057-8064.

Wang, Q., Zhou, C., Johnson, K.E., Colgrove, R.C., Coen, D.M. & Knipe, D.M. 2005, "Herpesviral latency-associated transcript gene promotes assembly of heterochromatin on viral lytic-gene promoters in latent infection", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 102, no. 44, pp. 16055-16059.

Wang, S., Shih, Y., Ko, W., Wei, Y. & Shih, C. 2008, "Cadmium-induced autophagy and apoptosis are mediated by a calcium signaling pathway", *Cellular and Molecular Life Sciences,* vol. 65, no. 22, pp. 3640-3652.

Wang, X., Flynn, A., Waskiewicz, A.J., Webb, B.L.J., Vries, R.G., Baines, I.A., Cooper, J.A. & Proud, C.G. 1998, "The Phosphorylation of Eukaryotic Initiation Factor eIF4E in Response to Phorbol Esters, Cell Stresses, and Cytokines Is Mediated by Distinct MAP Kinase Pathways", *Journal of Biological Chemistry,* vol. 273, no. 16, pp. 9373-9377.

Wang, X., Li, W., Parra, J., Beugnet, A. & Proud, C.G. 2003, "The C Terminus of Initiation Factor 4E-Binding Protein 1 Contains Multiple Regulatory Features That Influence Its Function and Phosphorylation", ***Molecular and cellular biology,*** **vol. 23, no. 5, pp. 1546-1557.**

Waskiewicz, A.J., Flynn, A., Proud, C.G. & Cooper, J.A. 1997, "Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2", ***The EMBO journal,*** **vol. 16, no. 8, pp. 1909-1920.**

Waskiewicz, A.J., Johnson, J.C., Penn, B., Mahalingam, M., Kimball, S.R. & Cooper, J.A. 1999, "Phosphorylation of the Cap-Binding Protein Eukaryotic Translation Initiation Factor 4E by Protein Kinase Mnk1 In Vivo", ***Molecular and cellular biology,*** **vol. 19, no. 3, pp. 1871-1880.**

Watson, R.J. & Clements, J.B. 1980, "A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis", ***Nature,*** **vol. 285, no. 5763, pp. 329-330.**

Wheeler, C.E. 1958, "The Effect of Temperature upon the Production of Herpes Simplex Virus in Tissue Culture", ***The Journal of Immunology,*** **vol. 81, no. 2, pp. 98-106.**

Whitley, D., Goldberg, S.P. & Jordan, W.D. 1999, "Heat shock proteins: A review of the molecular chaperones, ,", ***Journal of Vascular Surgery,*** **vol. 29, no. 4, pp. 748-751.**

Wigdahl, B., Scheck, A., De Clercq, E. & Rapp, F. 1982, "High efficiency latency and activation of herpes simplex virus in human cells", ***Science,*** **vol. 217, no. 4565, pp. 1145-1146.**

Wigdahl, B.L., Isom, H.C. & Rapp, F. 1981, "Repression and Activation of the Genome of Herpes Simplex Viruses in Human Cells", ***Proceedings of the National Academy of Sciences of the United States of America,*** **vol. 78, no. 10, [Part 2: Biological Sciences], pp. 6522-6526.**

Wigdahl, B.L., Ziegler, R.J., Sneve, M. & Rapp, F. 1983, "Herpes simplex virus latency and reactivation in isolated rat sensory neurons", ***Virology,*** **vol. 127, no. 1, pp. 159-167.**

Wilcox, C.L. & Johnson, E.M.,Jr 1988, "Characterization of nerve growth factor-dependent herpes simplex virus latency in neurons in vitro.", ***The Journal of Virology,*** **vol. 62, no. 2, pp. 393-399.**

Wilcox, C.L. & Johnson, E.M.,Jr 1987, "Nerve growth factor deprivation results in the reactivation of latent herpes simplex virus in vitro.", ***The Journal of Virology,*** **vol. 61, no. 7, pp. 2311-2315.**

Willey, D., Trousdale, M. & Nesburn, A. 1984, "Reactivation of murine latent HSV infection by epinephrine iontophoresis", ***Investigative Ophthalmology Visual Science,*** **vol. 25, no. 8, pp. 945-950.**

Williams, D.D., Price, N.T., Loughlin, A.J. & Proud, C.G. "Characterization of the Mammalian Initiation Factor eIF2B Complex as a GDP Dissociation Stimulator Protein", ***Journal of Biological Chemistry,*** **vol. 276, no. 27, pp. 24697-24703.**

