MOLECULAR VIROLOGY OF KSHV:
ELUCIDATING VIRF2 AND VIRF4 FUNCTION

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ABSTRACT

The innate type I interferon antiviral response is the first line of defence invoked to limit the spread of viral infections. Hence a number of viruses including Kaposi's sarcoma-associated herpesvirus (KSHV) have evolved defence strategies against this antiviral response. KSHV is the aetiologic agent of KS and almost one quarter of the KSHV genes specify either demonstrated or potential immunomodulatory activity including the four viral interferon regulatory factors (vIRFs). vIRFs 1, 2 and 3 have previously been shown to inhibit type I IFN signalling, whereas the inhibitory role of vIRF4 is yet to be reported. A previous stable isotope labelling of amino acids in cell culture (SILAC) study coupled to LC-MS/MS identified USP7 and ribosomal proteins as binding partners of both vIRF2 and vIRF4. The aim of the present study was to investigate the role of these binding partners in type I IFN signalling and to determine the regulatory mechanisms behind the effects of these partner proteins on the functions of the vIRF2 and vIRF4 proteins. Polysome profiling and microarray studies were carried out on vIRF4 expressing cells and suggested a novel regulatory role for vIRF4 in translation. USP7 was also characterised as a positive regulator of IFN signalling and the mechanism behind this effect was explored.
I am grateful to God for the gift of life which paved way for the opportunity to do as much as I have done so far. "So then it is not of him that wills, nor of him that runs, but of God that shows mercy" (Romans 9: 16 AKJV).

I am eternally grateful to Prof David Blackbourn for accepting to supervise me for a Ph.D. in his Lab. Thank you for believing in me and being fully supportive of me in all situations. The desire not to disappoint you has been the greatest drive and motivation for my Ph.D. I also thank Dr Nicolas Locker my co-supervisor for his help at every stage of this work and also for helping me to find my feet in the lab during the early stage. I am grateful to him for always finding time to help out with my work despite his heavy load of responsibilities.

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I am fortunate to be married to my best friend, Akinola who has not only kept the home base alive but continuously reassured me of his love and care while I pursued my dreams far away from our home. I feel immensely blessed to be a mother to Ayomikun Esther, Onaopemipo Grace and Oluwadarasimi Emmanuel, all of whom have consistently remained on top of their game despite having lacked motherly attention for this period.
DEDICATION

This thesis is dedicated to my daughters; Ayomikun Esther and Onaopemipo Grace and all female African children, so you know that no one except yourself, can put a ceiling on your ambition as a girl-child.
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<tr>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSHV</td>
<td>Kaposi's Sarcoma-Associated herpesvirus</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr Virus</td>
</tr>
<tr>
<td>RRV</td>
<td>Rhesus Rhadinovirus</td>
</tr>
<tr>
<td>HHV</td>
<td>Human Herpesvirus</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>KS</td>
<td>Kaposi's Sarcoma</td>
</tr>
<tr>
<td>PEL</td>
<td>Primary effusion lymphoma</td>
</tr>
<tr>
<td>MCD</td>
<td>Multicentric Castleman's disease</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>vIRF</td>
<td>Viral Interferon Regulatory Factor</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer And Activator Of Transcription</td>
</tr>
<tr>
<td>ISGF3</td>
<td>Interferon Stimulated Gene Factor 3</td>
</tr>
<tr>
<td>ISGs</td>
<td>Interferon Stimulated Gene</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon Stimulated Response Element</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HVS</td>
<td>Herpesvirus saimiri</td>
</tr>
<tr>
<td>FLIP</td>
<td>FLICE inhibitory protein</td>
</tr>
<tr>
<td>LUR</td>
<td>Long unique region</td>
</tr>
<tr>
<td>TR</td>
<td>Terminal repeats</td>
</tr>
<tr>
<td>HS</td>
<td>heparan sulphate</td>
</tr>
<tr>
<td>GSK</td>
<td>Glycogen synthetase kinase</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>EpHA2</td>
<td>Ephrin-A2</td>
</tr>
<tr>
<td>X CT</td>
<td>Cysteine/glutamate transporter</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>LANA</td>
<td>Latency-associated nuclear antigen</td>
</tr>
<tr>
<td>v-Cyclin</td>
<td>Viral-cyclin</td>
</tr>
<tr>
<td>v-FLIP</td>
<td>Viral-Flice-inhibitory protein</td>
</tr>
<tr>
<td>vIL6</td>
<td>Viral-Interleukin 6</td>
</tr>
<tr>
<td>RTA</td>
<td>replication transcriptional activator</td>
</tr>
<tr>
<td>RBPJκ</td>
<td>recombination signal sequence-binding protein Jκ</td>
</tr>
<tr>
<td>IE</td>
<td>Immediate early</td>
</tr>
<tr>
<td>E</td>
<td>Early</td>
</tr>
<tr>
<td>L</td>
<td>Late</td>
</tr>
<tr>
<td>Mdm2</td>
<td>Murine double minute 2</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>IFNγR</td>
<td>Interferon gamma receptor</td>
</tr>
<tr>
<td>IFNαR</td>
<td>Interferon alpha receptor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>HOMEBOX</td>
<td>HOX</td>
</tr>
<tr>
<td>S</td>
<td>subunit</td>
</tr>
<tr>
<td>RP</td>
<td>Ribosomal protein</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>KCP</td>
<td>KSHV complement regulatory protein</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase R</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

1.1. Overview of Kaposi’s sarcoma-associated herpesvirus

Kaposi’s sarcoma-associated herpesvirus (KSHV) belongs to a family of large sized DNA viruses called the *Herpesviridae* (Ganem, 2007). The *Herpesviridae* consists of at least 12 different members known to infect animal species. They exhibit a large degree of similarity in their structure and mode of replication, as well as the potential to establish latent infections (Kemény *et al.*, 1997). The eight human herpesviruses (HHV) fall under three subfamilies; the alpha, beta and gamma herpesviruses. The gamma herpesviruses form the third subfamily and include Epstein Barr virus (EBV) and KSHV (Table 1-1).

The gamma herpesviruses have been further subdivided into two genera; the lymphocryptoviruses, which includes EBV and the rhadinoviruses to which KSHV belongs (Wong, 2005) (Liao, 2006). Both EBV and KSHV have been strongly implicated in the aetiology of some human tumours. KSHV has a plethora of gene products that encode proteins with immune regulatory activity (reviewed in Aresté & Blackbourn, 2009). These viral gene products subvert the normal cellular pathways involved in cell cycle progression, immune surveillance, apoptosis and other antiviral responses (Wen & Damania, 2010).

This chapter will focus on KSHV in relation to its associated disease forms, and its immunomodulatory effects. Additional details on USP7 structure and interactions as well as its role in antiviral immunity will be discussed. Lastly, viruses and modulation of translational control will be described.
Table 1-1: Classification of Herpesviruses

This table shows the human herpesviruses in their respective sub-families (Based on Davison et al, 2009)

<table>
<thead>
<tr>
<th>FAMILY</th>
<th>SUBFAMILY</th>
<th>SPECIFIC EXAMPLES</th>
<th>ALTERNATE NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herpesviridae</td>
<td>Alphaherpesvirinae</td>
<td>Herpes simplex virus 1 &lt;br&gt; Herpes simplex virus 2 &lt;br&gt; Varicella zoster virus</td>
<td>HSV-1 &lt;br&gt; HSV-2 &lt;br&gt; VZV;HHV-3</td>
</tr>
<tr>
<td></td>
<td>Betaherpesvirinae</td>
<td>Cytomegalovirus &lt;br&gt; Human herpesvirus 6a &lt;br&gt; Human herpesvirus 6b &lt;br&gt; Human herpesvirus 7</td>
<td>CMV;HHV-5 &lt;br&gt; HHV-6a &lt;br&gt; HHV-6a &lt;br&gt; HHV-7</td>
</tr>
<tr>
<td></td>
<td>Gammaherpesvirinae</td>
<td>Epstein Barr virus &lt;br&gt; Kaposi’s sarcoma-associated herpesvirus</td>
<td>EBV;HHV-4 &lt;br&gt; KSHV;HHV-8</td>
</tr>
</tbody>
</table>

1.1.1. Discovery of KSHV

Dr Moritz Kaposi, a Hungarian dermatologist, was the first to describe Kaposi’s sarcoma (KS) in 1872 (Kaposi, 1872). The rising prevalence of KS among homosexual and heterosexual HIV/AIDS patients strongly suggested the possibility of an infectious aetiology. Valerie Beralt, (reviewed in Peterman et al., 1993) studied the epidemiology of KS in HIV-infected people and suggested a possible viral aetiology of KS. The hypothesis of a possible viral aetiology for KS led many to search for a causative agent. In 1994, Chang and Moore, identified new fragments of DNA in KS tissue using representational difference analysis (Chang et al., 1994). These fragments of DNA belonged to a virus later named KSHV, now recognized as the causative agent for KS and primary effusion lymphoma (PEL) (Bouvard et al., 2009). PEL is a rare form of lymphoid hyperplasia often seen in patients with end stage AIDS. Subsequent to this discovery, further studies have confirmed that 95% of KS lesions contain KSHV sequences (Ganem, 2006). A form of multicentric angiofollicular lymphoid hyperplasia was also later found to contain KSHV DNA and thus referred to as multicentric Castleman’s disease (MCD) (Soulier et al., 1995). These three malignancies are discussed further in 1.2.
1.1.2. Structure of KSHV

All herpesviruses share the same morphology comprising: the DNA core, the capsid, the tegument and the outer envelope with glycoprotein spikes. Electron microscopy of the KSHV virion reveals an icosahedral protein capsid surrounding the DNA core. The capsid is made up of 162 capsomeres and measures approximately 100nm in diameter (Figure 1-1). It is surrounded by a protein coat called the tegument. More recently it was discovered that the virion tegument layer, is not an amorphous protein layer but is made up of organised proteins that interact with each other and with other virion capsid and envelope proteins. The KSHV tegument proteins perform essential functional roles in virus host interactions (reviewed in Sathish et al., 2012; Sathish et al., 2012). The outermost layer is a lipid envelope decorated with glycoprotein spikes which facilitate KSHV entry (see section 1.1.4).

**Figure 1-1: Electron microscopy of KSHV virion**

The KSHV virions showing the nucleocapsid surrounded by the viral envelope. Reproduced from Milligan et al., 2004.
1.1.3. **KSHV genome**

The KSHV genome is a double-stranded DNA molecule ranging in size from 165 to 170 Kilo base (kb) pairs. The long unique region (LUR) measures about 140kb and contains all the open reading frames (ORF). It is flanked by multiple terminal repeat (TR) sequences at both ends (Figure 1-2) (Wen & Damania, 2010).

The KSHV genes can be classified into three major categories: 1) the genes similar to those of the other members of the herpesviridae; 2) the genes designated “K” which are peculiar to the KSHV; 3) the genes that share homology with cellular genes and may be found in other herpesviruses (Wong, 2005). The KSHV-specific ORFs are designated K1 to K15 in relation to their loci on the KSHV genome (Figure 1-2). Different KSHV isolates have varied numbers of TRs, which range from 61 to 75, and this variation accounts for the disparity in genome sizes (Duprez et al., 2007).

About 50% of the KSHV genome is committed to modulation of the host environment to facilitate the survival of the virus. The various genes encode for proteins that interfere with such processes as the host cell cycle regulation, apoptosis, signal transduction and immune responses (reviewed in Rezaee et al., 2006). Studies have also identified a total of 12 microRNAs (miRNA) within the KSHV genome (Cai et al., 2005; Pearce et al., 2005; Pfeffer et al., 2005a; Samols et al., 2005). Two of the miRNAs are located within the K12 ORF, while the rest are confined to the non-coding region between ORF K12.1 (Kaposin) and ORF K13/ORF (v-FLIP). Recent ribo sequencing data has revealed a large cluster of sequence reads from outside of the existing coding regions. Two of these newly discovered regions include the 10 kb antisense RNA to the latent transcripts (ALT) and the 17 kb K1-ORF11 antisense (K1/11-AS) (Arias et al., 2014).
The map shows that the genome of KSHV consists of a long unique region (LUR) which measures about 140kb and contains all the open reading frames (ORF). It is flanked by multiple terminal repeat (TR) sequences at both ends. The sequence is depicted here as U followed by a single copy of TR, which is shown in a broader format. Protein-coding regions are indicated by coloured arrows grouped according to the key, with gene nomenclature below and names of the encoded immunomodulatory proteins. This figure was reproduced from Areste & Blackbourn, 2009.
1.1.4. **KSHV replication**

The initial step in KSHV replication is the entry of the virus into the host cell which is facilitated by the binding of its envelope glycoproteins to the heparan sulfate receptor. Three major envelope glycoproteins have been shown to be important in the membrane fusion process, these are gB, gH and gL. The KSHV virion envelope glycoproteins H and L (gH/gL) form a dimeric complex that facilitates their entry into the host cell (Hahn et al., 2012). The KSHV complement regulatory protein (KCP), encoded by ORF4, also participates in cell infection (Spiller et al., 2006). A cysteine/glutamate transporter, xCT, has been considered as a possible receptor site for the KSHV (Kaleeba & Berger, 2006). This observation was based on the fact that non-permissive cell lines have their permissivity restored following the use of recombinant xCT expressing cells (Kaleeba & Berger, 2006). Furthermore, antibodies developed against xCT successfully prevented KSHV infection (Kaleeba & Berger, 2006). xCT forms a heterodimer with CD98 to form a complex which associates with α3β1, a cell surface factor thereby further supporting the fact that integrins also play a role in facilitating KSHV entry (Ganem, 2007). CD98 is a multifunctional protein previously referred to as fusion regulation protein 2 (FRP-2). FRP-2 interacts with integrin α3(FRP-1) to play important roles in fusion between two cells (Veettil et al., 2008). There is a possibility that multiple entry pathways exist for KSHV, including the possibility of binding to cellular integrin α3β1 as a potential receptor (Akula et al., 2002). DC-SIGN, a C-type lectin, has also been identified as an entry receptor for KSHV on dendritic cells and macrophages. Thus, the expression of Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN is important for infection and replication of KSHV in B cells (Rappocciolo et al., 2006). KSHV envelope-associated gB contains a tripeptide Arg-Gly-Asp (RGD) motif that binds the integrin α3β1 during virus entry. The role of α3β1 in KSHV binding, entry and downstream signalling is controversial. More recently it was demonstrated that αVβ3 rather than α3β1 binds gB and mediates both the cell adhesion and entry of
KSHV into target cells (Garrigues et al., 2008). The phosphorylation and activation of focal adhesion kinase (FAK) and ephrin receptor tyrosine kinase A2 (EphA2) are important in KSHV entry (Hahn et al., 2012). KSHV induces the interaction of integrins with EphA2 and the adapter protein c-Cbl. The increased rates of KSHV infections upon overexpression of EphA2 supports a vital role of EphA2 in the infection of endothelial cells (Boshoff, 2012; Hahn et al., 2012).

Following entry, the viral capsid is released into the cytoplasm and transported to the nuclear membrane, where the capsid releases the viral genome into the nucleus (Chandran, 2010). KSHV undergoes both latent and lytic cycles of replication. During the latency period, there is restricted viral gene expression that allows the virus to persist in a state of dormancy. The viral genome is, still maintained with a potential for possible virion production subsequently as a result of lytic replication. Latency is discussed in more detail below (section 1.2.1).

The lytic phase of KSHV replication is characterised by host cell lysis during the production of new progeny viruses that necessitates the expression of a much greater range of KSHV gene products than latency. During the lytic phase the expression of viral transcripts occurs in an ordered format. The immediate early (IE) genes are first produced followed by the delayed-early (DE) and the late (L) genes (Dittmer, 2003; Fakhari & Dittmer, 2002; Jenner et al., 2001). Among the IE genes, there is a switch protein known as replication and transcriptional activator (RTA), which is encoded by ORF50 and is a key inducer of lytic replication. This protein is discussed in more detail below (section 1.3.1). The DE genes are responsible for preparing the cells for viral DNA replication and protein synthesis. The L genes are expressed following viral DNA replication and mediate the assembly and release of viral products. The lytic phase of the KSHV lifecycle is completed when viral DNA replication, packaging of the capsid, virion maturation and egress occur.
Co-infection with other herpesviruses such as HSV 1, CMV and HHV-6 has been postulated to cause KSHV reactivation (Lu et al., 2005; Qin et al., 2008; Vieira et al., 2001). One mechanism by which this reactivation can occur is by activating Toll-like receptors 7 and 8 (TLR 7/8) (Gregory et al., 2009). Both the lytic and latent phases contribute significantly, albeit in varied measures, to the pathogenesis of KSHV tumorigenesis and are discussed below.

1.2. Viral genes involved in maintenance of KSHV latency

Three of the latency genes, latency associated nuclear antigen (LANA), v-Cyclin and v-FLIP are encoded by one transcription unit while the others comprising 25 matured miRs, three kaposin transcripts, a bicistronic transcript for vCyclin and vFLIP, are encoded from a second independent promoter (Ganem, 2006; Ye et al., 2011).

1.2.1. Latency associated nuclear antigen (LANA)

LANA, a product of ORF 73 is the most widely used marker of KSHV infection (Kellam et al., 1997; Rainbow et al., 1997). This multifunctional protein is present in KS spindle cells, see Figure 1-3, as well as in B-cells of MCD and PEL (Dupin et al., 1999). LANA tethers the viral episome to the host chromosome during the mitotic phase to ensure partitioning of the viral genome into daughter cells (Ballestas et al., 1999). LANA preferentially binds KSHV terminal repeats (TR) to form a DNA /protein complex. The LANA-TR complex is sufficient for episomal DNA replication and possibly recruits the cellular DNA replication machinery to complete viral DNA replication (Fejér et al., 2003; Ye et al., 2011). LANA also interferes with the function of the tumour suppressor genes Rb and P53 by binding to and inactivating the products of these genes (Ye et al., 2011). The tumour suppressor genes are discussed in more detail in section 1.5.1.

The β-catenin protein has been found to play a role in cell-to-cell adhesion and the Wnt signal transduction pathway (Olmeda et al., 2003). Other functions of β-Catenin include stimulation of cell proliferation in different tumours, as well as modulation of programmed cell death. The ability of LANA to sequester the β-catenin inhibitor,
glycogen synthetase kinase 3β (GSK-3β), appears to be responsible for its enhancing entry of cells into the synthetic phase (Fujimuro et al., 2005). This is because GSK-3β targets and inactivates β-catenin through ubiquitin mediated proteolysis. LANA plays a major role in KSHV associated neoplasia through the maintenance of latency, inhibition of replication and interference with cell cycle control.

Figure 1-3: LANA expression in the spindle cells of KS lesions

KS biopsy from a nodular lesion was dual stained to show the nuclear staining of KSHV LANA (N) along the endothelial cell cytoplasm (E) which is stained red for vascular endothelial growth factor (VEGF-C). This Figure was reproduced from Colman and Blackbourn, 2008.

1.2.2. V-FLIP

Apoptosis is also known as programmed cell death, and it plays a critical role in maintaining tissue homeostasis in multicellular organisms (Krueger et al., 2001). Death receptors such as Fas ligand, TNF alpha and TNF-related apoptosis-inducing ligand (TRAIL) are responsible for transmitting apoptotic signals initiated by specific
ligands. The TNF-R1-associated death domain protein (TRADD) is an adaptor protein that interacts with TNFR1/p55 and mediates programmed cell death signalling and NF-Kappa B activation (Khwaja & Tatton, 1999). The death domain of TRADD associates with the Fas associated protein with death domain (FADD) (Muzio et al., 1996). FADD, via its death effector domain (DED) triggers the activation of caspase 8, resulting in a cascade of events and eventually apoptotic cell death (Muzio et al., 1997).

The viral Flice inhibitory protein (v-FLIP) also known as K13 is encoded by ORF 71 (Dittmer et al., 1998; Fakhari & Dittmer, 2002; Jenner et al., 2001). v-FLIP is a homologue of the host FLICE inhibitory protein. FLICE is the acronym for FADD-like-interleukin-1 beta converting enzyme. The FLIPs block early signalling events of the death receptors by binding to adaptor proteins (TRADD and FADD) of the Fas/TNFR signalling pathway. This interaction inhibits the activation of caspase 8, and therefore Fas-mediated death induction (Ganem, 2006). v-FLIPs also bind to the IKappa B kinase (IKK) and heat shock protein (hsp 90), thereby promoting phosphorylation of IKB and release of the antiapoptotic transcription factor, NF-kB.

1.2.3. V-cyclin

Viral Cyclin (v-Cyclin), encoded by ORF72, is a viral homologue of Cyclin D. It binds and inactivates CDK 6 by forming a v-Cyclin-CDK6 complex. This complex phosphorylates the pRb protein, as well as a host of other unique cellular substrates resulting in aberrant cell proliferation and activation of DNA damage response and chromosomal instability (Koopal et al., 2007; Ye et al., 2011). The v-Cyclin-CDK6 complex has reduced sensitivity to CDK inhibitors such as p16, p21 and p27. Another action of v-Cyclin-CDK6 kinase complex is the phosphorylation of nucleophosmin (NPM), a nuclear phosphoprotein, thereby stabilising the LANA-nucleophosmin interaction. The interaction between nucleophosmin and LANA enhances the silencing of KSHV lytic genes, thereby ensuring that a latent state is maintained.
Previous studies have shown that NPM recruits histone deacetylase (HDAC 1) as a corepressor during differentiation (Liu et al., 2007). The HDACs interact directly and indirectly with viral sequences to prevent the expression of lytic genes (Gwack et al., 2001).

1.2.4. Kaposins

The Kaposin transcripts A, B and C are the most abundantly expressed viral transcripts during KSHV latency (Wen & Damania, 2010). Kaposin is expressed as a latent transcript in KS and PEL cells and is also strongly induced during lytic replication by RTA due to the presence of at least one affinity binding site for the lytic switch protein. The 25-bp homologous region of the Kaposin promoter shares remarkable homology to the polyadenylated RTA response element (PAN RRE) and has been identified as a binding site for the RTA (Sadler et al., 1999; Staskus et al., 1999). Kaposin B binds to and activates the kinase, MAP kinase-activated protein kinase 2 (MK2), a target of the p38 mitogen-activated protein kinase signalling pathway thus facilitating mRNA decay (McCormick & Ganem, 2005).

1.2.5. Latent KSHV microRNAs

miRNAs are small non-coding RNAs which regulate gene expression leading to degradation and repression of targeted mRNAs (Cullen, 2006). As described above, ten of the twelve KSHV miRNAs are found between v-FLIP and Kaposin, while the remaining two are found within the coding region (miR-K10) and 3’ UTR of ORF K12. The twelve miRNAs are expressed during latency phase. However, both K10 and K12 expression is further increased during lytic induction (Cai et al., 2005; Pfeffer et al., 2005b; Samols et al., 2005). An in vivo study was conducted in a strain of inbred laboratory mice that lack mature T and B cells, natural killer (NK) and are also deficient in multiple cytokine signaling pathways (NOD/LtSz-scid IL2Rnull ) mice. The finding from this study revealed that mice transplanted with foamy virus vectors expressing the miRNAs from human hematopoietic progenitors proved that miR-K12-
11 is a functional mimic of an oncogenic human miRNA and functions as a key regulator of hematopoiesis and B-cell differentiation (cellular miR-155). miR-155 is an miRNA with tumourigenic ability (Skalsky et al., 2007). The finding of sequence identity between these two miRNAs, one viral and one cellular, could explain in part KSHV tumourigenesis (Boss et al., 2011).

1.3. Lytic cycle and pathogenesis of KS

Lytic replication provides the platform for initiation of KSHV infection and dissemination of the virus in the host (Rezaee et al., 2006). In KS lesions, lytic replication ensures continuous maintenance of the population of latently infected cells that otherwise are quickly lost by segregation of latent viral episomes during the division of spindle cells (Grundhoff & Ganem, 2004). KS development involves deregulated cell proliferation, inflammation and angiogenesis. KSHV-infected cells during the lytic cycle produce signalling molecules whose paracrine effects result in inflammation and angiogenesis. The signalling molecules include the chemokines and vIL6. The chemokines belong to a large family of cytokines that mediate leukocyte chemotaxis during inflammation (reviewed by Rot & Von, 2004). The chemokines act by binding to a family of specific G protein-coupled receptors (GPCRs) (see review by Murphy, 2002). The viral GPCR upregulates many host genes including VEGF, which mediates angiogenesis and increased vascular permeability (Pati et al., 2001; Schwarz & Murphy, 2001). The vIL6 protein also stimulates angiogenesis and inhibits STAT2 phosphorylation by type 1 interferon receptors (Aoki et al., 2003).

1.3.1. Replication and transcription activator (RTA)

The switch from latent to lytic cycle is mediated by RTA, a product of ORF 50, an IE gene. RTA binds directly to the DNA of several KSHV promoters (Bu et al., 2007). It is necessary and sufficient to complete the lytic reactivation process (Guito & Lukac, 2012; Lukac et al., 1998). RTA binds most avidly to a 16bp sequence found in the
PAN and Kaposin promoters as well as to ori-Lyt, the origin of lytic DNA replication. These are duplicate copies, nuclear ori-Lyt (L) and (R) and are found between KSHV ORFK4.2 and K5 and between K12 and ORF71, respectively (AuCoin et al., 2002; Lin et al., 2003). The binding of RTA to ori-Lyt mediates transactivation and the formation of a replication complex which is required for DNA replication. RTA can also induce transcription through its interaction with transcriptional control proteins RBPJκ, C/EBPα, API and HMGB, all of which recruit additional co-activators of transcription (Guito & Lukac, 2012).

1.4. KSHV transmission

The highest viral load of KSHV, as measured by DNA levels was present in samples of saliva than in other body fluids sample. Hence KSHV is principally spread in saliva secretions (Pauk et al., 2000). Oral sex and deep kissing increase the chance of acquiring the virus (Casper et al., 2004). The finding of KSHV DNA in tissue biopsy from the urogenital tract as well as in male ejaculates suggests the possibility of a sexual mode of transmission (Martin et al., 1998). Other researchers have also confirmed the sexual route as a possible means of KSHV spread (Levy, 1997). Blood transfusion is another remote but possible route of acquiring KSHV infection, particularly in immunosuppressed persons (Blackbourn et al., 1997; Lefrere et al., 1997). Vertical transmission in infected pregnant women occurs infrequently and HHV-8 sequences have been discovered in the peripheral blood mononuclear cells (PBMC) of infected mothers (Brayfield et al., 2003; Gessain & Duprez, 2005). Organ transplantation is also a viable means of acquiring the KSHV virus due to post transplant immune suppression (Schulz, 2006).

Primary KSHV infection is not well recognised because of its low incidence among studied populations. KSHV seroconversion states have been associated with varied clinical manifestations such as fatigue, diarrhoea, mild lymphadenopathy, and localised rashes (Edelman, 2005).
1.5. Viruses and cancer

The history of oncogenic viruses dates back to the 19th century (Moore & Chang, 2010; reviewed in Moore & Chang, 2010). One of the earliest publications on tumour viruses by Ellerman and Bang described the putative viral transmission of avian erythroblastosis (Ellerman V., 1908). In 1964, Epstein et al. discovered the first human tumour virus which was named EBV (Epstein et al., 1964).

About 15% of all human cancers have been attributed to viral agents. In 2008, two million new cases of cancer worldwide were ascribed to infectious aetiology (Danaei, 2012). The incidence of infection associated cancer is higher in developing countries compared with the developed nations (Ferlay et al., 2010). The interplay between viral infections, genetic factors and somatic mutations against a background of immunosuppression and exposure to carcinogens all contribute to the development of cancers (de Martel et al., 2012; Parkin, 2011). Table 1-1 below summarises the viral agents that have been linked with human tumours.
Table 1-2: Viruses causing human cancer

<table>
<thead>
<tr>
<th>NAME OF VIRUS</th>
<th>ASSOCIATED CANCER TYPE</th>
<th>YEAR OF VIRUS DISCOVERY</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epstein Barr virus (EBV)</td>
<td>Burkitts’ lymphoma, Hodgkin’s lymphoma, Nasopharyngeal carcinoma</td>
<td>1964</td>
<td>Epstein et al., 1964</td>
</tr>
<tr>
<td>Hepatitis B virus (HBV)</td>
<td>Hepatocellular carcinoma</td>
<td>1980</td>
<td>Beasley et al., 1981</td>
</tr>
<tr>
<td>Human papilloma virus (HPV)</td>
<td>Epidermodysplasia verruciformis</td>
<td>1970</td>
<td>Orth et al., 1980</td>
</tr>
<tr>
<td>Human T lymphotropic virus (HTLV)</td>
<td>Adult T cell Leukemia</td>
<td>1980</td>
<td>Poiesz et al., 1981</td>
</tr>
<tr>
<td>Human papilloma virus (HPV)</td>
<td>Cervical, anal, penile, vulva, vagina and oropharyngeal cancer</td>
<td>1984</td>
<td>Boshart et al., 1984</td>
</tr>
<tr>
<td>Hepatitis C virus (HCV)</td>
<td>Hepatocellular carcinoma</td>
<td>1987</td>
<td>Durst et al., 1987</td>
</tr>
<tr>
<td>Kaposi’s sarcoma associated Herpes virus (KSHV)</td>
<td>Kaposi sarcoma</td>
<td>1990</td>
<td>Chang et al., 1994</td>
</tr>
<tr>
<td>Merkel cell polyomavirus (MCPyV)</td>
<td>Merkel cell carcinoma</td>
<td>2008</td>
<td>Feng et al., 2008</td>
</tr>
</tbody>
</table>

The seven human oncogenic viruses with their years of discovery and the associated malignancy

¹ The causative association between MCPyV and Merkel cell carcinoma has not yet been established.
1.5.1. The cell cycle and cancer

In their study of root cells, Howard and Pelc, described the cell cycle as a continuous four-stage process (Howard & Pelc, 1986). These stages are serial and made up of the following phases; the Gap phase (GAP1), the synthetic phase, the Gap 2 phase and the mitotic phase. The mitotic phase is also further subdivided into the prophase, prometaphase, metaphase, anaphase and telophase stages (Figure 1-4). The G0 phase (not shown in the figure below) is a resting stage and damaged cells could be forced to enter the G0 phase rather than progress through the entire cycle.

Two restriction points exist between G1 and S phase as well as between G2 and M phase. Damaged cells with potential for tumourigenesis are checked and prevented from crossing the restriction points. The products of the tumour suppressor genes, p53 and retinoblastoma gene (Rb1) guard the restriction points. Mutation of the p53 gene can result in the passage of cells with damaged DNA through the restriction points, hence their progression to tumourigenesis (Chow, 2010). Viral proteins are capable of activating the genes that encode cell cycle regulatory proteins (Flint et al., 2009). This activation results in the proliferation of cells that would normally be in the resting phase. The role of p53 in antiviral immunity is further discussed in section 1.9.3 below.

The orderly progression of cells through the cell cycle is mediated by the cyclins D, E, A and B, which are activated by binding to their specific cyclin dependent kinases (CDK 6, 4, 2 and 1). The CDK inhibitors exert a negative control on the cell cycle to maintain homeostasis. The CDK inhibitors belong to three main families: the INK4 family ;( P15, P16, P18 and P19), cip/kip (P21, P27 and P57), and the pRb protein family (p107 and p130).
1.5.2. KSHV and cancer

KSHV has been associated with malignancies including KS, MCD and PEL (Cesarman et al., 1995; Du et al., 2001; Soulier et al., 1995).

1.5.2.1. Kaposi’s sarcoma

KS is a highly vascularised malignancy of endothelial, lymphoepithelial, or macrophage origins (Edelman, 2005). Following the discovery of KS by Moritz Kaposi in 1872, much clinical work has been done to describe the clinical forms and epidemiology of KS. The tumour presents with reddish brown or purple pigmented lesions. These may be found on the skin, mucosal surfaces or in visceral organs such as the liver spleen and kidney. Unusual sites include the eyes, salivary gland, musculoskeletal system and the larynx. Histology sections of KS lesions reveal...
extravasation of erythrocytes, infiltration of inflammatory cells and neo-angiogenesis. Four different subtypes of KS have been described. These are classical (sporadic), endemic (African), epidemic (AIDS associated) and iatrogenic (post transplantation) (Edelman, 2005). The classical KS, described by Moritz Kaposi, runs an indolent course in elderly males of Mediterranean descent as well as Eastern European origin (Edelman, 2005). Lesions of classical KS are mostly confined to the upper and lower extremities. Progression from classic KS to Non-Hodgkin’s lymphoma has been reported (Friedman-Birnbaum et al., 1990; Iscovich et al., 1999).

Endemic KS may be aggressive or indolent and usually involves the lymph nodes in contrast to the classical KS. Pre-pubescent children from Eastern Europe are at a higher risk (Dutz & Stout, 1960). It is a more aggressive form in younger individuals and is often fatal.

Iatrogenic KS has been reported following long term immunosuppressive therapy. This form of KS is more common among patients who undergo renal transplant (Eltom et al., 2002; Wen & Damania, 2010).

Epidemic KS is the most common type of KS and it also represents the most common HIV associated malignancy both in developed and developing nations. Its appearance in HIV-infected persons suggests a stage IV HIV disease and it is considered as one of the AIDS-defining illnesses.

The HIV epidemic has been associated with a rising incidence of KS, particularly in younger HIV infected patients (Thomas, 2001). The advent of highly active antiretroviral therapy (HAART) for HIV has led to a decreased incidence of KS in Europe and the US (Grulich et al., 2001; Jackson et al., 2010a; Mocroft et al., 2004). Nevertheless, the emergence of HAART-resistant HIV strains raises concern for the re-emergence of KS (Cortez & Maldarelli, 2011; Paydary et al., 2013).
In developing countries like Africa, there is need to assess the impact of HAART particularly in areas where the prevalence of KSHV has been found to be high (Maskew et al., 2005). In San Francisco, the incidence of KS among white men peaked at 32.1 per 100,000 in 1987 and fell to 2.8 per 100,000 about a decade after (Eltom et al., 2002). Contrary to the finding in the US, the incidence of KS among HIV-infected persons seems to be on the rise in sub-Saharan Africa (Bohlius et al., 2014). A recent study in Africa confirmed this trend by reporting an increase in the incidence rate of KS among the HIV infected group (Akarolo-Anthony et al., 2014).

1.5.2.2. Primary effusion lymphoma

KSHV has been strongly associated with PEL (body cavity–based lymphoma) and is now recognised as the causative agent (Bouvard et al., 2009; Cesarman et al., 1995). PEL is a lymphomatous effusion tumour arising from clonally expanded malignant B cells (Wen & Damania, 2010). It is mainly found in body cavities such as the pericardium, peritoneum and pleurum. Unusual forms of PEL have been reported as solid masses in lymph node and other organs. In virtually all cases of PEL, every cell harbours the KSHV DNA. PEL cells may be KSHV infected or KSHV/EBV double-positive. In KSHV/EBV co-infection, it is believed that KSHV is the predominant tumourigenic factor as almost five KSHV genes are expressed in contrast to the limited expression of EBV genes (Carbone et al., 2009). KSHV episomes are found in PEL cell lines at a high copy number of 50-150 viral genomes per infected cell (Cesarman et al., 1995; Renne et al., 1996; Staudt et al., 2004).

1.5.2.3. Multicentric castleman’s disease

MCD is a rare polyclonal B cell angiolymphoproliferative disorder that has been linked with KSHV. Two variants of this disease have been identified. The plasmablastic variant runs an aggressive course and progresses rapidly with resultant fatality (Wen & Damania, 2010). Unlike the plasmablastic form the hyaline form is not associated with KSHV and it is mainly found in HIV negative patients. For the plasmablastic
variants, KSHV genomes are detectable in about 50% of HIV negative MCD cases but are almost always present in all HIV positive MCD cases. The KSHV infected B cells are mainly found in the mantle zone of the lymph node follicle (Katano et al., 2000). MCD is often characterized by sustained fever, sweats, weight loss, lymphadenopathy and splenomegaly (Guihot et al., 2005). In MCD/HIV co-infection the symptoms characteristically fluctuate over 24–48 hours at regular intervals (Aaron et al., 2002; Oksenhendler et al., 1996). The clinicopathology of MCD can be partly attributed to the dysregulation of IL-6. This dysregulation might partly be due to the activity of a KSHV vIL-6 (Wen & Damania, 2010). There have been reports of plasmablastic lymphoma complicating MCD in HIV-infected patients. This is usually associated with a poor prognostic outcome (Oksenhendler et al., 2002).

1.6. Immune response to viral infection
The host immune system responds to invasion of pathogens including viruses. Both the innate and adaptive immune system provide powerful antiviral mechanisms to inhibit and neutralise viral particles (Lee et al., 2010). Viruses have evolved a vast range of immunomodulatory proteins and diverse immune evasion strategies to ensure their survival. KSHV is no exception as its genome comprises of at least 50% of immunomodulatory genes (reviewed in Rezaee et al., 2006). My project focuses on innate immunity hence I shall elaborate on it in the following sections.

1.7. The innate immune system
The first line of defence against viral infection is the innate immune system. The effector mechanisms for innate immunity consist of the complement cascade, type 1 interferon production, inflammatory cytokines, natural killer cell immunity, apoptosis, autophagy and the Toll like receptor (TLR) pathway (Lee et al., 2010). KSHV has been shown to regulate the interferon and autophagy pathways. This section will focus on IFN signaling pathways.
1.7.1. Interferon signalling pathway

Interferon (IFN) is produced by the host cells in response to the presence of pathogens including viruses. Human interferons have been classified into three major types: I, II and III. The type I IFNs include 5 different subtypes namely, IFNα and IFNβ as well as IFNω, IFNε and IFNκ. IFNγ is the only Type II IFN (Pestka et al., 2004), while type III IFNs consist of IFN-λ1, -λ2, and -λ3 that are also called interleukin-29 (IL-29), IL-28A, and IL-28B, respectively (Kotenko et al., 2002).

1.7.2. Production of interferon

The IFN production pathway is activated in the presence of viral stimuli detected by specific sensors. The IFN α and β genes are induced through the activation of pattern recognition receptors (PRR) which include the TLRs, retinoic inducible gene-1 (RIG-1) and melanoma differentiation-associated gene-5 (Mda-5) (Kawai et al., 2005; Takeda & Akira, 2005). Both RIG-1 and Mda-5 are the major cytoplasmic sensors and are members of the RIG-1-like (RLR) family, that recognises double stranded RNA (Yoneyama et al., 2004).

IFN production is transcriptionally controlled by the cellular interferon regulatory factors (IRFs). The IRF family members contain a well conserved DNA binding domain (DBD) characterized by five conserved tryptophan repeats (section 1.6.4). The KSHV genome encodes four viral interferon regulatory factors (vIRFs 1-4) that are homologues of the host IRFs. These are discussed further in section 1.7.2.

A multi-protein transcription complex referred to as IFNβ enhanceosome binds to the IFNβ promoter, in response to the IFNβ pathway activation. The IFNβ promoter is made up of four positive regulatory domains (PRDs). IRF proteins bind to PRDI and III, NFκB to PRDII, and ATF-2/c-Jun to PRDIV (Figure 1-5). A fourth protein, HMG-I, assists in stabilising the complex by promoting inter-protein interactions. The assembled enhanceosome recruits histone acetyl transferases (HAT) and other
transcriptional machinery such as RNA polymerase II and the CREB-binding protein (CBP)/p300 (Panne et al., 2007).

1.7.3. TLRs

TLRs are PRRs whose origin dates back to the TLR in Drosophila where it was found to instruct dorsal-ventral patterning in early embryo development (Seth et al., 2006). The TLRs recognise varied microbial signatures (Seth et al., 2006). TLRs 3, 7, 8 and 9 are the major PRRs for viral particles. The Toll/IL-1 receptor (TIR) shows a high degree of similarity with the cytoplasmic domain of TLRs and IL-1 receptors. Each TLR associates with a different combination of four TIR domain containing adapters.
(MyD88, TIRAP/MAL, TRIF and TRAM) through homophilic interaction of TIR domains (Kato et al., 2005). The most important of these is the myeloid differentiation factor 88 (MyD88) which is a key protein utilised by all the TLRs except TLR3 (reviewed in (Takeuchi & Akira, 2010). Both TLR 7 and 9 signalling leads to production of IFNs and pro-inflammatory cytokine (Figure 1-6).
1.7.4. Interferon regulatory factors

The interferon regulatory factor (IRF) family of transcription factors play a vital role in regulation of the entire type I interferon (IFN) system. In addition the IRFs are involved in most PRR signalling events thus facilitating the establishment of a platform that links the innate and adaptive immunity (Ozato et al., 2007).
The IRF family are made up of a well conserved DBD characterised by five conserved tryptophan repeats (Liang et al., 2008b). There are presently nine recognised members of the IRF family of transcription factors: IRF1 to 9 (Taniguchi et al., 2001)). These factors modulate the cellular response to IFNs and also regulate cellular processes such as cell growth, apoptosis, establishment of T cell immune response and antiviral defence (Harada et al., 1998). IRF1, IRF3, IRF5, and IRF7 are positive regulators of type I IFN gene transcription (Honda & Taniguchi, 2006b). The amino-terminus of all IRF family members contains a highly conserved 120 amino acid DNA-binding domain that recognises ISRE consensus DNA sequences that are found in promoters of type I IFNs, type I IFN-induced genes, and many other genes involved in immunity and oncogenesis. Previously, IRF1, IRF3, IRF5, and IRF7 have been shown to be positive regulators of type I IFN gene transcription (Honda & Taniguchi, 2006b).

The next section will focus on IRF1, IRF2, IRF3, IRF7 and IRF9 because of their involvement in IFN signalling pathways. My project focuses on how KSHV encoded homologues of cellular IRFs, specifically, vIRF2 and vIRF4 subvert the IFN response.

1.7.4.1. IRF1 and IRF2

IRF1 was the first member of the family of IRFs to be discovered, as a specific binding partner of the upstream regulatory region of the human IFNβ gene regulator, where it functions as a virus-inducible enhancer-like element (Miyamoto et al., 1988). The N-terminal DNA binding domain of IRF1 binds to the ISRE of target interferon stimulated genes thus mediating their expression.

IRF1 and IRF2 show 62% homology in the first 154 residues of amino-terminal regions and only 25% homology in the rest of the molecule (Fujita et al., 1988). Over expression of IRF1 upregulated induction of endogenous IFN α and β (Fujita et al., 1988; Harada et al., 1990). In contrast, IRF2 competitively inhibits the IRF1-mediated transcriptional activation of interferon α and β, thus suggesting that IRF1 and IRF2
function as a transcriptional activator and repressor, respectively. (Taniguchi et al., 2001). Both IRF1 and 2 function as transcription factors in the regulation of cell cycle and apoptosis. IRF1 recruits p300 and trans activates the p53 (Dornan et al., 2004). The deletion of the p300-binding sites in IRF1 inhibits IRF1-mediated stimulation of p53 acetylation and associated p53 activity (Dornan et al., 2004). The deletion or rearrangement IRF1 gene has been associated with the development of leukaemia, myelodysplastic syndrome, and gastrointestinal tract tumours (Boulthrop et al., 1993; Tamura et al., 1996).

1.7.4.2. IRF3 and IRF7

IRF3 and IRF7 share a high degree of similarity in terms of their primary structures and play a vital role in virus induced type 1 Interferon response (Taniguchi et al., 2001). Both IRF3 and IRF7 are activated following TLR activation. Upon virus infection, IRF3 is post-translationally activated by kinases TBK1 and IKKε (Hiscott, 2007). This C-terminal phosphorylation results in its dimerisation and concomitant involvement in the transcription of the type I interferon genes that drive the antiviral response.

IRF3 expression stimulates the antiviral response through its role in the synthesis of IFNβ thus playing a critical role in mediating antiviral response (Lowther, Moore et al. 1999). Conversely, the IRF7 gene is expressed at a very low level in multiple endothelial fibroblasts (MEFs), and is strongly induced by type I IFN through the activation of ISGF3; this induction is completely absent in ISGF3-deficient murine MEFs (Marie, Durbin et al. 1998; Sato, Hata et al. 1998). IRF7 is synthesised in response to IFN and activated by phosphorylation in virus infected cells.

1.7.4.3. IRF9

IRF-9, STAT1, and STAT2 make up the ISGF3 complex (discussed further in section 1.6.5.4 below). The DNA binding unit of ISGF-3 is IRF-9, also called ISGF3y/p48. IRF9 forms a DNA binding complex with STAT1 homodimers or with STAT2 alone
(IRF9-S2C and IRF9-S1C). These complexes bind to DNA with the same specificity as ISGF3 (Kraus et al., 2003b). A fusion protein consisting of IRF9 and the transactivating domain (TAD) of STAT2 recapitulates the interferon biological response in HEK 293T cell lines (Kraus et al., 2003b). IRF9 alone and the IRF9 fused with the TAD of STAT1 (IRF9-S1C), also transactivate the ISRE promoter independent of IFN stimulation, but at a much lower level than IRF9-S2C. Taken together, IRF9 functions to target STAT activation domains to the IFN-responsive promoter thus ensuring rapid and specific activation (Kraus et al., 2003b).

Many viruses including KSHV have evolved various strategies for subverting host immune responses through interference with the activities of cellular interferon regulatory factors particularly IRF3 and IRF7. Virus-encoded proteins that bind directly to and interfere with the transactivating ability of IRF3 and IRF7 include: the E6 oncoprotein of the human papillomavirus (Ronco et al., 1998), the NSP1 of the rotavirus (Graff et al., 2002) and RTA protein of Kaposi’s sarcoma-associated herpesvirus (Yu et al., 2005). Further immunomodulatory effects of KSHV on the IRF is discussed in section 1.7.2 below.

1.7.4.4. ISGF3 complex

The ISGF3 complex is made up of IRF9 and unphosphorylated STAT1 and STAT2. ISGF3 mediates an efficient and robust type I IFN-activated response through regulated control of transcription of antiviral ISGs. The ISGF3 components exist in an inactive/unphosphorylated state in unstimulated cells. However their activation occurs as an early event in the type I IFN signalling pathway. ISGF3 translocates into the nucleus to activate the ISRE promoters of IFNα responsive genes (Blaszczyk et al., 2016).

The STAT2 component of ISGF3 is required for ISGF3-mediated transcription and contributes a potent TAD while the STAT1 provides additional DNA contact thus stabilising the ISGF3 complex (Qureshi et al., 1996; Shearer et al., 2003). The
carboxyl terminus of STAT2 contains an intrinsic nuclear export signal that facilitates its continuous shuttling in and out of the nucleus (Banninger & Reich, 2004b). The IRF9 contributes most of the DNA-binding specificity by recognising the core sequence of the ISRE (Veals et al., 1992).

1.7.5. NF-KAPPA B SIGNALLING PATHWAY

The NF-kappaB (NF-κB) proteins refers to a family of structurally-related transcription factors including p65 (RelA), RelB, c-Rel, p50 and p52 (Fan et al., 2013a). The NF-κB pathway is crucial in the innate immune response and also controls a diverse array of cellular processes such as inflammation, cellular growth, and apoptosis.

As shown above in figure 1-6, the NF-κB pathway is triggered via the adaptor protein MyD88 in response to activation of all of the TLRs, except TLR3 (Kawai & Akira, 2010). Four IL-1 receptor associated kinases (IRAK) genes exist in the human genome (IRAK1, IRAK2, IRAK3, and IRAK4) (Rhyasen et al., 2013). Activated MyD88 adaptor molecule dimerises and recruits IRAK1 and IRAK2 to form the; MyD88: IRAK4: IRAK2 death domain (DD) complex (Rhyasen et al., 2013). This complex facilitates the recruitment of IRAK4 which subsequently phosphorylates IRAK1 (Akira et al., 2006; Rhyasen et al., 2013). IRAK1 undergoes a cascade of autophosphorylation that facilitates its dissociation from the complex to bind and activate TNFα receptor-associated factor 6 (TRAF6) (Jain et al., 2015). The activated TRAF6 interacts with NF-κB essential modulator (NEMO), transforming growth factor-β (TGF-β)-activating kinase 1 (TAK1), TAK1/MAP3K7 binding proteins; TAB1, TAB2 and TAB3. TAK1 undergoes phosphorylation and the activated TAK1 triggers the activation of the canonical I-kappa-B kinase (IKK) complex, which consists of IKKα and IKKβ and the regulatory subunit NEMO (also called IKKy)(Szili, 2014).

An alternate route for activation of the NF-κB signalling pathway is via the RIG-I-like receptors (RLRs). TRAF6 interacts with mitochondrial antiviral-signalling protein (MAVs) and subsequently recruits a complex containing MAVs, TNF receptor
associated death domain protein (TRADD), Fas-associated protein with death domain (FADD), caspase 8 and caspase 10 (Takahashi et al., 2006). The resultant complex activates the canonical IKK complex; IKKα/IKKβ/NEMO (Kumar et al., 2006; Takahashi et al., 2006).

1.7.6. JAK STAT SIGNALLING

Type 1 IFNs share a large degree of amino acid homology (Pestka et al., 1987; Stark et al., 1998). They bind to the same receptors (IFNAR). The Type I IFNAR is made up of two distinct subunits; the IFNAR1 and the IFNAR2 (Novick et al., 1994). The intracellular domain of IFNAR1 is associated with tyrosine kinase 2 (tyk2) and that of IFNAR2, the tyrosine kinase, jak1 (Takaoka et al., 2005a).

In the canonical JAK STAT signalling pathway, the binding of IFNα/β to the IFNAR results in a conformational change. Tyrosine 466 on the IFNAR1 becomes phosphorylated by tyk2 and triggers the phosphorylation of the STAT2. Figure 1-7 illustrates how phosphorylated STAT2 attracts STAT1, which is subsequently phosphorylated by JAK1. The association of both STAT1 and STAT2 with IRF9 forms the ISGF3 complex, which translocates to the nucleus where it binds to the ISRE, upstream of IFN sensitive genes.

In the non-canonical JAK STAT pathway, other 'non canonical' complexes besides the ISGF3 complex are formed. The activation of IFNAR can induce the formation of STAT1, STAT2 and STAT3 homodimers and heterodimers as well as activated STAT4, STAT5 and STAT6 in some cell types (Steen & Gamero, 2013). Additionally, a number of studies have demonstrated that STAT2/IRF9 complex is sufficient to induce the activation of ISGs in the absence of STAT1 (Blaszczyk et al., 2016; Ousman et al., 2005).

Interestingly, an unphosphorylated ISGF3-like complex (U-ISGF3) made up of; IRF9 as well as unphosphorylated STAT1 and STAT2, has been shown to prolong the
expression of a subset of ISGs. The U-ISGF3 is formed as a result of prolonged IFNβ stimulation, which induces an increase in concentration of IRF9, STAT1 and STAT2 with consequent activation of a second, delayed response after the initial phosphorylation-dependent ISGF3 signalling (Cheon et al., 2013; Cheon & Stark, 2009). Furthermore, combined chromatin immunoprecipitation (ChIP) and expression analysis indicates that non phosphorylated STAT2 play a role in activation of ISRE promoters of ISGs (Morrow et al., 2011; Testoni et al., 2011).