Willis, A.E. 1999, "Translational control of growth factor and proto-oncogene expression", ***The international journal of biochemistry & cell biology,*** **vol. 31, no. 1, pp. 73-86.**

Wilson, J.E., Pestova, T.V., Hellen, C.U.T. & Sarnow, P. 2000, "Initiation of Protein Synthesis from the A Site of the Ribosome", ***Cell,*** **vol. 102, no. 4, pp. 511-520.**

Wysocka, J. & Herr, W. 2003a, "The herpes simplex virus VP16-induced complex: the makings of a regulatory switch", ***Trends in biochemical sciences,*** **vol. 28, no. 6, pp. 294-304.**

Xu, Z., Zhang, Y., Jiang, J., Yang, Y., Shi, R., Hao, B., Zhang, Z., Huang, Z., Kim, J. & Zhang, G. 2010, "Epidermal growth factor induces HCCR expression via PI3K/Akt/mTOR signaling in PANC-1 pancreatic cancer cells", ***BMC Cancer,*** **vol. 10, no. 1, pp. 161.**

Yang, Q., Inoki, K., Ikenoue, T. & Guan, K. 2006, "Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity", ***Genes & development,*** **vol. 20, no. 20, pp. 2820-2832.**

Young, S.K., Rowe, N.H. & Buchanan, R.A. 1976, "A clinical study for the control of facial mucocutaneous herpes virus infections : I. Characterization of natural history in a professional school population", ***Oral Surgery, Oral Medicine, Oral Pathology,*** **vol. 41, no. 4, pp. 498-507.**

Yueh, A. & Schneider, R.J. 1996, "Selective translation initiation by ribosome jumping in adenovirus-infected and heat-shocked cells.", ***Genes & development,*** **vol. 10, no. 12, pp. 1557-1567.**

Yueh, A. & Schneider, R.J. 2000, "Translation by ribosome shunting on adenovirus and hsp70 mRNAs facilitated by complementarity to 18S rRNA", ***Genes & development,*** **vol. 14, no. 4, pp. 414-421.**

Yuhasz, S.A. & Stevens, J.G. 1993, "Glycoprotein B is a specific determinant of herpes simplex virus type 1 neuroinvasiveness.", ***The Journal of Virology,*** **vol. 67, no. 10, pp. 5948-5954.**

Zaborowska, I. & Walsh, D. 2009, "PI3K Signaling Regulates Rapamycin-Insensitive Translation Initiation Complex Formation in Vaccinia Virus-Infected Cells", ***The Journal of Virology,*** **vol. 83, no. 8, pp. 3988-3992.**

Zachos, G., Clements, B. & Conner, J. 1999, "Herpes Simplex Virus Type 1 Infection Stimulates p38/c-Jun N-terminal Mitogen-activated Protein Kinase

Pathways and Activates Transcription Factor AP-1", *Journal of Biological Chemistry,* vol. 274, no. 8, pp. 5097-5103.

Zhang, P., McGrath, B.C., Reinert, J., Olsen, D.S., Lei, L., Gill, S., Wek, S.A., Vattem, K.M., Wek, R.C., Kimball, S.R., Jefferson, L.S. & Cavener, D.R. 2002, "The GCN2 eIF2{alpha} Kinase Is Required for Adaptation to Amino Acid Deprivation in Mice", *Molecular and cellular biology,* vol. 22, no. 19, pp. 6681-6688.

Zhang, Y., Feigenblum, D. & Schneider, R.J. 1994, "A late adenovirus factor induces eIF-4E dephosphorylation and inhibition of cell protein synthesis.", *The Journal of Virology,* vol. 68, no. 11, pp. 7040-7050.

Zhu, X.X., Chen, J.X., Young, C.S. & Silverstein, S. 1990, "Reactivation of latent herpes simplex virus by adenovirus recombinants encoding mutant IE-0 gene products.", *The Journal of Virology,* vol. 64, no. 9, pp. 4489-4498.

Zhu, Z., Cai, W. & Schaffer, P.A. 1994, "Cooperativity among herpes simplex virus type 1 immediate-early regulatory proteins: ICP4 and ICP27 affect the intracellular localization of ICP0.", *The Journal of Virology,* vol. 68, no. 5, pp. 3027-3040.