Figure 1-7: The IFN signalling pathway
This figure shows the cascade of events following the binding of IFN α and β to their specific receptors. IFN α and β binding triggers the JAK-STAT signalling pathway. Tyk 2 phosphorylates IFNAR1 leading to the phosphorylation of STAT2. STAT2 recruits STAT1, which is phosphorylated by JAK1. STAT1 and STAT2 associate with IRF-9 to form the ISGF-3 complex. The ISGF-3 complex translocate to the nucleus and binds to ISRE sequences in IFN sensitive genes
1.7.7. INTERFERON STIMULATED GENES (ISG)

The binding of IFN to the IFNAR activates the JAK-STAT signalling pathway with resultant transcriptional regulation of many downstream effector genes (Der et al., 1998). IFN induces hundreds of ISGs, some of which may have antiviral effector functions. The following sections will detail some of these genes and the effects of the proteins they encode.

1.7.7.1. The IFIT gene family

Four members of the IFIT family have been described in humans, namely ISG56/IFIT1, ISG54/IFIT2, ISG60/IFIT3, and ISG58/IFIT5 (reviewed in Fensterl & Sen, 2011). The four human genes are found in a cluster on chromosome 10q23.3 (reviewed in Fensterl & Sen, 2011). The IFIT1 (ISG56) genes share some similarity in their gene architecture and promoter structure. They are made up of two exons and their promoters have two ISRE as the only identifiable cis-acting elements. The ISG58 is an exception as it consists of three ISREs (Levy et al., 1986). IFIT expression is induced by both type I and type III IFNs. RNA and DNA viruses induce the activation of IFIT genes including; Sendai virus, Respiratory syncytial virus, Vesicular stomatitis virus, West Nile virus, LCMV, Influenza viruses, Reovirus, HSV, Cytomegalovirus, and Adenovirus (Daffis et al., 2010; Zhou et al., 2013). The antiviral effect of IFIT in Hepatitis B and C has been shown in vitro to be linked to the inhibition of translation initiation. IFIT1 and IFIT2 directly binds eukaryotic translation initiation factor 3 (eIF3) and suppresses the transcription of virus genes (Zhou et al., 2013). Alongside IFIT3, both IFIT1 and IFIT2 form a complex in the cytoplasm that recognises and destroys triphosphate RNA. IFIT5 is also stimulated in response to triphosphate RNA directly (Zhou et al., 2013).
1.7.7.2. Mx

The human MxA and MxB proteins are members of the interferon-induced myxovirus (influenza virus) resistance family of MX proteins (Horisberger, 1995). The MxA gene is encoded on the long arm of chromosome 21 together with several other genes which are important in the interferon signalling pathway. The Mx proteins are guanosine triphosphate (GTP)-binding proteins with intrinsic GTPase activity (Verhelst et al., 2012). The antiviral activity of MxA depends on both oligomerisation and GTPase activity (Gao et al., 2011). The Mx proteins inhibit viral replication by preventing nucleocapsids from entering into the nucleus. Mx has antiviral activities against both RNA and DNA viruses including: Semliki Forest virus and classical swine fever virus (Zhao et al., 2011). In vitro studies in mice have shown that Mx acts as an antiviral effector and inhibits viral RNA synthesis (Schneider-Schaulies et al., 1994).

1.7.7.3. 2’, 5’ Oligoadenylate Synthetase (OAS) and RNase L

The OAS genes encode the 2’-5’ oligoadenylate synthetase family and play a key role in the innate immune response. The four members of the human OAS gene family; OAS1, OAS2, OAS3, and OAS-like (OASL) are located in a cluster on chromosome 12 (Hovanessian & Justesen, 2007). The OAS1, OAS2 and OAS3 proteins can generate 2’–5’-linked oligoadenylates (see review, Hornung et al., 2014). OASL is devoid of 2’–5’-linked oligoadenylate synthase activity, but has potent antiviral activity (Zhu et al., 2014).

The OAS encoded proteins exist in the inactive form prior to stimulation in response to a PAMP, which is a double stranded RNA molecule. Activated OAS utilises ATP in the production of 2’, 5’-linked oligoadenylates, which binds to and activate RNase L. The antiviral effect of RNase L occurs through cleavage of both viral and cellular mRNA, thus resulting in the inhibition of viral replication. The murine coronavirus, mouse hepatitis virus (MHV), inhibits the type I interferon response by blocking the OAS-RNase L pathway and promotes hepatitis. The MHV accessory protein, ns2,
cleaves 2’,5’ OAS thereby preventing viral RNA degradation, a mechanism believed to facilitate hepatitis development (Zhao et al., 2012).

**1.7.8. IFN GAMMA PATHWAY**

The type II IFN pathway is regulated by IFN gamma (γ) which shares some antiviral properties of type 1 IFN (Zhou et al., 2007). However IFNγ is the only IFN in the type II IFN pathway and is produced by activated T cells and NK cells (Platanias, 2005b). IFNγ binds to the specific IFN-gamma receptor (IFNGR), which is made up of two subunits, IFNGR1 and IFNGR2, and is expressed on surfaces of nearly all cells. The binding of IFNγ induces oligomerisation of IFNGR, thus resulting in trans-phosphorylation of the receptor-associated JAK1 and JAK2. Both JAK1 and JAK2 pre-associate with the intracellular domains of IFNGR1 and IFNGR2 respectively (Gambin et al., 2013). The activated JAKs induce tyrosine phosphorylation at residue 440 of the intracellular domain of the receptor that serves as a docking site for STAT1. Further downstream, STAT1 homodimerises following tyrosine 701 phosphorylation then translocate to the nucleus where it binds to gamma-activated sequence (GAS) elements in the promoters of IFN-gamma-regulated genes (Pestka et al., 2004; Ramana et al., 2002). IFNγ exerts pleiotropic effects including; antiviral immunity, antigen processing and presentation, cellular proliferation and apoptosis (Schroder et al., 2004).
The type II IFN receptor is composed of two subunits; IFNGR1 and IFNGR2, which are associated with the JAKs 1 and 2. IFNγ binds and phosphorylates STAT1 with resultant formation of STAT1–STAT1 homodimers that translocate to the nucleus and bind elements that are present in the promoter of certain ISGs, thereby initiating the transcription of these genes.

**Figure 1-8: Type II IFN pathway**
1.8. Viral evasion of JAK STAT signalling pathway

Viruses have evolved to target all stages of JAK STAT signalling, from IFNAR phosphorylation to transactivation of the ISRE by the ISGF3 complex, in order to mitigate against the antiviral effects of the IFN response. The ISGF3 complex is a key target for manipulation by a number of viruses. The C proteins of Sendai virus induce proteasome-mediated degradation of STAT1 thus preventing JAK-STAT signalling (Garcin et al., 2002). Furthermore the P protein encoded by Nipah virus inhibits nuclear translocation of STAT1 by sequestering the inactive STAT1 in the cytoplasm (Ciancanelli et al., 2009). The non-structural protein 5 (NS5) protein of Dengue Virus (DENV) binds to STAT2 and inhibits the phosphorylation of STAT2 and also causes the degradation of STAT2 thus inhibiting IFNα-mediated signalling (Mazzon et al., 2009). The E7 protein of human papillomavirus (HPV) 16 targets IRF9, the third member of the ISGF3 complex, by preventing the translocation of IRF9 into the nucleus thus inhibiting the activation of ISGs following IFNα stimulation (Barnard and McMillan, 1999). Both STAT1 and IRF9 have been identified as the proximal targets of KSHV vIRF activity. KSHV vIRF2 attenuates the accumulation of IRF9 and phosphorylated STAT1 thus inhibiting IFNα-induced ISRE activation (Mutocheluh et al., 2011). VZV immediate early gene product ORF63 had earlier been shown to prevent ISG-induction (Sen et al., 2010). More recently, Simian varicella virus (SVV) ORF63 was shown to cause a proteasome-dependent degradation of IRF9 (Verweij et al., 2015).

Indeed, KSHV has adapted various strategies for evading the host immune response. This is achieved mainly through interference with signalling pathways. In the next section I will elaborate on KSHV interference with interferon signalling.
1.8.1. INTERFERENCE WITH INTERFERON SIGNALLING

The IRFs have been discussed earlier in section 1.6.6. They help to control transcription and production of IFN α and β production (Honda et al., 2006; Honda & Taniguchi, 2006a; Honda et al., 2005). The four KSHV encoded vIRFs are homologues of the host IRFs. These are discussed further in section 1.8.2.

The viral protein encoded by ORF 45 binds to IRF7, therefore inhibiting its phosphorylation and translocation (Ganem, 2007). This IE gene is incorporated into the KSHV genome during replication and helps to ensure that IFN α induction is prevented prior to viral gene expression (Zhu & Yuan, 2003). RTA has been shown to target IRF7 for proteasome-mediated degradation, preventing IFN induction by this regulatory factor (Yu et al., 2005). The K-bZIP protein encoded by ORFK8, binds directly to the PRD I and III regions of IFNβ which inhibits IRF3 from binding to the IFNβ promoter. This action results in low levels of IFNβ and also prevents the formation of the enhanceosome (Lefort et al., 2007). LANA competes with IRF3 for binding, to the IFNβ promoter thus preventing the expression of this cytokine (Cloutier & Flamand, 2010).

1.8.2. THE KSHV VIRAL INTERFERON REGULATORY FACTORS

The KSHV vIRF genes are encoded in a cluster within the KSHV genome between ORFs 57 and 58 (Russo et al., 1996). vIRFs 1-3 inhibit the induction of type 1 IFN genes and ISGs. Both vIRF1 and 2 target IRF3-mediated IFN signalling while vIRF3 interacts with IRF7 DNA binding thereby preventing IFNα production (Joo et al., 2007a). The precise mechanism of action of vIRF4 is still being debated (Liang et al., 2008a) and is one of the subject of the present thesis. The majority of the vIRFs, excluding vIRF3 are expressed during the lytic phase.
1.8.2.1. vIRF1

The vIRF1 protein, encoded by ORF K9, downregulates both the IFN production and the JAK STAT signalling pathways. vIRF1 binds to the transcriptional co-activator CBP/p300 thereby preventing the transactivation of IFNα (Burysek et al., 1999b). vIRF1 competes with CBP/p300, for IRF3-binding, thus resulting in reduction of IRF3-CBP/p300 complexes (Lin et al., 2001). IRF3-CBP complexes are required for the formation of the IFNβ enhanceosome. The complex derived from the binding of vIRF1 to CBP/300 disrupts the formation of this transcription complex on the IFN-β promoter (Lin et al., 2001). The IFNβ enhanceosome is responsible for recruiting HATs as well. The vIRF3/CBPp300 complex inhibits the HAT activity (Liang et al., 2008b). vIRF1 also interferes with apoptosis by inhibiting the acetylation of p53 by p300. In addition vIRF1 inhibits Ataxia Telangiectasia-Mutated (ATM) kinase. ATM activation is required for the phosphorylation of Ser15 on p53 and Ser395 on Hdm2. The phosphorylation prevents Mdm2-mediated ubiquitination of p53 thus resulting in its stabilization (Baresova et al., 2014)

1.8.2.2. vIRF2

vIRF2 is encoded by an inducible 2.2-kb spliced transcript K11/11.1. The protein down-regulates the activation of the IFNα and IFNβ promoters (Burysek et al., 1999b). It forms a multi-protein complex with IRF3 and caspase-3 that leads to the turnover of IRF3, meaning that the IFNβ promoter cannot be effectively transactivated (Areste et al., 2009; Fuld et al., 2006). vIRF2 inhibits IFN inducible genes that are controlled by IRF1, IRF3 and ISGF3 (Fuld et al., 2006). Its interference with the JAK STAT signalling pathway results in decreased levels of the ISGF3 components of phosphorylated STAT1 and IRF9 (Fuld et al., 2006; Mutocheluh et al., 2011). This action prevents the formation of functional ISGF3 (Mutocheluh et al., 2011). By inhibiting the kinase activity of PKR and consequent downregulation of protein
synthesis, KSHV has evolved a mechanism by which it can overcome the interferon-mediated antiviral effect (Burysek & Pitha, 2001).

1.8.2.3. vlRF3

vlRF3 is encoded by K10.5/10.6 and is also known as LANA 2. It mainly targets and inhibits the DNA binding domain of IRF7, thereby suppressing the activation of the IFNα promoter (Joo et al., 2007b). IRF5 can be activated by DNA damage, as well as by TLR7 and TLR9 signalling pathways resulting in the production of pro-inflammatory cytokines (Takaoka et al., 2005b). vlRF3 prevents the binding of IRF5 to DNA, thereby inhibiting its transcriptional activity (Wies et al., 2009). vlRF3 further represses the activation of ISRE and IFNβ promoter elements by abrogating the binding of IRF5 to them (Wies et al., 2009).

The vlRF3 protein targets PKR and decreases the PKR-mediated inhibition of protein synthesis (Esteban et al., 2003). It further interferes with translation initiation by decreasing the phosphorylation of eIF-2α (Esteban et al., 2003). vlRF3 is classed as an oncogene as it directly interacts with p53 leading to the inhibition of p53 transactivation (Rivas et al., 2001). vlRF3 activates the transcription of the cellular oncogene c-Myc and also blocks the IRF5 mediated activation of p21. Both p21 and c-Myc control important stages in cell growth and proliferation. This interaction thus increases the oncogenic potential of KSHV (Lubyova et al., 2004).

1.8.2.4. vlRF4

The role of vlRF4 protein in IFN pathway modulation is yet to be reported, as no reduction in IFNβ promoter activity was observed following activation with Sendai virus in the presence of vlRF4 (Kanno et al., 2006).

KSHV vlRF4 interacts with p53 and down-regulates its immune surveillance activities (Lee et al., 2009b). The central region of vlRF4 acts as a stabilising factor for Mdm2. Mdm2 is an E3 ubiquitin ligase for p53 and targets it for proteasome-mediated
degradation. In the presence of vIRF4, p53 levels are kept low partly from Mdm2 mediated degradation of p53. vIRF4 interacts with Ubiquitin specific protease 7 (USP7), a direct antagonist of Mdm2 and protects p53 from Mdm2-mediated degradation (Lee et al., 2009b).

1.9. Ubiquitin-specific protease 7

USP7 was first described as Herpes-associated ubiquitin specific protease (HAUSP) following the discovery of its interaction with the immediate early protein, infected cell protein (ICP0) of HSV 1 (Everett et al., 1997). ICP0 activates viral genes during the early stages of infection and is crucial for efficient lytic viral replication and regulation of the latent to lytic switch states of HSV 1. In this regard HSV 1 ICP0, plays a similar role to to KSHV RTA (Smith et al., 2011). USP7 stabilises ICP0 thus protecting it from self-mediated auto-ubiquitination (Canning et al., 2004). Among other functions, USP7 is also involved in apoptosis, epigenetics, cell proliferation, tumour suppression, neural stem cell maintenance and DNA damage (Du et al., 2010; Huang et al., 2011; Li et al., 2002b; Meulmeester et al., 2005; Oh et al., 2007; Schwertman et al., 2012; Song et al., 2008; van der Horst et al., 2006). USP7 deubiquitinates cell cycle regulatory genes such as p53, PTEN, FOXO and claspin as well as MdM2, an E3. Hence USP7 is recognised as an important drug target for cancer therapy (Nicholson & Suresh Kumar, 2011).

1.9.1. USP7 STRUCTURE AND ORGANISATION

The USP7 gene is located on chromosome 16p13.3 and encodes a 1102 amino acid protein with a molecular weight of approximately 130 kDa (Pfoh et al., 2015a). A catalytic domain links the N-terminal tumour necrosis factor receptor associated factor (TRAF) domain to the five C-terminal ubiquitin-like domains (CTD) (Pfoh et al., 2015a). Many cellular proteins including; minichromosome maintenance complex binding protein (MCM-BP) P53, HdM2, HdMx, Ubiquitin-conjugating enzyme E2 E1 (UbE2E1) and bind to the USP7 N-terminal domain (Jagannathan et al., 2014; Sarkari
et al., 2010; Sarkari et al., 2013; Sheng et al., 2006). The USP7-MCM-BP interaction is important for DNA replication. (Jagannatha et al., 2014). USP7 play vital roles in oncogenesis due to its interaction with p53 and Mdm2. More details on this interaction can be found in section 1.9.2.

A number of cellular proteins such as Guanidine monophosphate synthetase (GMPS), DNA methyltransferase 1 (DNMT1) and Forkhead box protein O4 (FOXO4) bind to the C-terminal domain of USP7. USP7 interacts with, and deubiquitinates, GMPS (Cheng et al., 2015a; van der Knaap et al., 2005). This interaction is important for the contributory role of USP7 to epigenetic silencing. Both GMPS and USP7 act in the same biological pathway, as GMPS is critical for deubiquitination of histone H2B and also regulates modulation of H2B deubiquitination by USP7, thus playing a role in chromatin silencing (van der Knaap et al., 2005). USP7 also stabilises DNMT1, a primary enzyme in DNA methylation, through acetylation and ubiquitination (Cheng et al., 2015b). USP7 interaction with DNMT1 stabilised DNMT1 and protected it from acetylation by the acetyltransferase Tip60 (Du et al., 2010).

FOXO4 belongs to a family of FOXO transcription factors which play key roles in the regulation of cellular metabolism, cell-cycle progression and cell death. USP7 reverses the monoubiquitination of FOXO4 in response to oxidative stress thus suggesting a role for USP7 in regulation of FOXO4-mediated stress responses (van der Horst et al., 2006).
Figure 1-9: Organisation of USP7

The N-terminal domain is joined to the C-terminal region by the catalytic domain. The interacting proteins are shown at the specific position of interaction. See text for relevant references. * represents viral proteins interacting with USP7.
1.9.2. USP7 AND P53

As described above (section 1.5.1), p53 is a short-lived tumour suppressor gene that plays a prominent role in tumourigenesis and normal cell growth (Guo et al., 2014). p53 levels are regulated essentially through ubiquitination, phosphorylation and acetylation hence the importance of the interaction between p53 and USP7 (Cummins et al., 2004a; Haupt et al., 1997). Ubiquitination involves enzymes that conjugate chains of ubiquitin to a protein substrate (Lecker et al., 2006). The polyubiquitinated proteins are consequently recognised and targeted for degradation by the proteasome. Ubiquitination involves three main activities: activation, conjugation, and ligation, and depends on ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s), respectively (Lecker et al., 2006).

MDM2 and p53 mutually regulate each other through a feedback loop (Wu et al., 1993). Activate p53 increases the transcription and translation of Mdm2 mRNA and protein. The Mdm2 protein binds to N-terminal transactivation domain of the p53 thus inhibiting its function through three major mechanisms. Firstly, Mdm2 functions as an E3 ligase, and targets p53 for proteasomal degradation; secondly Mdm2 binds to p53 and promotes its export out of the nucleus thus preventing it from binding to its specific nuclear target (Zhao et al., 2015). Lastly Mdm2 forms a tight complex with p53 thus preventing its transactivity (Momand et al., 1992).

USP7 interacts with and deubiquitinates p53, protecting it from Mdm2-mediated ubiquitination (Li et al., 2002b). The USP7-p53 interaction occurs between the residues 357–382 of the C-terminal regulatory region of p53 and USP7 N-terminal TRAF domain (53–208), as shown in figure 1-7 (Hu et al., 2002b). Studies have shown that overexpression of USP7 stabilised p53, and conversely decreased USP7 levels destabilised p53 (Li et al., 2002b).

The role of USP7 in p53 regulation is paradoxical as the disruption of the human USP7 gene by targeted homologous recombination results in an increased
expression of p53, as opposed to the expected destabilization of p53 (Cummins et al., 2004a).

1.9.3. P53 AND ANTVIRAL IMMUNITY

Viral infection triggers the activation of p53 with consequent induction of apoptosis thus suggesting a key role for p53 in the antiviral defence of the host (Takaoka et al., 2003). The p53 promoter contains a functional ISRE sequence hence p53 acts as a downstream transcriptional target of type I interferon (IFN) signalling (Munoz-Fontela et al., 2008). The mechanism behind this effect has been linked to binding of p53 to IRF9 component of the ISGF3 complex (Munoz-Fontela et al., 2008). The ISGF3 complex binds to the ISRE promoter of ISGs leading to the transactivation of the ISGs, and the induction of an antiviral state. A significantly increased replication titre of recombinant vesicular stomatitis virus (VSV) expressing GFP (VSV-GFP), was observed in the absence of p53 (Munoz-Fontela et al., 2008).

A number of viruses, including SV40, human papillomavirus, KSHV, adenoviruses, and even RNA viruses such as polioviruses, have evolved mechanisms designated to abrogate p53 responses. The EIA protein of adenovirus as well as the E7 protein of HPV (types 16 and 18), inhibits formation of Rb-E2F complexes, thereby leading to inappropriate entry of cells into the synthetic phase (Flint et al., 2009). The immunoevasive strategies of KSHV was discussed in section 1.8.1 above.

1.9.4. USP7 AND ANTVIRAL IMMUNITY

Several viral proteins target USP7 in order to promote survival and enhance their ability to infect susceptible host cells. USP7 appears to be a host target for human herpesviruses to support their lifecycle and pathogenesis, thus making USP7 a potential target for anti-viral therapy. The viral proteins, specifically EBV EBNA1 and KSHV vIFRF4, competitively bind with at least 10-fold higher affinity to the TRAF domain of USP7 compared with endogenous binding substrates (Lee et al., 2011; Saridakis et al., 2005).
HSV 1 ICP0 targets USP7 for proteasome-dependent degradation. ICP0 interaction with USP7 stabilises ICP0 protein levels during early viral infection and this is the dominant outcome of the interaction between the two proteins (Boutell et al., 2005b). HSV 1 ICP0-null mutants that no longer bind USP7 are significantly impaired in their ability to replicate, thus confirming the importance of USP7 for HSV 1 (Sacks & Schaffer, 1987). ICP0 induces the translocation of USP7 from the nucleus to the cytosol, where they jointly deubiquitinate TRAF6 and IKKβ resulting in attenuation of TLR-mediated immunity through inhibition of NF-κB (Daubeuf et al., 2009).

The EBNA1 protein of EBV plays a vital role in ensuring successful replication, segregation, and transcriptional activation of latent EBV genomes (Holowaty et al., 2003). EBNA1 and p53 bind the same pocket of the USP7 TRAF domain (Holowaty et al., 2003). Mutant forms of EBNA1 that lacked the USP7 binding sequence were used to show that USP7 binding was not required for replication or transcriptional activation of EBV. However, EBNA1 competitively binds to USP7 N-terminal TRAF, thus blocking the p53-USP7 interaction. Hence EBNA1, but not the mutants with deficient USP7-binding domains, showed increased survival of cells that were otherwise meant to undergo apoptosis due to DNA damage or overexpression of p53 (Saridakis et al., 2005).

KSHV vIRF4 and more recently vIRF1 have been shown to interact with the TRAF domain of USP7 (Lee et al., 2011). vIRF4 antagonizes p53-mediated tumour suppressor activity by regulating both Mdm2 and USP7 (Lee et al., 2011). The KSHV vIRF4-Mdm2 interaction occurs through the C-terminal region of Mdm2, resulting in the inhibition of Mdm2 auto-ubiquitination. The stabilisation of Mdm2 levels enhances Mdm2-mediated p53 degradation (Lee et al., 2009b). This mechanism suggests that vIRF4 circumvents host growth surveillance to enhance survival of KSHV in infected cells in a similar manner to HSV ICP0 and EBV EBNA.
In addition to inhibiting p53 acetylation, KSHV vIRF1 competitively binds USP7-NTD through its EGPS sequence and decreases availability of USP7 for binding and deubiquitination of p53 (Chavoshi et al., 2016b). KSHV ORF45 and LANA also bind to and inhibit multiple members of the USP7-p53-Hdm2 pathway indicating the crucial role of p53 degradation for long term survival of KSHV in host cells (Jager et al., 2012; Lee et al., 2011; Shin et al., 2006). The present thesis is focused on establishing a role for USP7 in the type I IFN response and further exploring the interaction between KSHV vIRF2 and vIRF4.

1.10. Viruses and translational control

Our lab has demonstrated KSHV vIRF2 and vIRF4 inhibit the type I IFN response. Proteomics analyses on cells engineered to inducibly express these two viral genes have been done with the aim of identifying the cellular partners of these viral proteins and to understand the ways in which they inhibit the IFN response. Ribosomal proteins were among those that interacted with these viral proteins (see Table 1-4). The mechanisms of such interactions are not fully understood, but suggest that these viral proteins exert translational control of the host.

We therefore hypothesise that KSHV modulates the IFN-mediated type I antiviral response and manipulates the profile of mRNA species that are translated; and that vIRF2 and vIRF4 therefore mediate the translational control of the host. This section summarises the translation processes and how viruses exert translational control of the host.

The 5’ end-independent translation involves an internal initiation and may function alongside the cap dependent translation in eukaryotes. This type of translation is initiated at the internal ribosomal entry site (IRES), which is made up of highly structured elements within the 5’-UTR of the transcript (Malys & McCarthy, 2011). IRES are RNA factors that are responsible for end-independent ribosomal recruitment to internal locations in mRNA (Jackson et al., 2010a).
Translation initiation depends largely on the presence of initiation factors (IF), that are large multimeric complexes (Jackson et al., 2010b; Kozak, 1999; Sonenberg & Hinnebusch, 2009). Eukaryotes have an increased number of IFs compared with prokaryotes. The eukaryotic IFs can be divided into the core factors eIF1, eIF1A, eIF2, eIF2B, eIF3, eIF4A, eIF4B, eIF4E, eIF4G, eIF4H, eIF5, eIF5B, and the auxiliary factors eIF6, PABP and others (Malys & McCarthy, 2011). A large number of translation eukaryotic initiation factors (eIFs) are involved in translation initiation (Kuang et al., 2011). The eIF4F cap binding complex is a heterotrimeric structure made up of eIF4E, which is the cap binding protein. The other two parts are the RNA helicase referred to as eIF4A, and a scaffolding protein (eIF4G) (Kuang et al., 2011). The mRNAs initiate translation by arranging the cap-binding complex on the 5’-cap structure (Kuang et al., 2011). A ternary complex is formed made up of eIF-2-GTP-Met-tRNA. The formation of the complex and the delivery of the Met-RNA to the 40S ribosomal subunit are considered as rate limiting steps. The α subunit of the eIF2 (eIF2α) needs to be phosphorylated by specific PKR (Clemens, 1994; Merrick & Hershey, 1996). The phosphorylation stage represents a major point of control over the translation initiation stage. The eIF2 plays a vital role of directing the ternary complex to the 40S ribosomal unit to form the 43S pre-initiation complex (Merrick & Hershey, 1996; Pain, 1996). The 43S pre-initiation complex associates with the mRNA to begin the process of ribosome scanning, which starts from the 5’ end of the mRNA or the site of ribosome entry for the cap-independent translation (Gale et al., 2000). The ribosomal scanning continues until the Met-tRNA interacts with the AUG codon (Gale et al., 2000).

Ribosomes play a significant role in the translation initiation process as they catalyse the translation of mRNA. The eukaryotic ribosome is made up of four RNA species and 79 ribosomal proteins (RP) (Barna, 2013). The two major steps in translation
initiation are the formation of 48S initiation in the P-site of the 40S ribosomal subunits and secondly the joining of 48S complexes with 60S subunits (Jackson et al., 2010a).

Viruses rely solely on the translational machinery of the host cell for the synthesis of viral proteins (Gale et al., 2000). As obligate intracellular parasites, viruses compete with the endogenous transcripts of the host for the translation of viral mRNA and have evolved diverse strategies for enhancing translational efficiency of viral mRNA at the expense of cellular mRNA (Gale et al., 2000). The dependence of viruses on the translational machinery of the host allows the recruitment of cellular ribosomes in order to translate viral mRNAs, consequently allowing the virus to seize control of regulatory cellular translation factors and host signalling pathways.

The translation process is subdivided into three stages; initiation, elongation and termination. These three stages are regulated by specific factors, which are targeted by different viruses, thus interfering with the various steps (Walsh & Mohr, 2011). The translation initiation stage, is the rate limiting stage hence its regulation is often more manipulated than the other two stages (Walsh & Mohr, 2011). Viruses manipulate important eukaryotic translation factors to the evolution of specialized cis-acting elements that recruit ribosomes or modify genome-coding capacity (Walsh & Mohr, 2011). Table 1-3 below summarises host translation factor targets of some viruses.
Table 1-3: Viral subversion of host protein translation machinery

<table>
<thead>
<tr>
<th>Viral protein</th>
<th>Target</th>
<th>Effects on targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV (E6)</td>
<td>4EBP1</td>
<td>Phosphorylates 4EBP1 (via PDK1 activation and TSC2 degradation) (Spangle &amp; Munger, 2010)</td>
</tr>
<tr>
<td>KSHV (v-GPCR)</td>
<td></td>
<td>Phosphorylate 4EBP1 (via PI3K–AKT–mTOR pathway activation) (Bhatt &amp; Damania, 2012; Martin et al., 2011)</td>
</tr>
<tr>
<td>EBV (LMP2A)</td>
<td></td>
<td>Phosphorylate 4EBP1 (via TSC2 inactivation) (Chuluunbaatar et al., 2010) Dephosphorylates 4EBP1 (PP2A dependent) (Perez et al., 2011b)</td>
</tr>
<tr>
<td>HSV-1 (US3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCMV (UL38)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSV-1 (ICP6)</td>
<td>eIF4G</td>
<td>Binds eIF4G and increases its interaction with eIF4E (Walsh &amp; Mohr, 2006) Binds eIF4G, dephosphorylates eIF4E (via competitive displacement of MNK1) and promotes ribosome shunting on viral mRNAs (Cuesta et al., 2004)</td>
</tr>
<tr>
<td>Adenoviruses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(100K)</td>
<td>eIF4A</td>
<td>Binds eIF4A (consequence unknown) (Lenarcic et al., 2014) Binds eIF4A and either eIF4H or eIF4B, and this targets vhs endoribonuclease activity to mRNAs, accelerating mRNA turnover (Walsh et al., 2013b)</td>
</tr>
<tr>
<td>HCMV (UL69)</td>
<td>eEF1A</td>
<td></td>
</tr>
<tr>
<td>HSV-1 (vhs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCMV (UL97)</td>
<td>eRF1</td>
<td>Binds eRF1 and inhibits translation at its own stop codon to regulate translation of the downstream HCMV ORF (Janzen et al., 2002; Matheisl et al., 2015)</td>
</tr>
<tr>
<td>KSHV SOX and K10</td>
<td>PABP</td>
<td>Bind PABP and causes its nuclear accumulation (Janzen et al., 2002)</td>
</tr>
<tr>
<td>HCMV UL69</td>
<td></td>
<td>Binds PABP (consequence unknown) (Aoyagi et al., 2010)</td>
</tr>
<tr>
<td>HSV-1 ICP27</td>
<td></td>
<td>Binds PABP and stimulates translation of a viral mRNA subset (Dobrikova et al., 2010a)</td>
</tr>
<tr>
<td>HCMV UL47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV (E6)</td>
<td>eIF2</td>
<td>Binds GADD34–PP1α to dephosphorylate eIF2α (Kazemi et al., 2004)</td>
</tr>
<tr>
<td>HSV-1 γ34.5</td>
<td></td>
<td>Inhibits PERK (Lee et al., 2009a) Regulates eIF2α phosphatase (Wilcox &amp; Longnecker, 2016)</td>
</tr>
<tr>
<td>HCMV (UL97)</td>
<td>eEF1A and eEF1B</td>
<td>Phosphorylate eEF1Bα (Gill et al., 2012; Kato et al., 2001)</td>
</tr>
<tr>
<td>EBV (BGLF4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KSHV (ORF57)</td>
<td>Ribosome</td>
<td>Binds PYM to recruit 40S ribosomes to viral mRNAs (Boyne et al., 2010)</td>
</tr>
</tbody>
</table>
1.11. Ribosomal regulation of gene expression

Ribosomal proteins have been found to exert regulatory control in translating mRNAs (Kondrashov et al., 2011). In this instance RPL38 ribosomal protein was found to be involved in transcript-specific translational control thereby suggesting a probable role for ribosomal proteins in control of gene expression.

The phosphatidylinositol 3-kinase (PI3K) signalling pathway is activated by growth factors and other mitogenic stimuli (Bjornsti & Houghton, 2004; Gingras et al., 2001). The activation of the PI3K-signalling pathway regulates activation of mammalian target of rapamycin (mTOR) through one of its downstream effectors, AKT (Lekmine et al., 2003; Platanias, 2005a). IFN-dependent activation of mTOR and downstream activation of p70 S6 kinase (p70 S6K) phosphorylates the S6 ribosomal protein leading to initiation of mRNA translation and induction of type I IFN responses (Lekmine et al., 2003). The finding of a significant number of ribosomal proteins as vIRF2 and vIRF4 interactome components (see Table 1-4 below) led to the hypothesis that the vIRF2 and vIRF4 proteins interact with a specific ribosomal protein(s), which then possibly recruit the rest of the ribosomal complex. As ribosomal proteins have earlier been linked with translation of ISGs, this thesis sought to investigate if KSHV vIRF2 and vIRF4 associate with ribosomal proteins in order to exert translational control of ISGs.
<table>
<thead>
<tr>
<th>vIRF2 &amp; vIRF4 partner</th>
<th>protein Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>40S S6</td>
<td>May play an important role in controlling cell growth and proliferation through the selective translation of particular classes of mRNA</td>
<td><a href="http://www.uniprot.org/uniprot/P62753">http://www.uniprot.org/uniprot/P62753</a></td>
</tr>
<tr>
<td>60S P2</td>
<td>Plays an important role in the elongation step of protein synthesis</td>
<td><a href="http://www.uniprot.org/uniprot/P05387">http://www.uniprot.org/uniprot/P05387</a></td>
</tr>
<tr>
<td>40S S3</td>
<td>Identified in a mRNP granule complex; Identified in a HCV IRES-mediated translation complex; HSP90 associates with S3 preventing its ubiquitination and degradation and thereby retaining the integrity of the ribosome.</td>
<td><a href="http://www.uniprot.org/uniprot/P23396">http://www.uniprot.org/uniprot/P23396</a></td>
</tr>
<tr>
<td>40S 18S</td>
<td>Located at the top of the head of the 40S subunit, it contacts several helices of the 18S rRNA</td>
<td><a href="http://www.uniprot.org/uniprot/P62269">http://www.uniprot.org/uniprot/P62269</a></td>
</tr>
<tr>
<td>40S S19</td>
<td>Required for pre-rRNA processing and maturation of 40S ribosomal subunits</td>
<td><a href="http://www.uniprot.org/uniprot/P39019">http://www.uniprot.org/uniprot/P39019</a></td>
</tr>
<tr>
<td>40S S14</td>
<td>Undefined? Or various</td>
<td><a href="http://www.uniprot.org/uniprot/P62263">http://www.uniprot.org/uniprot/P62263</a></td>
</tr>
<tr>
<td>40S S13</td>
<td>Undefined? Or various</td>
<td><a href="http://www.uniprot.org/uniprot/P05756">http://www.uniprot.org/uniprot/P05756</a></td>
</tr>
</tbody>
</table>

Proteins that showed an > 2-fold change in abundance compared to the control were included in this table (Hindle & Blackbourn, unpublished observation).
1.12. Aims and Objectives

SILAC proteomics studies of vIRF2 and vIRF4 revealed a possible interaction of USP7 with vIRF2 and vIRF4. This effect was confirmed by an immunoprecipitation assay. USP7 is targeted by a number of viral proteins to enhance their replication and survival. As we had earlier shown, both vIRF2 and vIRF4 were negative regulators of IFN signalling. We thus hypothesised that USP7 possibly plays a role in the interferon response.

Similarly, 23/56 of the proteins identified as binding to vIRF2, and 12/23 of the proteins identified as binding to vIRF4 were ribosomal proteins, based on the less stringent approach (Table 1-4). Previous studies suggest that ribosomes play a role in transcript-specific translational control. We thus hypothesised that vIRF2 and vIRF4 proteins interact with ribosomal proteins in order to exert translational control of host mRNA.

The two main aims are to:

1. Understand whether vIRF4 exert translational control given the apparent association of ribosome-associated proteins with these viral proteins.

2. Understand the role of USP7 in the IFN response, including why vIRF2 and vIRF4 might have evolved to bind to USP7.

The objectives concerning KSHV proteins are to:

1. Confirm the effect of vIRF2 and vIRF4 on the JAK-STAT signalling pathway.

2. Investigate the effect of vIRF2 and vIRF4 on the transcription of IFN responsive genes.

3. Investigate the control of gene expression at translational level in vIRF4-expressing cells, in the presence and absence of IFN, to determine whether vIRF4 exerts translational control on the host.
The objectives concerning USP7 are to:

1. Determine the effect of USP7 on the IFN response.
2. Assess the effects of USP7 on the JAK-STAT pathway.
3. Assess the biological significance of USP7 on the JAK STAT pathway
4. Investigate the functional significance of binding of USP7 to vIRF2 and vIRF4
CHAPTER 2

MATERIALS AND METHODS
2. CHAPTER 2

The experimental methodologies used throughout this research project will be outlined and described in this chapter, with reference to background literature where applicable. The appendices include a comprehensive list of commonly used chemicals and reagents (Appendix A), details of reagent constituents (Appendix B), primer sequences (Appendix C).

2.1. Tissue culture methods

The cells used in the present study are listed in Table 2.2 below. All cell lines were grown and maintained at 37°C in a humidified environment supplied with 5% CO₂. Cells were grown in T75 flasks, T25 flasks, 6-well plates, 24-well plates, 12-well plates and 96-well plates depending on the type of experiment being conducted. The culture media for the different cell lines are also described in Table 2.2. For passage of confluent adherent cells, the existing medium was removed, cells were then washed with phosphate buffered saline (PBS) and covered in 10% Trypsin-verseine for three minutes to allow cells to detach. Culture medium was added to quench the trypsin and the cells were pelleted by centrifugation (5 minutes, x1300 rpm) in a TX-75 swinging bucket rotor for the Hereaus Megafuge 40R (Thermofisher Scientific Inc., Waltham, MA, USA). Pellets were re-suspended in fresh media, plated at the required number and stored in the incubator. For cryopreservation, aliquots of the culture cells in 10% DMSO were stored in liquid nitrogen (-196°C) for long-term storage and -80°C for short-term storage. Cells were counted prior to seeding the culture plates (section 2.1.1).
<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s Modified Eagle’s Medium (DMEM) with low glucose (1g/dL)</td>
<td>Sigma-Aldrich (D6429)</td>
</tr>
<tr>
<td>Minimum essential Medium (MEM)</td>
<td>Sigma-Aldrich (M0446)</td>
</tr>
<tr>
<td>McCoy 5A medium</td>
<td>Sigma-Aldrich (M4892)</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Sigma-Aldrich (D9170)</td>
</tr>
<tr>
<td>Phosphate buffered saline (PBS)</td>
<td>Sigma-Aldrich (P4417)</td>
</tr>
<tr>
<td>Foetal Bovine Serum (FBS) , certified heat inactivated, US origin</td>
<td>GIBCO (10082147)</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>Invitrogen (15070063)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Invitrogen (25030123)</td>
</tr>
<tr>
<td>Trypsin EDTA (0.25%), phenol red</td>
<td>Thermo Fisher Scientific (25200056)</td>
</tr>
<tr>
<td>Non-essential amino acids (NEAA)</td>
<td>Sigma (M7145)</td>
</tr>
<tr>
<td>Blasticidin</td>
<td>Merck Millipore (203351)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Sigma-Aldrich (T7660)</td>
</tr>
<tr>
<td>Freezing media</td>
<td>90% FCS, 10% Dimethyl sulfoxide (DMSO)</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Sigma-Aldrich (F0895)</td>
</tr>
<tr>
<td>Trypan blue</td>
<td>Sigma-Aldrich (T8154)</td>
</tr>
<tr>
<td>Recombinant human IFNa2B</td>
<td>PBL Interferon source. Stratatech (11115-1)</td>
</tr>
</tbody>
</table>
Table 2-2: Cell lines used in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Suspension/ Adherent</th>
<th>Source</th>
<th>Origin</th>
<th>Culture media</th>
</tr>
</thead>
<tbody>
<tr>
<td>293</td>
<td>Adherent</td>
<td>Embryonic kidney</td>
<td>Human</td>
<td>DMEM with 10% Foetal bovine serum and 1% Penicillin streptomycin</td>
</tr>
<tr>
<td>293T</td>
<td>Adherent</td>
<td>Embryonic kidney</td>
<td>Human</td>
<td>DMEM with 10% Foetal bovine serum and 1% Penicillin streptomycin</td>
</tr>
<tr>
<td>HeLa</td>
<td>Adherent</td>
<td>Cervical carcinoma</td>
<td>Human</td>
<td>DMEM with 10% Foetal bovine serum and 1% Penicillin streptomycin</td>
</tr>
<tr>
<td>Saos2</td>
<td>Adherent</td>
<td>Osteogenic sarcoma</td>
<td>Human</td>
<td>McCoy 5A with 1%FBS and 1% penicillin streptomycin</td>
</tr>
<tr>
<td>L929</td>
<td>Adherent</td>
<td>Fibroblasts</td>
<td>Mouse</td>
<td>DMEM with 10% Foetal bovine serum and 1% Penicillin streptomycin</td>
</tr>
<tr>
<td>Vero</td>
<td>Adherent</td>
<td>Kidney epithelial cells</td>
<td>Monkey</td>
<td>DMEM with 10% Foetal bovine serum and 1% Penicillin streptomycin</td>
</tr>
<tr>
<td>EV-NTAP, vIRF2-NTAP, vIRF4-NTAP</td>
<td>Adherent</td>
<td>T-Rex-293 cells stably transfected (Polyclonal)</td>
<td>Human</td>
<td>DMEM with 10% Foetal bovine serum and 1% Penicillin streptomycin complete with 5μg/mL Blasticidin and 200μg/ml Zeocin</td>
</tr>
</tbody>
</table>
2.2. Cell counting

Cell suspensions were diluted in 0.5% Trypan blue solution and PBS at a ratio of 1:1:8 and 10μL of this mixture was transferred to each chamber of a haemocytometer (Neubauer chamber). The cells were counted under an inverted microscope. Since non-viable cells take up Trypan blue dye it is possible to distinguish between non-viable and viable cells. Each square of the haemocytometer with 13mm cover slip in place represents a total volume of 0.1 mm$^3$. 1 cm$^3$ is equivalent to 1 mL, so that cell concentration per mL was determined by multiplying the average number of cells per square by the dilution factor and also by the area covered by the grid lines ($10^4$) using the formula below:

Cells per mL = the average count per square x dilution factor x $10^4$ (a constant).

For example: if the average count per square is 50 cells x $5 \times 10^4 = 2.5 \times 10^6$ cells/mL.

2.3. Transfection of cells

Transfection is a means of delivering DNA into a cell using a lipid based medium for the delivery. Cells were transiently transfected using a lipid-based system, Lipofectamine$^{TM}2000$ (Invitrogen). The Lipofectamine$^{TM}2000$ was used according to the manufacturer’s instructions. The amount of Lipofectamine$^{TM}2000$ and Opti-MEM (Life Technologies) used were dependent on the size of the cell culture plate containing cells to be transfected. The required amount of DNA was added to 1 volume of Opti-MEM, while at the same time adding to another 1 volume of Lipofectamine$^{TM}2000$ to Opti-MEM. The Opti-MEM-DNA was incubated for 5 minutes at room temperature, then added to the Opti-MEM-lipid formulation. The lipid-DNA complex was further incubated for 20 minutes at room temperature, before being added drop wise onto cells. Transfection efficiency was assessed using fluorescent microscopy (data not shown).
2.4. siRNA transient transfection

ON-TARGET plus Smart pool siRNAs directed against human USP7 was obtained from Dharnacon Inc, GE Healthcare Limited, UK. The ON-TARGET SMARTpool has been modified to reduce off-target effects and targets four mRNA regions at once. It was used at a final concentration of 30µM using Lipofectamine™2000 (Invitrogen) transfection reagent according to manufacturer's instruction. Briefly, for reverse transfection, the indicated volume (usually 2µL) of Lipofectamine™2000 reagent was mixed with 50µL of Opti-MEM reduced serum media (Invitrogen) per well and allowed to stand at room temperature for 5 minutes. Separately, the required concentration of siRNA was prepared in 50µL of Opti-MEM reduced serum media per well, after which the Lipofectamine™2000 mix was added to the siRNA mix and incubated at room temperature for 20 minutes. Freshly trypsinised cells were counted and seeded at a concentration of 2x10⁴ in 24 well plate format. Knockdown efficiency was assessed at 48 hours by qPCR (section 2.8.5) and western blot (section 2.9) at 72 hours.

2.5. Dual luciferase reporter assay

The dual luciferase reporter assay (DLA) system (Promega E1910) sequentially measures the activities of two distinct luciferase enzymes, firefly (Phontinus pyralis) and Renilla (Renilla reniformis), expressed from the same sample. After a specific time (dependent on the experiment), the cell medium was removed and the cells were washed gently with PBS. Cells were lysed using 1X Passive Lysis Buffer (Promega E1010) for a minimum of 10 minutes with agitation on a Stuart® SSL3 gyro-rocker set to 30 rpm (Bibby Scientific Ltd., Stratfordshire, UK).

The firefly luciferase reporter is expressed by the promoter of interest and is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a stabilised luminescent signal. The constitutively expressed Renilla luciferase signal acts as an internal control and normalises changes in luminescence due to factors other than the transcriptional control being studied, for example transfection efficiency between
samples. After quantifying the firefly luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is initiated by adding Stop & Glo® Reagent to the same tube. Cells were transfected with reporter plasmids containing either the firefly luciferase gene (driven by the promoter of interest), or the constitutively active *Renilla* luciferase plasmid (pRLSV40).

To obtain results, samples were analysed in duplicates on POLARstar Omega (BMG LABTECH) spectrophotometer. A blank reading was taken each time prior to the sample reading. The programme for assaying samples is shown in Table 2.3 below. The firefly luciferase values were normalised to their *Renilla* luciferase counterparts.

**Table 2-3: The programme for reading DLAs Reporter**

<table>
<thead>
<tr>
<th>plasmid</th>
<th>Information</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>LARII</td>
<td>50μL</td>
<td>Activate firefly luciferase</td>
</tr>
<tr>
<td>Measurement</td>
<td>10 seconds</td>
<td>Measure firefly luciferase</td>
</tr>
<tr>
<td>Stop &amp; Glo</td>
<td>50μL</td>
<td>Quenches firefly luciferase and Measures <em>Renilla</em> luciferase</td>
</tr>
<tr>
<td>Measurement</td>
<td>10 seconds</td>
<td>Measure <em>Renilla</em> luciferase</td>
</tr>
<tr>
<td>Reporter plasmid</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------</td>
<td>--------</td>
</tr>
<tr>
<td>p125-luc</td>
<td>Full length IFN-beta promoter driving expression of a firefly luciferase reporter gene, AmpR</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pISRE-luc</td>
<td>Luciferase reporter vector containing tandem repeats of the ISRE element driving expression of the firefly luciferase reporter gene, AmpR</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pRLSV40</td>
<td>Mammalian co-reporter vector for the strong constitutive expression of wild-type Renilla luciferase. Luciferase driven by the CMV promoter, AmpR</td>
<td>Addgene (E2231)</td>
</tr>
<tr>
<td>pCI-USP7</td>
<td>This contains the filled Ndel-HindIII USP7 fragment inserted into the Smal site of pClneo. The business bit will be: CMV promoter-Nhel-Xhol-EcoRI-Mul-Xba-Sall-Accl-(Sma/Nde)---USP7---(Hind/Sma)-Notl</td>
<td>A kind gift from Roger Everett (Glasgow)</td>
</tr>
<tr>
<td>PClneo</td>
<td>This contains the CMV promoter-Nhel-Xhol-EcoRI-Mul-Xba-Sall-Accl-(Sma/Nde)---(Hind/Sma)-Notl</td>
<td>A kind gift from Roger Everett (Glasgow)</td>
</tr>
<tr>
<td>pCI-USP7(C223S)</td>
<td>This is similar to pCI-USP7, but it has the C223S point mutation and also other silent changes that introduce a NarI site.</td>
<td>A kind gift from Roger Everett (Glasgow)</td>
</tr>
<tr>
<td>pEGFP-USP7</td>
<td>An EGFP fusion of USP7. The Ndel site was filled in and ligated to the filled BglII site in pEGFP-C1, using the normal HindIII sites of both at the 3'end.</td>
<td>A kind gift from Roger Everett (Glasgow)</td>
</tr>
<tr>
<td>pcDNA4/HisMax</td>
<td>Empty vector backbone for pcDNA4VIRF2/HisMax and pcDNA4VIRF4/HisMax, AmpR</td>
<td>Invitrogen (V86420)</td>
</tr>
<tr>
<td>pcDNA4VIRF2/HisMax</td>
<td>pcDNA4/HisMax vector encodes Xpress and Polyhistidine tag contiguous to vIRF2, AmpR</td>
<td>DJB Lab</td>
</tr>
<tr>
<td>pcDNA4VIRF4/HisMax</td>
<td>pcDNA4/HisMax encodes Xpress and Polyhistidine tag contiguous to vIRF4, AmpR</td>
<td>DJB Lab</td>
</tr>
<tr>
<td>pGAS-luc</td>
<td>GAS responsive promoter fused with firefly luciferase, AmpR.</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pcDNA4/TO IRF9-TAP</td>
<td>IRF9 with a C-terminal TAP tag, doxy-inducible, zeocin selection marker, AmpR.</td>
<td>A kind gift from Geoffrey Smith Lab</td>
</tr>
<tr>
<td>Construct Name</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>pcDNA4/TO STAT1-TAP</td>
<td>STAT1 with a C-terminal TAP tag, doxy-inducible, zeocin selection marker, AmpR.</td>
<td>A kind gift from Geoffrey Smith Lab (University of Cambridge)</td>
</tr>
<tr>
<td>pcDNA4/TO STAT2-TAP</td>
<td>STAT2 with a C-terminal TAP tag, doxy-inducible, zeocin selection marker, AmpR.</td>
<td>A kind gift from Geoffrey Smith Lab (University of Cambridge)</td>
</tr>
<tr>
<td>HA-VIRF2 (FL)</td>
<td>Codon optimised full length vIRF2 with N-terminal TAP tag (2 copies of the streptavidin-binding sequence and 1 copy of the FLAG epitope (Gloeckner et al., 2007), doxy-inducible, zeocin selection marker, AmpR</td>
<td>Constructed by A. Fowotade</td>
</tr>
<tr>
<td>HA-VIRF2 (N-TER)</td>
<td>Codon optimised N-terminal of vIRF2 with N-terminal TAP tag (2 copies of the streptavidin-binding sequence and 1 copy of the FLAG epitope (Gloeckner et al., 2007), doxy-inducible, zeocin selection marker, AmpR</td>
<td>Constructed by A. Fowotade</td>
</tr>
<tr>
<td>HA-VIRF2 (C-TER)</td>
<td>Codon optimised C-terminal of vIRF2 with N-terminal TAP tag (2 copies of the streptavidin-binding sequence and 1 copy of the FLAG epitope (Gloeckner et al., 2007), doxy-inducible, zeocin selection marker, AmpR</td>
<td>Constructed by A. Fowotade</td>
</tr>
<tr>
<td>pCDNA4TO-NTAP</td>
<td>N1 with a C-terminal TAP tag, doxy-inducible, zeocin selection marker, AmpR.</td>
<td>A kind gift from C. Maluquer de Motes (Maluquer de Motes et al., 2011)</td>
</tr>
<tr>
<td>pcDNA3 IRF9-S2C</td>
<td>Full length IRF9 fused to the N terminal 104 aa of STAT2, neomycin selection marker, AmpR.</td>
<td>Obtained from C. Horvath (Addgene #37544) (Kraus et al., 2003)</td>
</tr>
<tr>
<td>pcDNA3 IRF9-S1C</td>
<td>Full length IRF9 fused to the 38 C-terminal amino acids of STAT1, neomycin selection marker, AmpR.</td>
<td>Obtained from C. Horvath (Addgene #37543) (Kraus et al., 2003)</td>
</tr>
</tbody>
</table>
2.6. Plasmid DNA propagation and purification with endo free plasmid maxiprep

A single bacteria colony was inoculated into 5mL Luria Bertani broth (LB) containing 100μg/mL ampicillin. This was incubated overnight at 37°C on a shaker (165 rpm). The next day the bacterial broth was diluted down 1:1000 in LB broth containing 100μg/mL Carbenicillin (Sigma-Aldrich, D9170) or 5μg/mL of Kanamycin (Sigma-Aldrich, 60615) and further incubated overnight at 37°C on a shaker.

The bacterial cells were harvested by centrifugation at 6000 x g for 15 minutes at 4°C. Plasmid DNA was extracted from bacterial cultures with the Endo-free MaxiPrep kit (Qiagen, 12362) according to the manufacturer’s instructions. Briefly, the bacterial cells were lysed with 250μL of the lysis buffer P2. The released proteins were precipitated using chilled buffer P3. The precipitate was then poured into the barrel of the QIAfilter cartridge and the filtered lysate allowed to flow through a Qiagen tip under gravity. DNA was eluted from the tip by adding buffer QN and then centrifuged at 15,000 x g for 30 minutes at 4°C. The pellet was air-dried for 5–10 minutes, and re-suspended in a suitable volume of endotoxin-free Buffer TE.

Plasmid yield was determined by spectroscopy on Nanodrop 2000, UV-Vis Spectrophotometer (Thermo Scientific Inc) and the quality was further confirmed by resolving on 1% agarose gel.

2.7. Transformation reactions

A vial of frozen One Shot Chemically Competent E. Coli (Invitrogen) was thawed on ice. The plasmid was added to the competent cells, mixed, and kept on ice at room temperature for 30 minutes. The cells were subjected to a heat shock (42°C, 45 seconds), recovered on ice for 2 minutes and then transferred into 250μL of pre-warmed SOC media (Invitrogen, 15544034). A second non-inoculated vial was set up as a control for each transformation experiment. This mixture was then incubated at
37°C in a shaking incubator (225 rpm) for 1 hour. 100μL of the broth was then spread on labelled LB agar plates and incubated at 37°C overnight. Colony purification was carried out by selecting a single colony on LB agar plate and repeating this step twice.

2.8. PCR AND qPCR

2.8.1. RNA extraction

Cells were collected using cell scrapers (Corning, CLS3010) and centrifuged at 1000 x g for 4 minutes at 4°C, then washed once with PBS. The supernatant was discarded and the cell pellet lysed in RNA lysis buffer. RNA was extracted from cell lysates using the ZR MiniPrep™ kit (Cat No. R1064, ZymoResearch, USA) according to the manufacturer’s instruction. The RNA was DNAse treated using the Ambion TURBO DNA-free™ Kit (Life Technologies, AM1907). The RNA was eluted into DNAse/RNase free tubes using 50μL of RNase/DNAse free water. The extracted RNA was quantified spectrophotometrically and also subjected to RNA gel electrophoresis to assess the integrity of the RNA. Gel electrophoresis is described in section 2.8.4.

2.8.2. cDNA synthesis

cDNA synthesis utilises the reverse transcriptase enzyme for conversion of RNA into cDNA, which serves as a template for PCR and qPCR. This was performed using Transcriptor First strand cDNA synthesis kit (Roche, 04379012001) according to the manufacturer’s instruction. The anchored oligo dT primers bind at the beginning of the poly (A) tail and generates full length cDNA. A total of 1μg of the extracted RNA was mixed with 50pmol/μL of oligo dT primers and made up to a volume of 13μL with sterile distilled water. This was incubated at 65°C for 10 minutes, followed by a 2 minutes incubation on ice. To each sample, 4μL of Transcriptor reaction Buffer and 2μL of 10 mM deoxynucleotide mix was added along with 0.5μL of RNase inhibitor, to minimise degradation of the RNA. The contents were vortex mixed after addition of 0.5μL of M-MLV reverse transcriptase enzyme. Samples were incubated at 50°C
for 60 minutes. Inactivation of the reaction was achieved through heating at 85°C for 5 minutes followed by immediate cooling on ice. The resultant cDNA was stored at -20°C until it was used as a template for amplification in PCR or qPCR experiments.

2.8.3. Polymerase chain reaction (PCR)

PCR is the exponential amplification of a target DNA using a thermostatic Taq polymerase enzyme in the presence of a complementary DNA sequence. These reactions were carried out using GoTaq DNA Polymerase (Promega, M3001) in RNase/DNase-free PCR tubes (Invitrogen, AM12225). The desired amount of template cDNA was mixed with 5μL of 5X GoTaq Reaction Buffer (Promega, M7911), 2.5μL of dNTPs (Promega, U1511), 2.5μL of forward primer (200ng/μL), 2.5μL of reverse primer (200ng/μL) and 0.5μL of GoTaq DNA Polymerase and made up to 50μL with PCR grade water (Sigma-Aldrich, W1754). The tubes were spun for 5 seconds in a microcentrifuge and PCR was performed as indicated in Appendix Table 8-4. A list of genes used for PCR can also be found in Appendix C.

2.8.4. Agarose gel electrophoresis

Agarose gel electrophoresis uses an electric field to separate DNA fragments according to their sizes. Agarose (Sigma-Aldrich, A9539) was weighed and dissolved in a solution of 1 X Tris/Boric EDTA (TBE) by heating, resulting in a final concentration of 1- 2.0 % (w/v) depending on the size of fragments to be separated (details in Table 8-1). SYBR safe™ DNA (Invitrogen, S33102) was added at a dilution rate of 1:10,000. DNA or RNA samples were mixed with loading buffer (at a ratio of 5:1) and loaded on the gel. A corresponding DNA or RNA ladder (New England Biolabs) was also loaded each time to estimate the size of the desired bands. The gel was run in a Mini Sub Gel GT Electrophoresis Tank (BioRad, 1704487EDU) at 40-100V (dependent on gel percentage), for a time sufficient to separate desired bands in 1 X TBE. DNA bands were visualised with a Gel Doc™EZ system (BioRad, 1708270).
2.8.5. Real time quantitative PCR (qPCR)

A real-time PCR reaction utilizes the same principle of conventional PCR, but has the advantage of being able to amplify and simultaneously detect or quantify a targeted DNA molecule.

qPCR was performed on cDNA generated as described in section 2.8.2. Primer/probe sets for vIRF2 and vIRF4 genes were ordered from Sigma-Aldrich and designed to span intron-exon boundaries to ensure mRNA specific amplification (Appendix G). As an internal control for each reaction, a VIC labelled human GAPDH primer/probe set was used, to which data was normalised.

For the ISGs, primer/probe sets designed by PrimerDesign Ltd (Southampton, UK) were used and sequences are described in Appendix C. Primer and probes were used at 3mM and data was normalised to the housekeeping gene (Tubulin primer/probe) also used at 3mM. The primers for validation of the microarray qPCR validation were normalised against GAPDH genes. A Mastermix was generated based on the number of samples to be analysed and 15μL of this was pipetted into the wells of a MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems, 4306737). 5μL of cDNA template was then added separately, for each sample in the 96-well plate. Negative controls (no template and samples synthesised in the absence of reverse transcriptase) were included for each experiment. Samples were analysed on the Quantstudio™7 FLEX Real-Time PCR system (Life Technologies) using the following conditions; 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

2.9. SDS PAGE and Western blotting

Western blotting is an immunoassay that uses specific antibodies to identify proteins that have been separated based on size and charge by gel electrophoresis.
2.9.1. Cell lysate preparation

The cell culture plate was placed on ice and the cells rinsed with ice cold PBS. The cells were collected in an appropriate volume of ice cold radio immunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, R0728, see details in Appendix B) then centrifuged at 13,000 rpm for 5 minutes at 4°C to remove cell debris. Halt™ Protease inhibitor cocktail (100X) (ThermoFisher Scientific, 78420B) was added to lysis buffer at a dilution of 1:100. For immunoprecipitation, cells were lysed in IP lysis buffer (Table 2-7), the lysates were rotated in a cold room (30 minutes, 4°C) before being centrifuged (20 minutes, 13,000g 4°C) to remove cell debris. The supernatant was transferred into a fresh eppendorf tube and stored at -80°C until subsequent use.

2.9.2. Determination of protein concentration

Protein concentrations were determined using the Thermo Scientific Pierce BCA Protein Assay Kit (Catalogue No. 23225) according to the manufacturer’s instruction. Briefly, bovine serum albumin (BSA) standards of known concentrations were made up in distilled H₂O. The working reagent (WR) was prepared by mixing 50 parts BCA Reagent A with 1 part BCA Reagent B (50:1, Reagent A: B). 10μL of each standard and unknown were added to the 96-well microplate in triplicate. A 200μL volume of the WR was added to each well and mixed thoroughly on a plate shaker for 30 seconds. The plate was incubated at 37°C for 30 minutes and the absorbance was measured at 520nm, using POLARstar Omega (BMG LABTECH) spectrophotometer.

2.9.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, (SDS-PAGE)

Protein samples in 2 X loading buffer mixtures were loaded into wells of Precast TGX Mini-PROTEAN Tetra cell (BioRad, 165-8000) alongside a protein molecular weight marker (Novex protein ladder by Life Technologies, LC5800). Gels were run at 100V in 1 X SDS-PAGE running buffer for 90 minutes.
2.9.4. Western blotting

The resolved protein samples contained within the SDS-polyacrylamide gel were transferred to a Whatman protran nitrocellulose membrane using the Mini-Protean 3 Trans-blot Electrophoretic Transfer apparatus (BioRad, 170-3930). The gel was first removed from the gel cassettes and sandwiched with nitrocellulose between two layers of 3MM filter paper (Whatman). This sandwich was placed between two sponges in the transblot apparatus and run at 90 V for 2 hours. Post transfer the non-specific antibody binding sites on the blotted membrane were blocked for 1 hour at room temperature in 5% w/v Fluka analytical skimmed milk (Sigma-Aldrich, 70166 and 0.1% v/v Tween-20 (Sigma-Aldrich, P2287) in PBS. The membrane was probed overnight with the desired primary antibody (see Table 2-5) diluted in blocking buffer. Following primary antibody incubation, the membrane was washed (3 x 10 minutes) with PBS-Tween. The membrane was then incubated for at least 1 hour with the desired secondary antibody (see Table 2-6) diluted in blocking buffer. The membrane was again washed (3 x 10 minutes) with PBS-Tween. Immunoreactive bands were visualised using the Odyssey® CLx Imaging System infrared imaging system from LI-COR biosciences. In some instances, where indicated, the amount of protein on an immunoblot was assessed by quantification of protein bands using Odyssey software (LI-COR) and normalised to quantification of an appropriate control protein on the same membrane. The proteins for microarray validation were viewed using Enhanced Chemiluminescence (ECL) as recommended by manufacturer. Briefly, the membranes were incubated in Clarity Max™ Western ECL Blotting Substrates A and B (BioRad, 1705060) for 2 minutes before viewing on the Alpha Innotec® FLUORCHEM Q imaging system.

Antibodies were diluted in 5% (w/v) milk proteins in PBS containing 0.01% Tween-20 (TBST). For others depending on the manufacturers’ recommendation, antibodies
were diluted in 5\% (w/v) bovine serum albumin (BSA) in TBST. The complete list of antibodies used for immunoblotting in this study is shown in Table 2-5.
Table 2-5: Primary antibody used throughout this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse β-actin</td>
<td>1:2500</td>
<td>Sigma (A-5441)</td>
</tr>
<tr>
<td>Rabbit USP7</td>
<td>1:1000</td>
<td>Bethyl Laboratories (A300-033A)</td>
</tr>
<tr>
<td>Mouse Polyhistidine</td>
<td>1:1000</td>
<td>Sigma (H1029)</td>
</tr>
<tr>
<td>Rabbit RSAD2</td>
<td>1:200</td>
<td>Santa Cruz (0-24)</td>
</tr>
<tr>
<td>Rabbit EPSTI1</td>
<td>1:200</td>
<td>Santa Cruz (C-20)</td>
</tr>
<tr>
<td>Rabbit BRUNOL-4</td>
<td>1:100</td>
<td>Santa Cruz (A-4)</td>
</tr>
<tr>
<td>Rabbit TBP</td>
<td>1:2000</td>
<td>Abcam (Ab818)</td>
</tr>
<tr>
<td>Rabbit STAT1</td>
<td>1:100</td>
<td>Santa Cruz Biotechnology (sc-346)</td>
</tr>
<tr>
<td>Rabbit p-STAT1 Tyr 701</td>
<td>1:1000</td>
<td>Santa Cruz Biotechnology (9167)</td>
</tr>
<tr>
<td>Rabbit STAT2 (C-20)</td>
<td>1:100</td>
<td>Santa Cruz Biotechnology (sc-476)</td>
</tr>
<tr>
<td>Rabbit pSTAT2</td>
<td>1:1000</td>
<td>Millipore Catalogue (07-224)</td>
</tr>
<tr>
<td>Rabbit p48/IRF-9</td>
<td>1:100</td>
<td>Santa Cruz Biotechnology (sc-10793)</td>
</tr>
<tr>
<td>Rabbit Xpress</td>
<td>1:2000</td>
<td>Invitrogen (R910-25)</td>
</tr>
<tr>
<td>Mouse P53</td>
<td>1:1000</td>
<td>Santa Cruz Biotechnology (DO-1)</td>
</tr>
<tr>
<td>Mouse anti-FLAG</td>
<td>1:5000</td>
<td>Sigma-Aldrich (F1804)</td>
</tr>
<tr>
<td>Mouse GAPDH</td>
<td>1:4000</td>
<td>Ambion (6C5)</td>
</tr>
</tbody>
</table>

Table 2-6: Secondary antibody used throughout this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-rabbit IRDye800LT-conjugated</td>
<td>1:10,000</td>
<td>LI-COR Biosciences (926-68020)</td>
</tr>
<tr>
<td>anti-mouse IRDye680LT-conjugated</td>
<td>1:20,000</td>
<td>LI-COR Biosciences (926-32211)</td>
</tr>
<tr>
<td>anti-mouse HRP-conjugated</td>
<td>1:5000</td>
<td>DAKO (P0447)</td>
</tr>
<tr>
<td>anti-rabbit HRP-conjugated</td>
<td>1:5000</td>
<td>DAKO (P0448)</td>
</tr>
<tr>
<td>Solution</td>
<td>Composition</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>Non-ionic detergent lysis buffer</td>
<td>50mM Tris HCl pH 8.0, 50mM NaCl, 5 mM EDTA, 1% Triton X-100, 50mM sodium fluoride, 1mM sodium orthovanadate, 0.05% SDS, 10mM Sodium pyrophosphate, 1mM PMSF, (used at 1:100 dilution), Protease Inhibitor cocktail set III calbiochem (539134) (used at 1:100 dilution)</td>
<td></td>
</tr>
<tr>
<td>IP lysis buffer</td>
<td>50mM Tris-HCL (pH 7.5), 125mM NaCl, 5% Glycerol, 0.2% NP-40, Protease cocktail inhibitor set III calbiochem (539134) (used at 1:100 dilution)</td>
<td></td>
</tr>
<tr>
<td>10x Electrophoresis running buffer (1L)</td>
<td>Tris base (30.24 g), Glycerine (142.5 g), 1L distilled water (dH₂O), pH 8.4</td>
<td></td>
</tr>
<tr>
<td>10x Electrophoresis running buffer (1L)</td>
<td>100ml 10X electrophoresis buffer, 0.1% of 20% SDS, 1L dH₂O</td>
<td></td>
</tr>
<tr>
<td>10X Transfer buffer (1 L)</td>
<td>Tris 30.3g, Glycerine 142.5g &amp; 1 L dH₂O, pH to 8.4</td>
<td></td>
</tr>
<tr>
<td>1X Transfer buffer (1 L)</td>
<td>100ml 10X transfer buffer, 20% methanol, 700ml dH₂O</td>
<td></td>
</tr>
<tr>
<td>4X Tris-SDS-HCl, pH 6.8</td>
<td>0.5M Tris-Cl, 0.4% SDS</td>
<td></td>
</tr>
<tr>
<td>4X Tris-SDS-HCl, pH 8.8</td>
<td>1.5M Tris-Cl, 0.4% SDS</td>
<td></td>
</tr>
<tr>
<td>Tris Buffered Saline (TBS) (1 L)</td>
<td>NaCl 90g, Tris base 60g &amp; 1 L dH₂O</td>
<td></td>
</tr>
<tr>
<td>TBS-Tween (TBS-T) (1 L)</td>
<td>100ml TBS, 0.05% Tween-20, 900ml dH₂O.</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>10x PBS tablets (DULBECCO) dissolved in 1 L of milliQ water then autoclaved.</td>
<td></td>
</tr>
<tr>
<td>PBS-Tween (PBS-T) (1 L)</td>
<td>100ml PBS, 0.05% Tween-20, 900ml dH₂O.</td>
<td></td>
</tr>
<tr>
<td>2X Loading sample buffer (10ml)</td>
<td>1M Tris-HCl, pH 6.8, 4ml 10% SDS, 2ml glycerol, 2.5ml 2-β-mecaptoethanol, 500 μl bromophenol blue (1%)</td>
<td></td>
</tr>
<tr>
<td>Stripping Buffer</td>
<td>100mM Tris-HCl pH 6.8, 2% (w/v) SDS, 50mM 2-β-mercaptopoethanol.</td>
<td></td>
</tr>
<tr>
<td>5% Milk blocking buffer in TBS-T</td>
<td>5% (w/v) powdered milk dissolved in TBS-T, stored at 4°C.</td>
<td></td>
</tr>
<tr>
<td>5% Milk blocking buffer in PBS-T</td>
<td>5% (w/v) powdered milk dissolved in PBS-T, stored at 4°C.</td>
<td></td>
</tr>
<tr>
<td>Enhanced chemiluminescence</td>
<td>Amersham (RPN2106)</td>
<td></td>
</tr>
</tbody>
</table>
2.10. **Co-immunoprecipitation**

Cells lysates from transfected cells were prepared as in section 2.9.1. Some of the lysate was removed to be kept as ‘input’ lysate. 250µg of each sample was precleared by incubation with protein G-Sepharose 4B fast flow (Sigma-Aldrich, GE17-0618-01) or Thermo Scientific Pierce Streptavidin Agarose beads (Catalogue No. 20347) on a rotating wheel (5 hours, 4°C). Protein G-Sepharose 4B fast flow or Thermo Scientific Pierce Streptavidin Agarose beads was then added to these samples to allow immunoprecipitation and they were left to incubate further on the rotating wheel (overnight, 4°C). The beads were washed three times with IP lysis buffer by centrifugation (1 minute, 16,000g, 4°C) and the supernatants were collected in a fresh tube. Immunoprecipitates were analysed by western blot.

**Table 2-8: Antibodies used for immunoprecipitation**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xpress</td>
<td>1µL per sample</td>
<td>Invitrogen (R910-25)</td>
</tr>
<tr>
<td>Mouse IgG1 Isotype control</td>
<td>Matched to that of primary antibody</td>
<td>R&amp;D Systems (MAB002)</td>
</tr>
</tbody>
</table>

2.11. **Polysome profiling**

Polysome profiling is the spectrophotometric analysis of lysates through high velocity separation of translation complexes in linear sucrose gradients. Actively translating higher density polysomes are separated from the lower density monosomes, as well as free RNAs. Polysome profile analysis directly monitors the efficiency of translation.

Interferon treated vlRF4 expressing-stable cells and the empty vector control were collected and centrifuged at 1000 rpm for 4 minutes. The pellet was washed twice with PBS and cycloheximide to remove residual media. The cells were then resuspended in 500µL of polysome lysis buffer (Table 2.5). The lysate was further centrifuged (13,000 x g, for 5 minutes at 4°C). The supernatant was transferred into an RNase free ependorf, snap frozen on dry ice and stored at -80°C.
2.11.1. Sucrose gradient preparation and centrifugation

The monosome and polysome fractions come to rest when their density is equivalent to that of the surrounding sucrose, allowing cellular components to be analysed according to density. The various concentrations of sucrose were weighed and diluted in 1 X TMN buffer (Appendix D) to produce sucrose solutions at 10%, 18%, 26%, 34%, 42%, 50% and 60% sucrose in 20mL 1X TMN (Appendix A). To each of these, 1mg/mL cycloheximide was added to prevent polysome run off. The cycloheximide binds to the 60S ribosomal subunit and blocks translation elongation by preventing release of deacylated tRNA from the ribosome E site after translocation. 2µL of RNase inhibitor (Promega, N2611) was also added.

Sucrose solutions were layered in ultracentrifuge tubes (Beckman Coulter Inc., Brea, CA, USA) at a volume of 0.5mL for the densest portion, 60% concentration and 1.6mL for other concentrations. Each layer was frozen at -80°C for 30 minutes before the next layer was added. Gradients were stored upright at -80°C overnight then thawed at 4°C for 12 hours prior to use. The concentration of the cytoplasmic lysates were measured spectrophotometrically, before the lysates was carefully layered on the sucrose gradients and allowed to equilibrate. The thawed gradients were ultracentrifuged in a Beckman Coulter centrifuge (SW41.rotor) for 2 hours at 4°C (38,000 x g)

2.11.2. Fractionation and Data Acquisition

Previously ultracentrifuged lysates in the centrifugation tube were placed on a Tube Piercer of the density gradient fractionator (Teledyne ISCO, Lincoln, NE, USA) and tightly secured to the tube holder. A 65% sucrose solution was pushed through a 30mL syringe from the bottom of tube by switching on the syringe pump (kd Scientific Inc., Holliston, MA, USA)) to displace the gradient upwards at a flow rate of 1mL/min. The absorbance was continuously monitored at 254nm. Absorbance profiles were recorded by chart recorder, which is an integral part of the ISCO instrument and also
continuously recorded using the PeakTrak (Teledyne ISCO). Blank tubes containing extraction buffer were run first to determine the baseline. The absorbance values for the area under the curve for the test samples were calculated for the monosome and polysome fractions. These were used to determine the polysome-monosome ratio (P/M ratio) for the vIRF4 expressing cells, which was compared with the EV-NTAP (Figure 3-13).

RNA was extracted from the polysome fractions and stored at -80°C for cDNA synthesis and qPCR as earlier outlined in sections 2.8.2 and 2.8.5 respectively.
### Table 2-9: Buffers used for polysome profiling

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysome lysis buffer (10X)</td>
<td>3 M NaCl, 7.5 M Tris HCl, 150 mM MgCl₂</td>
</tr>
<tr>
<td>Polysome lysis buffer (1X)</td>
<td>0.5 mL polysome lysis buffer (10X), 0.5 mL Triton (10%), 50 µL Cycloheximide, 5 µL RNAse inhibitor, made up to 5 mL with RNAse-free water</td>
</tr>
<tr>
<td>10 X TMN</td>
<td>3 M NaCl, 150 mM MgCl₂, 150 mM Tris-HCl at pH 7.5, 1 mg/mL Cycloheximide</td>
</tr>
<tr>
<td>1 X TMN</td>
<td>10 X TMN buffer was diluted 1 in 10 to produce 1X TMN buffer</td>
</tr>
</tbody>
</table>

### Table 2-10: Composition of the Sucrose gradients

<table>
<thead>
<tr>
<th>Components</th>
<th>10%</th>
<th>18%</th>
<th>26%</th>
<th>34%</th>
<th>42%</th>
<th>50%</th>
<th>60%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>2 g</td>
<td>3.6 g</td>
<td>5.2 g</td>
<td>6.8 g</td>
<td>8.4 g</td>
<td>10 g</td>
<td>12 g</td>
</tr>
<tr>
<td>10XTMN Buffer</td>
<td>2 mL</td>
<td>2 mL</td>
<td>2 mL</td>
<td>2 mL</td>
<td>2 mL</td>
<td>2 mL</td>
<td>2 mL</td>
</tr>
<tr>
<td>RNAse inhibitor</td>
<td>2 µL</td>
<td>2 µL</td>
<td>2 µL</td>
<td>2 µL</td>
<td>2 µL</td>
<td>2 µL</td>
<td>2 µL</td>
</tr>
<tr>
<td>Cycloheximide(1 mg/mL)</td>
<td>200 µL</td>
<td>200 µL</td>
<td>200 µL</td>
<td>200 µL</td>
<td>200 µL</td>
<td>200 µL</td>
<td>200 µL</td>
</tr>
</tbody>
</table>

NB: Make each gradient up to 20 mL with RNAse free water and ensure even mix before filter sterilization with 0.22 µM.
2.12. Immunofluorescence assay

2.12.1. Plating, fixing and permeabilising

Adherent cells were grown on 16mm cover slips (Menzel-Gläser) in 12-well plates. Growth media was aspirated and cells washed with PBS, then fixed 4% (w/v) paraformaldehyde (20 minutes). 0.5% Triton-X was then used to permeabilise the cells (10 minutes) after which cells were blocked in blocking solution (30 minutes, room temperature), which comprised of heat inactivated neonatal calf serum (NCS), diluted to 10% (v/v) in PBS.

2.12.2. Immunofluorescence staining

Primary antibodies (see Table 2-12) were diluted in blocking solution and incubated on cells (30 minutes, room temperature). The slides were washed three times in PBS, before addition of the secondary antibody (see Table 2-13), also diluted in blocking solution, and incubated in the dark (30 minutes, room temperature). Slides were again washed three times and rinsed once in milliQ water (1 minute, room temperature). After a final wash, the coverslips were inverted onto glass slides using Mowiol® as the mounting agent (details of Mowiol® constituents is found in Appendix B). For nuclear staining, 4’,6-Diamidine-2’-phenylindole dihydrochloride (DAPI) was added to the Mowiol® 8-88 (Sigma-Aldrich, 81383) in 1:1000 dilution ratio (see Appendix B for more details on Mowiol® 8-88 and DAPI).

2.12.3. Cell imaging

Cells were viewed using a LSM 510 META confocal laser scanning microscope (Carl Zeiss). Either a 40X or 60X oiled objective was used and each laser was set to an equal optical slice and a pinhole diameter of approximately 1 Airy unit.
### Table 2-11: Primary antibodies used for Immunofluorescence

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-USP7</td>
<td>1:200</td>
<td>Bethyl Labs (A33-033A)</td>
</tr>
<tr>
<td>Mouse IgG Isotype control</td>
<td>1:200</td>
<td>R&amp;D (MAB002)</td>
</tr>
<tr>
<td>Anti-His</td>
<td>1:200</td>
<td>Sigma-Aldrich (H1029)</td>
</tr>
<tr>
<td>Anti-HA</td>
<td>1:200</td>
<td>Sigma-Aldrich (H3663)</td>
</tr>
</tbody>
</table>

### Table 2-12: Secondary antibodies used for Immunofluorescence

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate</td>
<td>1:200</td>
<td>Invitrogen (A-1001)</td>
</tr>
<tr>
<td>Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 568 conjugate</td>
<td>1:200</td>
<td>Invitrogen (A-1011)</td>
</tr>
</tbody>
</table>
2.13. Cell viability assay to measure toxicity of treatments

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, M2128) is based on the cellular reduction of MTT (Sigma-Aldrich) by the mitochondrial dehydrogenase of viable cells, to a blue formazan product that can be measured using a spectrophotometer. This assay determines the proportion of live cells based on metabolic activity within the wells. Living cells that are actively metabolising convert 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (a redox dye) into formazan, a fluorescent end product, whereas nonviable cells lose metabolic capacity and therefore cannot generate a fluorescent signal.

For siRNA assays, 72 hours post siRNA transfection, 50µL of MTT solution (5mg/mL) was added into each of the 24-well plates, then incubated for 4 hours at 37°C. The formazan was then solubilized with 500µL of DMSO. Optical density was measured using a spectrophotometer (Omega Fluorostar, Switzerland) at 540nm. Non-transfected cells were used as positive controls, while cells killed with 1% Triton X-100 were used as negative control. The percentage viability of each sample was derived by subtracting the negative control from each OD reading and further divided by the OD of the positive control.

2.14. Microarray

2.14.1. RNA Analysis

The integrity of the RNA extracted from polysomal and subpolysomal fractions were analysed using the Agilent RNA 6000 nano kit (G2938-90030). The results were analysed on an Agilent 2100 Bioanalyzer instrument. Agilent RNA kits contain chips made up of interconnected microchannels that allows electrophoretic separation of nucleic acid fragments according to their sizes.
2.14.1.1. Sample Preparation

1.5µL of the RNA was placed into RNase free tubes and heated at 70°C for 2 minutes. The sample was incubated on ice for 30 seconds then vortexed for 1 minute.

2.14.1.2. Preparing the Gel-Dye

The RNA dye concentrate (blue) was allowed to equilibrate to room temperature for 30 minutes and vortexed for 10 seconds. 1µL of dye was added into a 65µL aliquot of filtered RNA gel then vortexed well. The tube was spun at 13000 g for 10 minutes at room temperature.

2.14.1.3. Loading the Gel-Dye Mix

A new RNA chip was loaded on the chip priming station. 9µL of gel-dye mix was loaded in the well G. The plunger was positioned at 1mL and the chip priming station was closed. The plunger was pressed until it was held by the clip. The clip was released after 30 seconds and the plunger was finally pulled back after a further 10 seconds. The chip priming station was opened and 9µL of gel-dye mix was added into the two wells marked G.

2.14.1.4. Loading the Marker and the samples

5µL of RNA marker (green) was dispensed into all 12 sample wells and in the ladder well. 1µL of prepared ladder was loaded in ladder well and in the 12 sample wells. 1µL of sample was added into each of the 12 sample wells. 1µL of RNA Marker (green) was loaded into each unused sample well. The chip was vortexed for 1 minute and then analysed on the Agilent 2100 Bioanalyzer instrument within 5 minutes.

2.14.2. Qubit 2.0 Molecular probe fluorometer Invitrogen (Life Technologies)

Two assay tubes were set up for the standard, and one for each of the samples. The Qubit working solution was prepared by diluting the Qubit reagent 1:200 with the Qubit buffer. 200µL of working solution was prepared for each standard and sample (190µL of working solution was added to 10µL of the standard while 199µL of working solution
was added to 1µL of each sample). The tubes were vortexed for 3 seconds and incubated for 20 minutes at room temperature. Each tube was inserted into the Qubit fluorometer and the RNA concentration was measured.

2.14.3. Labelling of samples
Total RNA was amplified and labeled with Spike A (Cyamine 3-CTP) or Spike B (Cyamine 5-CTP) using the Agilent Low Input Quick Amplification kit according to manufacturer’s recommendation. This method uses T7 RNA Polymerase Blend and allows simultaneous amplification of target material and incorporation of Cyamine 3-CTP or Cyamine 5-CTP. Briefly, the Spike A and Spike B mixes were vortexed then heated for 5 minutes at 37°C. Both tubes were then spun for a minute at 13,000 rpm at room temperature.

Four dilutions were made from each sample with the first, second and third dilutions made in dilution ratios; 1:20, 1:40 and 1:16 respectively. The fourth dilution was derived using the formula

\[
\frac{1}{[\text{Total RNA (ng)} \times 0.005]}
\]

Following labeling, the cRNA was quantified by UV-Vis spectroscopy and 200ng each of Cy3 and Cy5 labeled targets were combined.

cDNA was synthesised by adding 1.8µL of T7 Primer Mix into each tube that contained 3.5µL of RNA and diluted spike-in controls. The reaction was incubated at 65°C for 10 minutes to denature the primer. The reaction was then placed on ice for 5 minutes. The 5X First Strand Buffer (Agilent Technology) was pre-warmed at 80°C for 4 minutes then briefly vortexed for a further minute. The cDNA Mastermix was prepared using 2µL 5X First Strand buffer, 1µL 0.1M DTT, 0.5µL 10mM dNTP and Affinity Script RNase Block Mix. The Mastermix was spun and added to each of the samples. The sample was heated to 40°C for 2 hours followed by 70°C for 15 minutes.
The sample was incubated on ice for 5 minutes then spun down and stored at -80°C till further use.

To make cRNA from the cDNA, two transcription master mixes were made, one with Cyanine 3-CTP and one with Cyanine 5-CTP in separate 1.5mL microcentrifuge tubes. The Master Mix was made from 0.75µL nuclease-free water, 3.2µL 5X Transcription Buffer, 10.6µL 0.1M DTT, 1µL NTP Mix, 0.21µL T7 RNA Polymerase blend and 0.24µL Cyanine 3-CTP or Cyanine 5-CTP. 6µL of Transcription Mastermix with Cyanine 3-CTP was added to the tube with Spike A Mix and similar volume into the tube containing the Spike B Mix. Samples were incubated at 40°C overnight.

2.14.4. Purification of cRNA

The RNeasy Mini Kit (Cat No. 74106) was used to purify the amplified cRNA samples. 84uL of nuclease-free water (Ambion) was added to the cRNA sample followed by 350µL of Buffer RLT and 250µL of nuclease-free absolute ethanol. The cRNA sample was transferred into an RNeasy Mini Spin Column in a collection tube and spun at 4°C for 30 seconds at 13,000 rpm. The flow-through was discarded and column transferred to a fresh collection tube and centrifugation step was repeated at 4°C for 30 seconds at 13,000 rpm. The cRNA was eluted into a fresh tube by adding 30µL of nuclease-free water and further centrifuged at 4°C for 30 seconds at 13,000 rpm. The cRNA was quantified using the NanoDrop ND-2000 UV-VIS Spectrophotometer version 3.2.1. The cRNA yield (µg) and specific activity were derived as outlined below using the; Cyanine 3 or Cyanine 5 concentration (pmol/µL), RNA absorbance (260/280nm) and cRNA concentration (ng/µL).
\[ \mu g \text{ of RNA} = (\text{Concentration of cRNA}) \times 30 \mu L \text{ (elution volume)} \]

Specific activity = Concentration of Cy3 or Cy5

Concentration of cRNA

The yield of cRNA required for labelling in a 4-pack is 0.825\(\mu\)g, this was derived for each of the sample prior to Hybridization.

2.14.5. Hybridization

The Fragmentation mix was prepared by adding 825ng of Cyanine 3-labelled cRNA to an equal volume of Cyanine 5-labelled cRNA followed by addition of 11\(\mu\)L 10X Gene Expression Blocking Agent, 2.2\(\mu\)L 25X Fragmentation Buffer and bringing up the volume to 55\(\mu\)L by adding 52.8\(\mu\)L nuclease-free water (Ambion). The mix was incubated at 60\(^\circ\)C for 30 minutes, then cooled on ice for 2 minutes. 55\(\mu\)L of the cRNA was added to 2X Hi-RPM Hybridization Buffer, then centrifuged at 13,000 rpm for 1 minute.

The slide was placed on either end, such that the “Agilent”-labelled barcode is facing down parallel to the SureHyb gasket slide, while the numeric barcode is facing up. The assembled chamber was placed in the oven rotator rack and the hybridization rotator was set to rotate at 10 rpm. The Hybridization was carried out at 65\(^\circ\)C for 17 hours.
2.14.6. Washing the Microarray slides

The Gene Expression Wash Buffer was pre warmed to 37°C. The hybridisation chamber was removed from the incubator and the array-gasket sandwich submerged in Gene Expression Wash Buffer 1. The microarray slides were removed carefully and placed on the slide rack in a different slide-staining dish containing the Gene Expression Wash Buffer 1 at room temperature for a minute then transferred into Gene Expression Wash Buffer 2 at elevated temperature and left to stir for a further minute. The rack was slowly removed to minimise droplet formation on the slides.

2.14.7. Scanning the slides

The microarray slide was scanned using the Agilent G2565CA Microarray Scanner System (Agilent Technologies). Briefly, the slides were placed in the slide holder, ensuring that the active microarray surface faced up, toward the slide cover. Scanning was carried out immediately to minimize the impact of environmental oxidants on signal intensities.

2.14.8. Data extraction using Agilent Feature Extraction Software

The Agilent Feature Extraction (FE) software version A7.5.1 was used to extract the data in a TIFF file format. Agilent's FE software is programmed to read up to 100 raw microarray image files. The software inserts microarray grids, rejects outlier pixels, and ensures accurate determination of feature intensities and ratios. It also derives relevant statistical confidences. Details of data analysis is described in chapter 4.
2.15. Chandipura plaque assay

Table 2-13: Solutions used for the plaque assay

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque assay medium</td>
<td>DMEM containing 1% penicillin-streptomycin</td>
</tr>
<tr>
<td>Overlay medium</td>
<td>Carboxymethylcellulose (CMC), 2X MEM (10% v/v Minimum Essential Medium (MEM) with 1% v/v L-glutamine, 2% v/v foetal calf serum heat inactivated, 1% v/v penicillin/streptomycin, 1% Sodium bicarbonate)</td>
</tr>
<tr>
<td>Crystal violet</td>
<td>0.5g crystal violet, 20ml ethanol, 0.9g NaCl, 100mL 40% formaldehyde, 880mL dH2O</td>
</tr>
</tbody>
</table>

2.15.1. Infection of cells with Chandipura virus

HEK 293 cells were reverse transfected with USP7 siRNA or the non-targeting control siRNA for 48 hours in 24-well plates at a density of 2 x 10^4 cells per well. After 48 hours, the culture medium was carefully removed and the cells were infected with Chandipura virus at a multiplicity of infection (MOI) of 0.1 in 500μL of culture medium (1 hour at 37°C). The medium was removed from all cells, washed twice with sterile PBS, and left in fresh culture medium with or without rIFNαB2 (300IU/mL). 24 hours later the supernatant was harvested in order to quantify Chandipura by plaque assay.

2.15.2. Plaque assay

To determine the titre of the Chandipura virus in the supernatants from Chandipura infected HEK 293 cell lines, plaque assays were performed on Vero cells. Vero cells were plated in 6-well plates at a density of 6 x 10^5 in DMEM complete medium and grown until a confluent monolayer of cells was formed. The supernatants from Chandipura infected EV-NTAP cells were diluted from 10^4 to 10^8 in plaque assay medium (Table 2-13). 500μL of each of each of these dilutions was then used to infect the Vero cells by carefully pipetting the volume onto the cells and incubating (30 minutes, 37°C). Overlay medium (Table 2-13) was mixed with 2X MEM at a ratio of
1:1. 2mL of this mixture was then added to each well of infected Vero cells. The plates were incubated at 37°C for 24 hours, so that plaques could form.

2.15.3. Counting
To visualise the plaques, the overlay media was removed and carefully washed once with PBS. The cells were then covered with 2% formal saline (see appendix A) for 20 minutes, after which plaques were stained with 2mL of crystal violet and incubated for 60 minutes at room temperature with gentle agitation. To remove the crystal violet the plates were carefully washed in tap water. The plaques were easily observed at the bottom of each well. To calculate the titre of virus in plaque forming units per mL (PFU/mL), the number of plaques per well was multiplied by 2 (to account for the 500µL of virus dilution used to infect each well, giving the titre per mL). This value was then multiplied by the dilution factor of the virus sample used to infect that particular well.

2.16. Cloning
2.16.1. Polymerase chain reaction (PCR)
The regions of interest were designed by Invitrogen, Thermo Fisher Scientific Inc. from sequences provided for vIRF2, the N-terminal and C-terminal mutants. The gene maps are included in Appendix D. Briefly, the plasmids encoding proteins of interest were generated with the desired epitope tags. The synthetic genes designated vIRF2-FL, vIRF2-N and vIRF2-C, respectively, were assembled from synthetic oligonucleotides and or PCR products. The vIRF2-FL fragments were inserted into pMA-RQ (ampR) to form a 4393bp plasmid, while the vIRF2-N and vIRF2-C were inserted into pMA-T to form 3367bp and 3454bp plasmids respectively. The plasmid DNA was purified from transformed bacteria and concentration determined by ultraviolet spectroscopy. The final construct was verified by sequencing and the sequence homology within the insertion sites was found to be 100%. The plasmids were received as 5µg lyophilized concentrations.
2.16.2. Restriction enzyme digestion

The plasmids on receipt were re-suspended in 5µL RNase–free water and 2µL was digested, while 2µL was transformed and purified to make glycerol stocks. Expression plasmids, into which vIRF2 and mutant genes were cloned, are described below and in more detail in Table 2-4. Restriction enzymes were purchased from New England Biolabs, Promega or Roche, and used according to the manufacturer’s instructions. The required plasmid backbones and the vIRF2 plasmids were digested using restriction enzymes. Digestion mixes contained 1.5µg of DNA, 0.5µL each of the required restriction enzymes (Xbal (R6431) and Not1 (R6181) Promega), 1µL of the appropriate 10X restriction enzyme buffer B (Roche) and water (up to 10µL). Reactions were incubated for 2 hours at 37°C. DNA products were then once again analysed by agarose gel electrophoresis and purified from the agarose gel as described in section 2.8.4.

2.16.3. Agarose gel electrophoresis and purification of DNA from agarose gels

PCR products were analysed by electrophoresis on a 0.8 % agarose gel run at 90V for 45 minutes in TBE buffer, and SYBR® Safe (Thermo Fisher) was used to visualise DNA with a Long wave ultraviolet light. Correctly sized DNA products were compared with a 1 Kb DNA ladder (Promega), then excised from agarose gels and DNA was purified from these gel slices using the QiaQuick Gel Extraction Kit (Qiagen, ID 28704) according to the manufacturer’s protocol.

2.16.4. Ligation of DNA products

Ligation of digested PCR products with the desired digested plasmid backbones was performed at a 2:1 molar ratio of insert: vector. Each ligation reaction consisted of DNA insert (6µL), recipient vector (2µL), T4 DNA ligase buffer (1µL) and T4 DNA ligase (1µL) (Promega) and water to a volume of 10µL. Ligation reactions were incubated overnight at 16°C.
2.16.5. Transformation of ligation products and bacterial colony screening

1μL of ligation product was transformed as described in section 2.7. Transformed bacteria were then spread on LB agar plates containing 100μg/mL Carbenicillin (Sigma-Aldrich). Plates were incubated overnight at 37°C to allow bacterial growth. Screening of the transformed bacterial colonies was done by restriction digest analysis of DNA from selected colonies.

Table 2-14: The expression plasmids used throughout this thesis

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCDNA4TO-NTAP</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>TAP-viRF2</td>
<td>viRF2-FL cloned into pCDNA4TO-NTAP</td>
</tr>
<tr>
<td>TAP-viRF2 (N-terminal)</td>
<td>viRF2-N-terminal cloned into pCDNA4TO-NTAP</td>
</tr>
<tr>
<td>TAP-viRF2 (C-terminal)</td>
<td>viRF2C-terminal cloned into pcDNA4/HisMax</td>
</tr>
<tr>
<td>pCDNA4TO-NTAP/HA</td>
<td>Provided by C. Maluquer deMotes (University of Surrey)</td>
</tr>
<tr>
<td>HA-viRF2</td>
<td>viRF2-FL cloned into pCDNA4TO-NTAP/HA</td>
</tr>
<tr>
<td>HA-viRF2 (N-terminal)</td>
<td>viRF2-N-terminal cloned into pCDNA4TO-NTAP/HA</td>
</tr>
<tr>
<td>HA-viRF2 (C-terminal)</td>
<td>viRF2-C-terminal cloned into pcDNA4TO-NTAP/HA</td>
</tr>
<tr>
<td>pCDNA4TO-NTAP/GFP</td>
<td>Provided by C. Maluquer deMotes (University of Surrey)</td>
</tr>
<tr>
<td>GFP-viRF2</td>
<td>viRF2-FL cloned into pCDNA4TO-NTAP/GFP</td>
</tr>
<tr>
<td>GFP-viRF2 (N-terminal)</td>
<td>viRF2-N-terminal cloned into pCDNA4TO-NTAP/GFP</td>
</tr>
<tr>
<td>GFP-viRF2 (C-terminal)</td>
<td>viRF2C-terminal cloned into pcDNA4TO-NTAP/GFP</td>
</tr>
</tbody>
</table>
CHAPTER 3

FUNCTIONAL ANALYSES OF STABLE CELL LINES EXPRESSING vIRF2 AND vIRF4 AND THE EFFECTS OF THESE VIRAL PROTEINS ON INTERFERON SIGNALLING
3. **CHAPTER 3**

3.1. **Introduction to chapter 3**

My studies centre on understanding the effect of the cellular partners of KSHV vIRF2 and vIRF4 on interferon signalling. To enable this understanding, this chapter describes the establishment of biological readouts to measure interferon induction. In addition, this chapter also investigates the effects of vIRF2 and vIRF4 on type I interferon signalling.

The interferon signalling pathway is fundamental in antiviral innate immunity (Stark & Darnell, 2012). Upon virus infection of a cell, the innate type I interferon antiviral response is invoked to limit the spread of infection. The pivotal component of the pathway orchestrating this response is the constitutively expressed interferon regulatory factor (IRF)-3 protein. The IRF3 is post-translationally activated by kinases TBK1 and IKKε following viral infection (Hiscott, 2007). This C-terminal phosphorylation results in its dimerisation and concomitant involvement in the transcription of the type I interferon genes that drive the antiviral response (Nakatsu et al., 2014).

Activation of the JAK STAT pathway drives the synthesis of over 300 ISGs that inhibit different stages of the viral lifecycle (Horner, 2014). These ISGs are usually not synthesized at the basal state, but are induced to express and mediate the antiviral effector functions of IFN upon viral infection or IFN treatment (Schoggins & Rice, 2011).

Many viruses, including KSHV, encode genes with immunomodulatory activities. This capacity enables them to evade the host immune response, thus ensuring their survival and replication in the host. Herpesviruses are generally known to encode numerous immunomodulatory genes, many of which are cellular homologues (Rezaee et al., 2006). KSHV utilises a number of mechanisms to subvert the host
innate immune response in order to establish lifelong latency in the host (Giffin & Damania, 2014). In this section, I will discuss how KSHV vIRF2 and 4 downregulate the interferon response.

KSHV is unique among the human herpesviruses as it encodes the vIRFs1-4, which are homologues of cellular IRFs (Cunningham et al., 2003b; Jacobs & Damania, 2011). The cellular IRFs including IRF3 are a large family of proteins that regulate expression of type I IFN (IFNα and β) (Paun & Pitha, 2007). The interference of vIRFs in the interferon production pathway, as well as their manipulation of cell cycle pathways, have been previously discussed in section 1.7.2. Their role in antiviral immunity may have a twofold function: first, to suppress the induction of an IFN-induced antiviral state against the establishment of KSHV de novo infection and second, to facilitate persistent infection of the host (Giffin & Damania, 2014).

Briefly, I will discuss the effects of the vIRFs on interferon signalling. vIRF1 downregulates IFNα and β as well as IFNγ-responsive reporter genes. One of the mechanisms behind vIRF1 inhibition of IFN induction of ISGs is through suppression of the transcriptional activity of IRF1 without competing for DNA binding with IRF1 (Gao et al., 1997). The vIRF2 protein has been studied in detail by our group and shown to downregulate transactivation of an ISRE-driven promoter (Mutocheluh et al., 2011). Additionally, vIRF2 expression also suppressed IRF1 induction of ISRE activation (Fuld et al., 2006). Our group has described a mechanism for vIRF2-induced inhibition of ISRE-containing promoter activation through targeting of phosphorylated STAT1 component of the ISGF3 complex (Mutocheluh et al., 2011). This chapter further investigates the role of vIRF2 on transcription of ISGs. vIRF3 expression inhibits IRF5-mediated ISRE activation and RNAi-mediated silencing of vIRF3 in PEL cells resulting in an increased binding of IRF5 to ISRE oligonucleotides (Wies et al., 2009). vIRF4 shares homology with the cellular IRFs and reportedly targets cellular IRF4 and the Myc gene to facilitate lytic replication (Lee et al.,
The role of vIRF4 on the IFN response has not yet been elucidated. In one study, it was shown to interfere with the IRF3 pathway and no reports exist of its analysis in the JAK STAT pathway. This thesis further investigates the function of vIRF4 in the interferon response.

The objectives of this chapter are to:

1) Develop and optimise reporter gene assay methods to quantify the expression of an interferon-inducible promoter (containing ISRE elements).

2) Develop and optimise qPCR methods to quantify the expression of a panel of interferon-responsive genes.

3) Confirm the expression of vIRF2 and vIRF4 from stable cell lines.

4) Investigate the effect of vIRF2 and vIRF4 on JAK-STAT signalling and therefore ISRE-containing promoter activation.

5) Confirm the effect of vIRF2 and vIRF4 on transcription of the interferon sensitive genes, IFIT1, OAS1 and ISG15.

This chapter details how plasmids expressing the ISRE promoter (pISRE-luc) were transiently transfected into HEK 293 cell lines, including those transfected into stable cell lines expressing vIRF2 and vIRF4 and then stimulated with interferon to activate the ISRE promoter. This ISRE promoter plasmid was used to measure interferon inducible gene expression in reporter studies. The aim was to investigate the roles of vIRF4 on the JAK-STAT signalling pathway (through examining activation of an ISRE-containing promoter). The vIRF2 were used as a positive control as it had been shown previously to inhibit ISRE activation (Mutochelu et al., 2011).

RNA from interferon treated mammalian cell lines were analysed by qPCR to measure interferon inducible gene expression. To study in more detail the effects of vIRF2 and vIRF4 on IFN signalling, the effect of both proteins on the transcription of
ISGs was further investigated using quantitative PCR, which measured the mRNA levels of IFIT1, OAS1 and ISG15. The aim was to test the hypothesis that vIRF2 and vIRF4 could decrease the expression of these ISGs.

3.2. Optimisation of dual luciferase reporter gene assay for measuring interferon-inducible gene expression.

Plasmid containing an ISRE promoter derived from ISG54 (pISRE-luc) was transfected into HEK 293 cells. The cells were treated with and without interferon. The binding of IFN to the IFNAR triggers the JAK STAT signalling pathway and results in the activation of the ISRE reporter (Doceul et al., 2014). Therefore, the effect of IFN induction was investigated using the dual luciferase assays (DLA) with an IFN-responsive luciferase reporter gene. The doses of reporter plasmid (pISRE-luc) and the plasmid pRLSV40, which constitutively expresses Renilla luciferase, were optimised (Figure 3-1). An interferon dose titration and a time course were then performed on HEK 293 cells. Normalised luciferase activity of the ISRE reporter plasmid peaked at a dose of 300IU/mL (Figure 3.2B) after 12 hours of rIFNaB2 treatment (Figure 3-2A). These parameters were used to evaluate the ISRE promoter activity in subsequent experiments.
Figure 3-1: Optimisation of pISRE-luc and pRLSV40 required to efficiently detect induction of the ISRE promoter.

HEK 293 cells were transfected with different concentrations of pISRE-luc and the constitutively expressing pRLSV40 (shown in the figure). Following 5 hours incubation, the cells were treated with rIFNαB2 (300IU/mL) for 12 hours, before being harvested and subjected to dual luciferase assay (DLA). pRLSV40-luc was added as an internal control to which the firefly luciferase levels were normalised. This figure represents the mean±/- standard deviation of three independent experiments.
HEK 293 cells were co-transfected with 250ng pISRE-luc and 1ng pRLSV40. Following 5 hours incubation, the cells were treated with rIFNα (300IU/mL) for different durations of time (A) or increasing amounts of rIFNα (B) before being harvested and subject to DLA. pRLSV40 was added as an internal control to which the firefly luciferase levels were normalised. This figure represents the mean+/ standard deviation of three independent experiments.

Figure 3-2: Optimization of length and dose of interferon treatment
3.3. Optimisation of a quantitative PCR method to measure interferon-inducible gene expression

To develop a qPCR method for assessing interferon induction, amplification primers for different interferon stimulated genes were tested using PCR and qPCR methods (described in section 2.8). The genes encoding IFIT1, OAS1 and ISG15 were validated for this purpose.

RNA was extracted from interferon treated and mock treated HEK 293 cells and analysed by agarose gel electrophoresis to verify integrity. RNA was reverse transcribed to cDNA as described in section 2.8.2, then subjected to both PCR and qPCR reactions using IFIT1, OAS1 and ISG15 primers (sequence details in Table 8-5). The PCR analysis showed that interferon treated cells produced a higher intensity band compared to the mock treated cells, suggesting interferon induction. Figure 3-4 shows the PCR with IFIT1 induction in HEK 293 cells, stimulated with 300IU/mL of IFN for 8 hours, as well as the unstimulated control. The 8 hours was determined as optimum for qPCR.

For the qPCR assays, HEK 293 and HeLa cells were seeded at 5x10^5 and 3x10^5 respectively in 6-well plates. Both cell lines were stimulated with 300IU/mL rIFNαB2 for 8 hours. RNA was harvested from cells and used for qPCR analysis of ISG induction. The Delta CT (ΔCt ) was determined using the mean Ct values for ISGs normalised against values derived for the housekeeping gene, tubulin in the same experiment. The Delta Delta Ct (ΔΔCt) was determined by further normalisation of the Ct values in the presence and absence of interferon treatment. IFNαB2 treatment of cells resulted in the induction of the ISGs examined; IFIT1, OAS1, and ISG15. The normalised expression ratio (NER) for the ISGs gene was derived using the formula NER = 2^{ΔΔCt}. In HEK 293 cells, (Figure 3-5A), the normalised expression ratio for IFIT1 was 47.5 indicating induction of IFIT1 gene by 47.5 fold. OAS1 showed a much higher fold induction of 392.8 while ISG15 was induced by 65.9 fold. In HeLa cells,
(Figure 3-5B) the IFIT1, OAS1 and ISG15 were induced by 156, 386.1 and 34.6 fold respectively.

**Figure 3-3: Agarose gel analysis of RNA.**

1μl of RNA extracted from HEK 293 and resolved on 1% agarose gel. 1 kb DNA ladder (L) was loaded in parallel. The gel was run at 100V for 40 minutes. The RNA were of good integrity and showed distinct 28S and 18S bands.
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**Figure 3-4: PCR on RNA extracted from 293 cell lines using the IFIT1 Primers**

Reverse transcription was performed followed by PCR using IFIT1 primers. Lane 1 is the 1Kb DNA ladder while lane 3 is the interferon treated and lane 5 is the mock treated. The gap between lanes 1 and 3 is because the samples were not loaded contiguously. Lanes 4 and 6 contain samples not treated with reverse transcriptase (RT). The red arrow is pointed towards IFIT1 band from the interferon treated cells and the blue arrow towards the non-interferon treated IFIT band.
Figure 3-5A: Optimisation of qPCR methods to quantify the expression of interferon-stimulated genes in HEK 293 cell lines.

1µg of cDNA was reverse transcribed from RNA extracted from HEK 293 cell lines following treatment with or without 300IU/mL of rIFNαB2. The cDNA was utilised for qPCR using IFIT1, OAS1 and ISG15 primers. Data represents the mean ± the SD of the three independent experiments which were each assayed in duplicate. The results are shown as normalized expression ratio of IFIT1 (47.5), OAS1 (392.8), and ISG15 (65.9), normalised to tubulin and further normalised between interferon treated and mock-treated cells.

Figure 3-5B: Optimization of qPCR methods to quantify the expression of interferon-responsive genes in HeLA cell lines.

1µg of cDNA was reverse transcribed from RNA extracted from HeLa cell lines following treatment with or without 300IU/mL of rIFNαB2. The cDNA was utilised for qPCR using IFIT1, OAS1 and ISG15 primers. Data represents the mean ± the SD of the three independent experiments which were each assayed in duplicate. The results are shown as normalised expression ratio of IFIT1 (156), OAS1 (386.1), and ISG15 (34.6), normalised to tubulin and further normalised between interferon treated and mock-treated cells.
3.4. Functional analyses of stable cell lines expressing vIRF2 and vIRF4

In order to use cell lines expressing vIRF2 and vIRF4 for subsequent studies, their ability to induce vIRF2 and 4 expression was assessed. The dose and length of tetracycline treatment required to induce vIRF2 and 4 expression was also optimised.

3.4.1. Optimisation of VIRF2 and VIRF4 expression using tetracycline

3.4.1.1. Optimising the amount of tetracycline necessary to induce vIRF2 & 4 protein expression

The stable cell lines have been previously derived, in the Blackbourn lab by Laura Hindle. The vIRF2 and vIRF4 genes were cloned into the empty vector pCDNA4TO-NTAP to form the pvIRF2-NTAP and pvIRF4-NTAP plasmids, respectively. These plasmids were stably transfected separately into a cell line with inducible tetracycline repressor gene; HEK 293 T-Rex cells, by selection with Blasticidin (5µg/mL) and Zeocin (200µg/mL).

The T-REx™ System consists of an inducible expression plasmid for expression of the gene of interest under the control of the strong human CMV promoter and two tetracycline operator 2 (TetO2) sites. Additionally, it is made up of a regulatory plasmid, pcDNA6/TR©, which encodes the Tet repressor (TetR) under the control of the human CMV promoter and a control expression plasmid containing the lacZ gene, which when co-transfected with pcDNA6/TR©, expresses β-galactosidase upon induction with tetracycline. The expression of the gene of interest is repressed in the absence of tetracycline and induced in the presence of tetracycline (Yao et al., 1998).

The pvIRF2-NTAP and pvIRF4-NTAP-transfected cell lines were subsequently evaluated for their ability to inhibit type I IFN signalling compared with the empty vector control (pCDNA4TO-NTAP), referred to in this thesis as EV-NTAP.
The pCDNA4TO-NTAP vector encodes an N-terminal tag contiguous to the vIRF2 and vIRF4 proteins, which enables the purification of the viral proteins alongside any of their interacting partner proteins. The NTAP tag contains protein G that binds any Immunoglobulin. In this thesis, the NTAP tag was detected by immunoblotting and probing with β-actin primary antibody. However, the β-actin band is easily differentiated from the NTAP tag based on their different expected sizes.

A tetracycline titration was performed over the range of 0–2μg/mL to induce expression of vIRF2 and 4. Cells were lysed 24 hours later and subjected to western blot analysis (Figure 3-6). The NTAP, VIRF2-NTAP and the VIRF4-NTAP bands correspond in size to 30kDa, 140kDa and 130kDa, respectively. In the three cell lines, 0.125μg/mL of tetracycline was sufficient to induce maximal expression of the respective proteins. (Figure 3-6 A, B and C, lanes 2). In subsequent experiments, 0.125μg/mL of tetracycline was used.

3.4.1.2. Optimising the tetracycline treatment time to induce vIRF2 & vIRF4 protein expression

The time course of tetracycline treatment of the vIRF2-NTAP, vIRF4-NTAP and NTAP proteins from their corresponding cell lines was investigated over a period of 72 hours to determine the peak expression of the induced proteins. The three cell lines were stimulated with 0.125μg/mL tetracycline and samples were obtained for western blot analysis at specific time points (0, 8, 24, 48 and 72 hours).

EV-NTAP expression was observed at the 0 time-point. For VIRF2-NTAP and VIRF4-NTAP cell lines, 8 hours of tetracycline treatment was sufficient to induce detectable expression of the VIRF2-NTAP and VIRF4-NTAP proteins, respectively (Figure 3-7B and C lane 2). Expression of these proteins peaked at 24 hours (lane 3) and was maintained until at least 72 hours post treatment. Based on these findings, further experiments were performed 24 hours post tetracycline treatment. In all cell lines,
weak expression of the NTAP protein was observed at time point 0, suggesting leaky expression in those cell lines.

Figure 3-6: Optimisation of concentration of tetracycline required to induce expression of the EV-NTAP, VIRF2-NTAP, and VIRF4-NTAP cell line.

EV-NTAP (A), vIRF2-NTAP (B) and vIRF4-NTAP (C) cells were treated with tetracycline at different concentrations ranging from 0.125-2 µg/mL tetracycline. Cells were harvested 24 hours later and lysates (20µg per sample) were analysed by western blot to detect the NTAP-tag and β-actin. Primary antibody: anti-β-actin. Secondary antibody: anti-mouse HRP-conjugated secondary antibody (for β-actin detection). The use of the anti-mouse HRP conjugated secondary antibody was sufficient to detect the NTAP-tag in all the cell lines as this tag contains protein G.
Figure 3-7: Optimisation of length of time required for tetracycline treatment to induce expression of the EV-NTAP, vIRF2-NTAP, and vIRF4-NTAP cell line.

EV-NTAP (A), vIRF2-NTAP (B) and vIRF4-NTAP (C) cells were treated with 0.125 µg/mL tetracycline. Cells were harvested at 0, 8, 24, 48 and 72 hour time points. The lysates (20µg per sample) were analysed by western blot to detect the NTAP-tag and β-actin. Primary antibody: anti-β-actin. Secondary antibody: anti-mouse HRP-conjugated secondary antibody (for β-actin detection). The use of the anti-mouse HRP conjugated secondary antibody was sufficient to detect the NTAP-tag in all the cell lines as this tag contains protein G.
3.5. Confirming the presence of vIRF2 and vIRF4 gene expression in the cell lines

PCR analysis was used to verify the presence of vIRF2 and vIRF4 mRNA in the stable cell lines following their induction with tetracycline.

3.5.1. Confirming the presence of vIRF2 and vIRF4 mRNA in the vIRF2-NTAP and vIRF4-NTAP expressing stable cell lines respectively

The EV-NTAP, vIRF2-NTAP and vIRF4-NTAP cell lines were treated with tetracycline for 24 hours, (as described in Figures 3-6 and 3-7, respectively) and harvested. PCR was performed on the cDNA samples using primers specific for either vIRF2 or vIRF4 (Appendix G). The vIRF2-NTAP cell line contained mRNA that was amplified with the vIRF2 specific primers (Figure 3-8 A, lane 3). No positive signals were observed for either the EV-NTAP or vIRF4-NTAP cell lines (lanes 1 and 5), indicating that these cell lines do not express the vIRF2 protein. There were no positive signals in the sample without reverse transcriptase (lanes 2, 4 and 6) confirming the signal was from reverse transcribed RNA and not contaminating genomic DNA. Lane 7 is a no-template control to rule out any contamination. Similarly, the vIRF4-NTAP cell line contained mRNA that was amplified with the vIRF4 specific primers (Figure 3-8 B, lane 5). Again, no positive signals were observed for either the EV-NTAP or vIRF2-NTAP cell lines (lanes 1 and 3) and there were no signals in the samples (lanes 2, 4, 6) lacking reverse transcriptase, again as expected. Lane 7 had no template and was set up to rule out contamination. These results further confirm the western blot results in Figures 3-6 and 3-7 that showed that the vIRF2-NTAP and the vIRF4-NTAP cell lines express the vIRF2 and vIRF4 proteins respectively.
Figure 3-8: The vIRF2-NTAP and vIRF4-NTAP cell lines contain vIRF2 or vIRF4 mRNA respectively.
EV-NTAP, vIRF2-NTAP and vIRF4-NTAP cells were treated with tetracycline (0.125µg/mL) for 24 hours and harvested. RNA was extracted and the samples were DNAse treated. Reverse transcription was performed followed by PCR using vIRF2 primers and vIRF4 primers. The vIRF2 and vIRF4 were amplified in lanes 3 and 5 respectively.

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Figure 3-8: The vIRF2-NTAP and vIRF4-NTAP cell lines contain vIRF2 or vIRF4 mRNA respectively.
EV-NTAP, vIRF2-NTAP and vIRF4-NTAP cells were treated with tetracycline (0.125µg/mL) for 24 hours and harvested. RNA was extracted and the samples were DNAse treated. Reverse transcription was performed followed by PCR using vIRF2 primers and vIRF4 primers. The vIRF2 and vIRF4 were amplified in lanes 3 and 5 respectively.
3.5.2. Optimisation of qPCR assay to detect vIRF2 and vIRF4 mRNA

In order to verify the conventional PCR data, qPCR was performed with a different specific primer and probes to target vIRF2 and vIRF4 mRNA in stable cell lines. The probes for each spanned an exon boundary in order to distinguish genomic DNA. cDNA dilutions were made in DNAse/RNAse-free water and ranged from $10^2$ – $10^6$ µm. The assays for both vIRF2 and vIRF4 worked best with primer concentrations of 3µM and probe concentrations of 5µM. Primer and probe efficiency values were measured using the Ct slope method. The ‘slope’ is a regression coefficient calculated from the regression line in the standard curve. The Ct values are plotted against the log cDNA concentration. With this method, the expected slope for a 10-fold dilution of cDNA template is approximately -3.32 with an amplification efficiency of 100%. The amplification efficiency was derived in singleplex reactions with vIRF2 and vIRF4 primers and in duplex reaction with GAPDH primers (Figure 3-9A) vIRF2 amplification efficiency in singleplex reaction was 105.8% and 91.3% in duplex reaction. For vIRF4 (Figure 3-9B), the amplification efficiency was 101.3% in the singleplex reaction and 96.1% in duplex reaction with GAPDH. Acceptable amplification efficiency reference values range from 90% to 110% (Rogers - Broadway & Karteris, 2015). No amplification was observed when water was substituted for cDNA.
Figure 3-9: vIRF2 and 4 and probe amplification efficiency validation study.

The HEK 293 cells were plated at a density of 5 x 10^5 cells in a 6-well plate and were treated with tetracycline 0.125μg/mL for 24 hours. RNA was extracted and cDNA synthesized from 500ng RNA with random primers and serially diluted 10 fold for five data points (1:10^-1-1:10^-5). The primers (see Table 2.24) were used at 3μM and the FAM-labelled probe (see Table 2.23) was used at 5μM. Primers and probes efficiency values (%) were measured using the Ct slope method. The amplification efficiency was calculated using the slope of the regression line in the standard curve (see 2.4.4). (A) vIRF2 amplification in singleplex and duplex with GAPDH. (B) vIRF4 amplification in singleplex and duplex with GAPDH. The dotted lines represent the regression line while the solid line depicts the plotted Ct values for vIRF2 and vIRF4 in A and B respectively.
3.6. Investigating the function of vIRF2 and vIRF4 expressed in the vIRF2-NTAP and vIRF4-NTAP cell lines.

3.6.1. The vIRF2 and vIRF4 expressing cell lines inhibit ISRE-containing promoter activation

The effect of vIRF2 and vIRF4 on the JAK STAT signalling pathways was measured using the pISRE-luc reporter plasmid containing an ISRE promoter. Promoter activity was quantified by the DLA. Plasmid concentration, IFN dose and length of treatment had been optimised in earlier experiments (section 3-1 and 3-2). Figure 3-10 showed that both vIRF2 and vIRF4 significantly downregulate the ISRE promoter.

![Figure 3-10: Effect of vIRF2 and vIRF4 proteins on ISRE promoter activity](image)

The empty vector (EV-NTAP), vIRF2-NTAP and vIRF4-NTAP cell lines were treated with 0.125μg/mL tetracycline for 24 hours to induce the viral genes, after which they were co-transfected with pISRE-luc (250ng) and the pRLSV40 plasmid (1ng). 5 hours post transfection, the cells were treated with rIFNαB2 (300IU/mL) and harvested after 12 hours. The pRLSV40 plasmid was added as an internal control to which firefly luciferase levels were normalised. The results are shown as fold change in the presence of IFN related to its absence. The data represent the mean ± the SD of the three independent experiments which were each assayed in duplicate. ** = p<0.001 ANOVA followed by a Dunnett post hoc test compared to EV-NTAP plus rIFNαB2.
3.6.2. The vIRF2 and vIRF4 expressing cell lines down regulate interferon-stimulated gene expression

Given that the previous data (Figure 3-10) demonstrated an inhibitory effect of vIRF2 and vIRF4 on a model gene system, that of an ISRE driven reporter gene, the effect of these viral proteins on the endogenous cellular genes ISG15, OAS1 and IFIT1 was measured by qPCR. RNA was extracted from tetracycline induced vIRF2, vIRF4 cells selected with Blasticidin (5µg/mL) and Zeocin (200µg/mL) and EV-NTAP cell lines following 8 hours treatment with 300IU/mL of rIFNαB2 according to the method described in section 2.8.1. Both vIRF2 and vIRF4 significantly down-regulated the expression of ISG15, OAS1 and IFIT1 gene in comparison to the EV-NTAP (Figure 3-11).
Figure 3-11: Effects of vIRF2 and vIRF4 proteins on interferon stimulated genes

1µg of cDNA was reverse transcribed from RNA extracted from tetracycline induced EV-NTAP, vIRF2 and 4 stable cell lines following treatment with 300IU/mL of rIFNaB2 for 24 hours. The cDNA was utilised for qPCR against ISG15, OAS1 and IFIT1 primers. The data represent the mean ± the SD of the three independent experiments which were each assayed in duplicate. The results are shown as normalised expression ratio (NER) of the ISGs in the stable cell lines normalised to Tubulin mRNA levels and further normalised to the presence of IFN. The NER of vIRF2 and vIRF4 cell lines were compared to EV-NTAP (column 1) *=p<0.05, ** = p<0.001 and ****=p<0.00001 ANOVA followed by a Dunnett post hoc test.
3.7. Discussion

The type I IFN response is the first line of defence against invading viruses and drives the development of specific antiviral immunity. Accordingly, many viruses, including KSHV, have evolved diverse strategies for circumventing the interferon response (Devasthanam, 2014). Studies in the present chapter investigated the role and mechanism of modulating the IFN response by two KSHV genes, vIRF2 and vIRF4, encoding proteins with homology to cellular IRFs.

To develop a DLA for measuring interferon-inducible gene expression, the dose of reporter plasmid, as well as the duration and dose of interferon treatment were optimised. Figure 3-1 showed that transfection of HEK 293 cell lines with 500ng of the reporter plasmid (pISRE-luc) and 1ng of the constitutively expressing Renilla luciferase plasmid, pRLSV40 produced the maximum activation of the IFN sensitive promoter. The pISRE-luc plasmid at dose of 250ng produced an efficient activation of the ISRE promoter, hence to achieve efficient transfection, this concentration was used in subsequent experiments. Figures 3-2A and B showed that 12 hours of treatment with 300IU/mL of rIFNαB2 produced the maximum interferon sensitive promoter response. This information formed the biologic readout for future experiments as subsequent DLA were performed using 250ng of the reporter plasmid and interferon treatments were carried out for 12 hours at a dose of 300IU/mL for DLA.

Stable cell lines expressing inducible vIRF2 and vIRF4 genes were derived previously (Hindle & Blackbourn, unpublished results) by cloning the vIRF2 and 4 genes into pCDNATO-NTAP to generate the pVIRF2-NTAP and pVIRF4-NTAP plasmids respectively. The HEK 293 stable cell lines were selected in Blasticidin (5µg/mL) and Zeocin (200µg/mL) and require tetracycline treatment to induce the expression of the viral proteins. The pCDNATO-NTAP (referred to as the EV-NTAP) served as a negative control for the vIRF2 and vIRF4 expressing cells. Optimisation experiments
were performed to determine the dose and length of time of tetracycline treatment required to maximise vIRF2 and vIRF4 expression of NTAP. Figures 3-6 and 3-7 showed that optimum expression of NTAP, vIRF2-NTAP and VIRF4-NTAP was achieved following treatment with 0.125μg/mL of tetracycline for 24-72 hours. Thus, future experiments utilising the stable cell lines were performed following 24 hours treatment with this concentration.

Furthermore, PCR was performed on cDNA from the stable cell lines to confirm the expression of the vIRF2 and vIRF4 genes. The absence of a band in the EV-NTAP and the vIRF4 lanes for vIRF2 primers confirms the identity of these cell lines and the specificity of the primers. (Figure 3.8A). A similar result was obtained for the vIRF4 primers (Figure 3-8B). The absence of bands in the no reverse transcriptase and the no template control also ruled out both genomic DNA contamination and reaction mix contamination respectively.

qPCR was done to quantify the vIRF2 and vIRF4 mRNA in the stable cell lines. vIRF2 and vIRF4 primers/probes binding efficiencies were measured by qPCR and TaqMan probe detection. Primers and probe efficiency values were measured using the Ct slope method and the result expressed as % efficiency. There was a linear correlation over all the data points plotted for each graph (Figure 3-9). Thus, vIRF2 amplification efficiency in singleplex reaction was 105.8% (Figure 3-9A) while in duplex reaction with GAPDH it was 91.3% (Figure 3-10A). For vIRF4, the amplification efficiency in singleplex reaction was 101.3% and 96.1% with GAPDH (Figure 3-9B). These data confirm the presence of vIRF2 mRNA in the vIRF2-NTAP cell line, and vIRF4 mRNA in the vIRF4-NTAP cell lines. They also verified that these primers and probes are suitable for measuring vIRF2 and 4 levels.

Having confirmed that the stable cell lines express the vIRF2 or vIRF4 proteins, the cells were used in further experiments to investigate the molecular mechanisms behind the action of vIRF2 and vIRF4 in regulating the type I interferon response.
It has been previously demonstrated that KSHV vIRF2 can inhibit the type I interferon response (Aresté & Blackbourn, 2009; Fuld et al., 2006; Mutocheluh et al., 2011). The present study confirmed the ability of vIRF2 to inhibit activation of the ISRE-containing promoter. Importantly, the data also demonstrate for the first time that vIRF4 also shares this capability. Figure 3-10 demonstrates that vIRF4 inhibits the ISRE activity by 74.5% compared with the 72.1% inhibition by vIRF2. The vIRF2 inhibition observed is in keeping with previous report from our group (Mutocheluh et al., 2011). This finding confirms that all the KSHV vIRFs have inhibitory effect on interferon signalling as previous studies have shown that viral interferon regulatory factors 1, 2 and 3 have anti-IFN activity (Burysek et al., 1999a; Lubyova & Pitha, 2000; Mutocheluh et al., 2011; Wies et al., 2009).

Our group had earlier observed that vIRF2 (Aresté & Blackbourn, 2009; Fuld et al., 2006) but not vIRF4 also downregulates IFNβ production (Fuld et al., 2006). vIRF2 stimulates the degradation of IRF3 via a mechanism involving caspase-3, and also inhibits IRF3-mediated transactivation of a full-length IFNβ reporter promoter (Aresté & Blackbourn, 2009; Fuld et al., 2006). Both vIRF1 and vIRF3 have been shown to inhibit IRF3-mediated transcriptional activation through the sequestration of p300/CBP hence they are negative regulators of the type I IFN production pathway (Burysek et al., 1999a; Lin et al., 2001). There is presently no published report on vIRF4-mediated inhibition of IFN production, however our group had shown that vIRF4 had no inhibitory effect on the IFNβ promoter activation (Hindle & Blackbourn, unpublished observation) thus making vIRF4 the only vIRF that does not inhibit the IFN production pathway.

The binding of IFNα/β to its receptor IFNAR triggers the JAK STAT signalling pathway with resultant transcription of a diverse set of genes referred to as IFN-inducible genes or ISGs (Randall & Goodbourn, 2008; Wong & Chen, 2016). This thesis has further teased out the role of vIRF2 in the negative regulation of the transcription of
The expression of ISGs mediate antiviral effector functions at different phases of the virus replication cycle. Consequently, viruses have evolved diverse strategies to subvert the IFN signalling pathway by blocking the antiviral functions of IFN (Horner, 2014). KSHV vIRF2 significantly downregulated the expression of ISGs, specifically ISG15, OAS1 and IFIT1 by 64.0%, 54.5% and 66.2% respectively (Figures 3-11).

The involvement of vIRF4 in this later stage of the type I IFN response has not been reported in the literature previously, hence this is a novel result. As we have shown in this chapter that vIRF4 downregulates the activation of ISRE promoter (Figure 3-11), the effect of vIRF4 on the expression of ISGs was also investigated. The vIRF4 protein significantly downregulated the expression of ISGs, specifically ISG15, OAS1 and IFIT1 by 74.9%, 33.2% and 55.5% respectively (Figures 3-11).

Several ISGs have been shown to have variable inhibitory activity against many viruses (Schoggins & Rice, 2011). A comprehensive screen of 380 genes tested for antiviral activity against HCV, HIV-1, yellow fever virus (YFV), WNV, Venezuelan equine encephalitis virus (VEEV), and chikungunya virus (CHIKV) revealed significant suppression of virus replication by approximately 25 of these genes (Schoggins et al., 2011). Hence it is understandable that KSHV vIRF2 and vIRF4 proteins have evolved to downregulate the expression of ISGs, possibly to enhance their survival and replication.

ISG15 is a ubiquitin-like modifier induced by type I interferon and binds to a large number of target proteins in a series of enzymatic reactions referred to as “ISGylation” (Morales & Lenschow, 2013). The antiviral activity of ISG15 was first observed in response to Sindbis viruses. Sindbis virus infected mice expressing ISG15 were found to survive more (Lenschow et al., 2005) compared with the ISG15 deficient control virus. Studies in human and mouse systems have also identified some viruses that are inhibited by ISG15: Influenza virus, vaccinia virus, vesicular stomatitis virus.
(VSV), Ebola virus-like particles (VLPs), dengue virus, and West Nile virus (WNV), HSV (strain 17) and γHV-68 (Lai et al., 2009; Lenschow et al., 2007; Osiak et al., 2005; Pincetic & Leis, 2009). One of the mechanisms behind the antiviral effect of ISG15 is by directly conjugation to viral protein and this has been observed with the NS1 protein of influenza B (Hsiang et al., 2009). As a counter mechanism, a number of viruses have evolved deubiquitinating enzymes capable of deconjugating ISG15 from ISGylated target proteins. The L proteins of Crimean-Congo hemorrhagic fever virus (CCHFV) and equine arteritis virus have been studied in this regard (Reyes-Turcu et al., 2009).

A recent study has shown that KSHV vIRF1 interacts with HERC5, the E3 ubiquitin ligase for ISG15 thereby altering ISG15 modification of cellular functions (Jacobs et al., 2015). Furthermore, KSHV-infected cells showed increased ISG15 conjugation and the knockdown of ISG15 in latent KSHV infected cells resulted in an increased level of KSHV replication as well as increased infectious titre (Jacobs et al., 2015). It is therefore interesting to find in this thesis that vIRF2 and vIRF4 significantly downregulate the expression of ISG15 (Figure 3-11).

The IFIT1 gene is made up of two exons and their promoter has two ISREs as the only identifiable cis-acting elements located upstream of the TATA box promoter (Xiao et al., 2006). The IFIT1 protein forms a multimeric complex with other members of the IFIT family. It binds and regulates the functions of viral proteins and RNA, thus inhibiting virus replication (Fensterl & Sen, 2015). IFIT1 inhibits IRES-driven translation in hepatitis C virus (Fraser & Doudna, 2007). IFIT1 binds and sequesters 5′-ppp-RNAs, consequently, they also inhibit the replication of viruses which produce such RNAs (Kumar et al., 2014). A number of viruses escape IFIT1 antiviral effect by active 2′-O-methylation of mRNAs. Studies in IFIT1−/− mice showed that mutant MHV68 viruses were virulent unlike in wild type mice because IFIT1 specifically binds and blocks the translation of mRNAs that are deficient in 2′-O-methylation of their
The non-structural protein, nsp16 of MHV68, is dedicated to 2′-O-methylating viral mRNAs. MHV68 mutants with D130A mutation in nsp16 and consequent compromise in its enzyme activity are unable to replicate in the spleen of wild type mice (Habjan et al., 2013).

Members of the OAS family exert their antiviral activity through the synthesis of 2-5A and subsequent activation of RNAse L (Ibsen et al., 2014). The OAS protein possess strong antiviral activity and inhibits viral replication (Kristiansen et al., 2010). The OAS proteins act through a common pathway; the RNAseL pathway, hence their antiviral function was investigated in RNAseL-deficient mice, which were found to show increased susceptibility to infection with RNA viruses from the Picornaviridae, Reoviridae, Togaviridae, Paramyxoviridae, Orthomyxoviridae, Flaviviridae and Retroviridae families (Zhou et al., 1997). The 2′-5′ OAS-directed RNAse L effector pathway blocks viral transcription, degrade viral RNA, inhibit translation, and modify protein function to control all steps of viral replication (Sadler & Williams, 2008).

The downregulation of the expression of the ISGs confirms the inhibitory effect of the vIRF2 and vIRF4 on ISRE promoter. In response to IFNα stimulation, both STAT1 and STAT2 are phosphorylated through the JAK STAT signalling pathway and they recruit the DNA-binding IRF9 subunit to form a multimeric transcription factor complex termed ISGF3 (Chang et al., 2004). ISGF3 binds the ISRE promoter with consequent transactivation of the interferon responsive genes. Previous experiments performed in our laboratory revealed that vIRF2 demonstrates pleiotropic activity of KSHV in inhibiting the type I IFN response (Mutocheluh et al., 2011). The mechanism behind these observations has not been fully identified. However vIRF2 was shown to inhibit IFNα-induced ISRE transactivation by attenuating the IRF9 and STAT1 component of the ISGF3 thus preventing the accumulation of functional ISGF3 (Mutocheluh et al., 2011).
In summary, results from chapter 3 were carried out to develop and optimise a biologic readout for assessing IFN stimulation. Both KSHV vIRF2 and vIRF4 inhibit the cellular response to type I IFNs, by inhibiting the transactivation of the ISRE promoter and also by inhibiting the transcription of the IFNα-induced ISGs at the mRNA levels. As we have identified a novel role for vIRF4 in type I IFN signalling, in the next chapter, we shall investigate the role of vIRF4 on the translation of host mRNAs.
CHAPTER 4

INVESTIGATING THE ROLE OF KSHV vIRF4

EXPRESSION ON TRANSLATION
4. CHAPTER 4

4.1. Introduction to chapter 4

Having identified both KSHV vIRF2 and vIRF4 as negative regulators of the type I interferon signalling pathway, the next step was to understand the mechanism behind the negative regulatory effect of these viral proteins on the JAK STAT signalling pathway. A previous SILAC-MS proteomics analysis carried out by our group had shown that 23/56 (41%) of the proteins identified as binding to vIRF2, and 12/23 (52%) of the proteins identified as binding to vIRF4 were ribosomal proteins (Hindle & Blackbourn, unpublished observations). Seven of such partner proteins were found in common between vIRF2 and vIRF4 (Table 1-4). The mechanisms behind these interactions are not fully understood but the large number of interacting cellular proteins suggest that these viral proteins may exert translational control over the host cell amongst other effects. Additionally, these interactions suggest that both vIRF2 and vIRF4 were probably interacting with a specific ribosomal protein(s), which then recruited the rest of the ribosomal complex. We therefore hypothesise that through vIRF2 and vIRF4, KSHV could modulate the antiviral response by manipulating the profile of mRNA species that are translated.

More recent reports have indicated that viruses have evolved strategies to customise the host translation machinery, ranging from evolution of specialised cis-acting elements that recruit ribosomes to modification of genome-coding capacity (Walsh et al., 2013a). As obligate dependants on the host translation system, viruses have presumably evolved diverse measures for customising the cellular translation apparatus in order to enhance their survival and replication (Walsh et al., 2013a).

To investigate the role of KSHV vIRF4 in the regulation of cellular translation, polysome profiling experiments were performed on stable cell lines expressing vIRF4. Polysome profiling has been discussed in detail in section 2.8. The technique allows
for assessment of translational efficiency by separating actively translating ribosomes and bound mRNAs on sucrose gradient. The efficiently translated mRNAs associated with heavy polysomes are separated from poorly translated ones associated with light monosomes (Gandin et al., 2014).

Studies in this chapter focus on vIRF4 because our group had earlier studied the impact of vIRF2 on the cell transcriptome in order to decipher the mechanism by which vIRF2 inhibits the type I IFN pathway (Mutocheluh & Blackbourn, unpublished observation).

The objectives of this chapter are to:

1) Investigate the effect of vIRF4 expression on translation efficiency using polysome profiling.

2) Investigate the modulation of the cell transcriptome profile in response to vIRF4 expression by DNA microarray analyses.

3) Validate the microarray data by qPCR and immunoblotting.
4.2. Investigating the effect of vIRF4 expression on translation

4.2.1. Polysome profiling

The polysome profiling experiments were designed to investigate the impact of vIRF4 expression on cellular translation efficiency. Having established that vIRF4 expression could suppress rIFNαB2 induction of pISRE-luc activity with consequent downregulation of expression of ISGs (chapter 3), the next step was to assess differential translation events in the presence of vIRF4 expression.

The EV-NTAP and vIRF4-NTAP stable cell lines described in chapter 3, were treated with tetracycline and 300IU/mL of rIFNαB2 for 8 hours. Cytoplasmic extracts from triplicate cultures were then harvested in polysome lysis buffer. Lysates were subjected to western blot analysis to verify vIRF4 expression prior to polysome profiling.

Polysome profiling allows separation of monosomes (poorly translated) from polysomes (efficiently translated) mRNAs. Monosomes consist of single ribosomal subunits that are not translation-competent and are found in the less dense sucrose gradient concentration while polysomes are multiple 80S ribosomes translating one mRNA and are found in the higher sucrose gradient. The method allows for the subsequent collection of fractions of sucrose gradients, thus allowing for isolation of mRNAs according to the number of ribosomes they are bound to. The monosomes are found in fractions 2/3 for the 40S subunit, fractions 4/5 for the 60S subunit, while the latter fractions contain the 80S subunit (Mašek et al., 2011). Polysome fractions are made up of actively translating mRNA and are found in fractions 6-10.

Monosome and polysome fractions can be pooled separately and mRNA levels are determined using qPCR, microarray or deep-sequencing. In the present study, monosome and polysome fractions pooled from the vIRF4 stable cell lines were used as a prototype and compared with the EV-NTAP. In Figure 4-1, vIRF4 expression
comparatively led to a reduction in the monosome and polysome peaks. The reduction in polysome peak suggests global suppression of active translation. A similar profile pattern was observed from EV-NTAP and vIRF4-NTAP cell lines that were unstimulated with IFN (data not shown). This confirms that the reduction in polysome peaks was a consequence of vIRF4 expression.

The area under the curve was analysed for the monosome fractions (1-5) and polysome fractions (6-10) using Graphpad software version 6.03. This analysis allowed for accurate determination of the Polysome/Monosome (P/M) ratio, which is shown in Figure 4-1B. The P/M ratio for the EV-NTAP was expressed as 100% and compared with the P/M ratio for vIRF4-NTAP. vIRF4 expression significantly reduced the P/M ratio by 22.3%, thus confirming that vIRF4 negatively regulates translation. The polysome profiling experiments were also repeated thrice in EV-NTAP and vIRF4-NTAP cell lines without IFN treatment and similar data obtained confirmed that reduction of the P/M ratio was a specific effect of vIRF4 expression (data not shown).
Figure 4-1A: Polysome profile pattern of the stable cell lines (EV-NTAP and vIRF4-NTAP cell lines).

Polysomal fractionation (as described in section 2.11) of EV-NTAP and vIRF4-NTAP cell lines treated with 0.125μg/mL tetracycline for 24 hours and then with 300IU/mL rIFNαB2 for a further 8 hours. (A) The absorbance at 254 nm was monitored during collection of the 10 fractions from the sucrose gradient of cytoplasmic lysates. The absorbance (Y-axis) is plotted against the number of fractions (X-axis). The 80S polysome peaks are reduced in the presence of vIRF4 compared to the EV. (B) The area under the curve (AUC) for the monosomes and polysomes was derived for both EV-NTAP and vIRF4-NTAP. The P/M ratio was determined for both cell lines and found to be significantly reduced in the presence of vIRF4 expression. This figure is representative of 3 replicates.
Figure 4-2B: Polysome profile pattern of the stable cell lines (EV-NTAP and vIRF4-NTAP cell lines).

Polysomal fractionation (as described in section 2.11) of EV-NTAP and vIRF4-NTAP cell lines treated with 0.125μg/mL tetracycline for 24 hours. (A) The absorbance at 254 nm was monitored during collection of the 10 fractions from the sucrose gradient of cytoplasmic lysates. The absorbance (Y-axis) is plotted against the number of fractions (X-axis). The 80S polysome peaks are reduced in the presence of vIRF4 compared to the EV. (B) The area under the curve (AUC) for the monosomes and polysomes was derived for both EV-NTAP and vIRF4-NTAP. The P/M ratio was determined for both cell lines and found to be significantly reduced in the presence of vIRF4 expression. This figure is representative of 3 replicates.
4.3. Investigating the modulation of the cell transcriptome profile in response to vIRF4 expression

4.3.1. Microarray studies

Microarrays are useful for assays comparing expression of a wide range of genes of two different cell types or tissue samples (Trevino et al., 2007). They are useful for studying expression profiles of mRNA in different conditions and provide insight into the regulation and integration of gene expression, as in the present study. Monosomal and polysomal fractions were pooled separately and RNA was prepared from one of each sample set for microarray transcriptome profiling as shown in the workflow below (Figure 4-2) (Arava et al., 2003; Ingolia, 2014; Schosserer et al., 2015). The objective is to analyse the data in terms of differential expression (DE) between the EV-NTAP control and vIRF4-NTAP samples.

RNA extraction from the pooled monosome and polysome fractions was carried out as previously described in section 2.5.1. Template RNA was reverse transcribed to cDNA using T7 Primer Mix as described in the manufacturer kit for the Agilent Two-Colour Microarray Gene Expression Analysis Quick Amp kit. cRNA was synthesised from cDNA then labelled and purified using Qiagen RNeasy kit. The cRNA was quantified using NanoDrop (ND-1000 UV-VIS Spectrophotometer version 3.2.1) and further confirmed by measuring concentration on the Qubit 2.0 Fluorometer (Life Technologies) (Table 4-1). Labelled cRNA was hybridised on whole human genome Agilent array2 4x44k using triplicate biological replicates with a dye swap each, totalling 12 microarrays (Table 4-1). Figure 4-3 shows the workflow for sample preparation and array process.

Template RNA integrity and labelled cRNA quality were determined using the NanoDrop UV-VIS Spectrophotometer and the Agilent 2100 bioanalyzer. The RNA Integrity Number (RIN) generated by the Agilent 2100 Expert software gives an index
of the total RNA quality while the A260/A280 and A260/A230 ratios determine the purity of the RNA sample. The RIN software algorithm classifies total RNA in a range from 1 to 10, with RIN 1 being the most degraded while RIN 10 is considered the most intact. The A260/A280 for high quality samples range from 1.8 to 2.0 while an A260/A230 ratio of >2.0 indicates the absence of organic compounds and cellular contaminants.

In this study all RNA samples with RIN of 8.6 or less were discarded and the RNA extraction process repeated. The acceptable cut-off for samples used in the microarray was set to 1.8 for A260/A280 ratio and 2.0 for A260/230. Figure 4-4 shows a representative electropherogram generated by the Agilent bioanalyzer. The RNA includes two distinct 18S and 28S ribosomal RNA peaks as well as that for 5S RNA.
Figure 4-3: Schematic representation of workflow from Polysome profiling to Microarray studies
Left; sucrose gradient, middle; pooled monosome and polysome fractions subjected to RNA extraction and right; microarray analysis of monosomal and polysomal RNA. LMW, low molecular weight; HMW, high molecular weight.
Figure 4-4: Workflow for sample preparation and array process
The steps followed from processing of template RNA to cRNA synthesis and purification and hybridisation are summarised above.
Table 4-1: Agilent 4 X 44K names and profile of the arrays

<table>
<thead>
<tr>
<th>Slide No.</th>
<th>Sample names</th>
<th>Treatment profile</th>
<th>cRNA concentration (ng/µL)</th>
</tr>
</thead>
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<td>A1</td>
<td>Cy3V4M</td>
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<td>344.7</td>
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<tr>
<td></td>
<td>Cy5EVM</td>
<td>Cy5-labelled Empty vector monosome</td>
<td>332.1</td>
</tr>
<tr>
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<td>306.3</td>
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<td>Cy3EVM</td>
<td>Cy3-labelled Empty vector monosome</td>
<td>308.9</td>
</tr>
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<td>Cy3-labelled Empty vector polysome</td>
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<td>Cy5-labelled vIRF4 polysome</td>
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<td>Cy3EVP</td>
<td>Cy3-labelled Empty vector polysome</td>
<td>183.6</td>
</tr>
</tbody>
</table>

Names were assigned to the Agilent 4 X 44K slides as shown in column 1 of the above table. The sample identity is presented in columns 2 and 3. The cRNA concentration is listed in column 4.
Figure 4-5: Electropherogram of total RNA analysis using the Bioanalyzer
The fluorescent unit (FU) is plotted against time (s) on the X-axis. Distinct bands are seen for the 5S, 28S and 18S and majority of signal fall into the time range from 20 to 50 seconds. The RIN in this instance was 9.0. This figure is representative of the 12 samples and suggests a high quality RNA.
4.4. Microarray analyses

The detailed steps for the microarray analyses can be found in section 2.10. Briefly, monosome and polysome lysates from EV-NTAP+IFN and vIRF4-NTPA+IFN cells (as described in 4.2.4.) were subjected to microarray analysis. Fluorescently labelled DNA probes were generated from equal proportions of RNAs (10μg) of pooled monosomal and polysomal fractions (fractions 1–4, Cy3) and pooled polysomal fractions (fractions 6–11, Cy5). cDNA synthesis and cRNA amplification were performed with an Agilent kit (low-RNA-input linear amplification kit, two colours) and hybridized on an Agilent 4×44K microarray slide. Assays were carried out in triplicate and in each case, polysomal RNAs were compared with monosomal RNAs with dye swaps to control for labelling bias. A schematic representation of steps from cRNA labelling down to microarray studies is summarised in Figure 4-5. Microarray slides were scanned using a GenePix 4200A microarray scanner and GenePix Pro 5.1 software (Axon Instruments, Union City, CA, USA).
Figure 4-6: Schematic representation of generation of cRNA for a two-colour microarray experiment.

Polysomal and monosomal RNA from EV-NTAP and vIRF4-NTAP cell lines were reverse transcribed to cDNA. The above also shows the flow through from cRNA synthesis and labelling to subsequent microarray analyses after a dye swap was performed.
4.5. VIRF4-regulated gene sets and signalling pathways. The data processing was performed by Dr Carla Moller-Levet (School of Biosciences and Medicine, University of Surrey).

4.5.1. Microarray pre-processings

Feature extraction files were read into R, a programming language and software environment for statistical computing and graphics (the R project website, http://www.r-project.org/), and the gene expression matrix (GEM) was generated (R Development Core Team, 2015). The GEM is an orderly profiling of genes in a tabular format where the genes are shown in rows while the columns show the different microarray samples. Individual values of the GEM were defined as log2 (signal) – log2 (control).

Quantile normalisation was performed using the “normaliseBetweenArrays” function in the R Bioconductor “limma” package (Ritchie et al., 2015). Background was corrected by removing the non-expressed genes from the analysis. The Control probes were filtered out while non-control replicated probes were averaged. Probes flagged by Agilent QC in more than 8 samples (out of 12) were filtered out leaving 20,408 probes out of 34,127.

4.5.2. Microarray analysis

Rank product (RP) analysis (Breitling et al., 2004) was used to identify probes that are found consistently at the top ranks of log2 ratios i.e. sample>control (S>C) across all samples and probes that are found at the bottom ranks of log2 ratios i.e. sample<control (S<C). Rank Product analyses were performed on 1) all microarray samples (monosome + polysome samples); 2) all monosome samples, 3) all polysome); 4) all samples comparing monosomes vs polysomes using “RP” function
from “RankProd” R package. Cut-off threshold for percentage of false positives (PFP) - output from Rank Products analysis - was set to 5%.

A total of 1,558 probes showed a percentage false positive (PFP) lower than 5% in the RP analysis of test sample vs control (in pooled monosome + polysome samples) and were thus considered reliable data points. Of these, 681 probes show a lower abundance of mRNA in control relative to sample (S<C) and a total of 877 probes show a higher abundance of mRNA in test samples relative to control (S>C).

The intersection between the significant probes (PFP <5%) in the three different analyses (based on pooled monosome + polysome samples, only monosome samples and only polysome samples) is shown in Venn Diagrams below (Figure 4-6).
Figure 4-7: Venn diagrams showing the intersection between significant probes
The intersection between the significant probes (PFP <5%) in the three different sets of samples (pooled monosome + polysome samples, only monosome samples and only polysome samples) is shown in the above Venn diagram. There were no shared probes between the monosome and polysome in both S<C and S>C showing that the analysis is accurate.
4.5.3. Modulation of the Cell transcriptome in response to vIRF4 expression

MetaCore from Thomson Reuters [MetaCore™ version 6.21 build 66768], an integrated software suite for functional analysis of experimental data, was used to perform enrichment analysis to provide a significant probes lists. Cellular and molecular processes identified by GO have been defined and annotated by Thomson Reuters scientists. Each process represents a preset network of protein interactions characteristic for the process. Process network sorting was done for the statistically significant networks and is shown below in Figure 4-7. The Enrichment analysis workflow in MetaCore was run using upregulated significant probes in pooled monosome + polysome samples (S>C, PFP <5% Metacore report) and downregulated significant probes in pooled monosome + polysome samples (S>C, PFP<5%).
Figure 4-8: Process networks sorting for the statistically significant networks regulated by vIRF4 expression.

Gene functional classification of top-enriched clusters under stringent setting using gene ontology terms (biological processes). Processes that are downregulated by vIRF4 expression are shown in Fig 4-7A while the upregulated processes are shown in 4-7B. The height of the histogram in –Log (pValue) corresponds to the relative fold enrichment value for a particular gene/protein found in these pathways.
4.5.4. Bioinformatics analysis of the translationally regulated genes by vIRF4

For each replicate we aimed at comparing the abundance of mRNA in the monosome fraction to that of the polysome fraction, which reflects translational efficacy (e.g. mRNAs that are more abundant in polysomes than monosomes are translationally upregulated), as a function of vIRF4 overexpression.

To identify the mRNAs that are translationally up- or downregulated by the expression of vIRF4, two approaches were used. Firstly, mRNAs that were upregulated in C>S in monosomes (n=80) or S>C in polysomes (n=122) were identified as being translationally upregulated while the mRNAs that were upregulated in S>C for monosomes (n=545) or C>S in polysomes (n=331) were identified as translationally downregulated mRNAs. The increase in the number of downregulated transcripts in the polysome fraction further validates the polysome profiling data (Figure 4-1A and B) which suggested that vIRF4 expression downregulates active translation.

In the second approach, monosome ratios (S/C) were compared to polysome ratios (S/C) via Rank Products. A total of 35 probes was found to be enriched in S/C in polysomes compared to monosomes (translationally upregulated) and a total of 10 probes were found to be enriched in S/C in monosomes compared to polysomes (translationally downregulated). Figure 4-8 shows a heatmap of the 45 significant translationally regulated probes as a result of vIRF4 expression. The 35 significantly upregulated probes identified with this approach were also among those found to be upregulated in the polysomes (n=122) in the first approach and similar observation was made for the 10 downregulated probes which correlates with those found to be downregulated in the polysomes using the first approach (n=331). However, the second approach appears to be more stringent and was thus considered for validation of the microarray data.
Figure 4-9: A heatmap of significant translationally-regulated genes as a result of vIRF4 expression.

A colour scale was used to represent the ratio of mRNA in monosome to polysome fractions. Significantly different (PFP<5%) probes based on rank product analysis of monosome ratios vs polysome ratios are shown here. In both cases, ratios are transformed from the log2 [polysome: monosome], in relation to vIRF4 expression. The blue indicates downregulated genes while red signifies upregulated genes.) The 35 mRNAs identified as being translationally upregulated are shown in while in (A) while the 10 mRNA that were translationally downregulated are shown in (B). The numbers depict the different replicates. ★ represents the genes selected for microarray validation.
4.6. Validation of vIRF4 deregulated gene sets

This chapter has described how microarray studies were performed on RNA from the EV-NTAP and vIRF4-NTAP stable cell lines and how some genes were found to be deregulated as a result of vIRF4 expression.

DNA microarrays present an unprecedented opportunity to carry out whole genome profiling. However, biological and technical variations impact the quality of gene expression data obtained from microarrays, hence further validation tests are indicated (Morey et al., 2006). Typically, microarray results are validated by a number of techniques, such as RT-PCR and Northern blotting to confirm at RNA level, and western blotting to confirm its effect at protein level (Dopazo et al., 2001). qPCR is commonly used as a validation because it provides reliable quantification of mRNA across a wide range of mRNA expression levels (Wurmbach et al., 2003).

Therefore, following the identification of candidate genes using the microarray platform described above, independent confirmation experiments were done using qPCR on total and polysomal RNA. A microarray experiment evaluates the expression levels of several genes simultaneously, thus making it impossible to verify each and every gene (Conway, 2003). Consequently, important genes are verified based on the purpose and scope of the experiment. In the present study, the choice of genes to be validated was based on the degree of expression change, p-value, and/or known relevance of the selected genes to the scope of the study. To this end, three genes identified from microarray analysis (Figure 4-8), were selected; RSAD2, EPSTI1 and CELF4 for validation. The transcripts chosen for validation here are genes found to play an important role in translation as well as in antiviral immunity. Both EPSTI1 and RSAD2 are interferon responsive genes and are discussed in detail in sections 4.6.1 and 4.6.2 below. CELF4 is a RNA binding protein and was also considered for validation because vIRF4, as well as vIRF2 bind to ribosomal proteins (Table 4-1) thus it could be hypothesised that vIRF4 upregulates CELF4 mRNA in...
order to exert control of cellular translation. Further details on CELF4 can be found in section 4.6.3.

Additionally, a major rationale for selecting these three genes is because KSHV is an oncogenic virus and the three selected genes have been shown to have prognostic relevance for different cancers. KSHV vIRF4-mediated upregulation of these genes suggests the possibility that they might have diagnostic or prognostic relevance for Kaposi’s sarcoma.

RT-qPCR was carried out on 1µg of total RNA extracted from total cytoplasmic lysates and polysomal lysates from EV-NTAP+IFN and vIRF4-NTAP+IFN cell lines. The CT values for the three genes were normalised against GAPDH and then further normalised between vIRF4 expression and the EV. The choice of GAPDH as a housekeeping gene to normalise the qPCR data set, was because it exhibited no change from microarray data. The qPCR showed upregulated expression of all three genes in the total as well as in the polysomal fractions (Figure 4-9). The result validates the microarray data (Figure 4-8) and also suggests that the genes are translationally upregulated overall due to vIRF4 expression, hence the abundant expression in the total as well as polysomal fractions. The primer sequences for the three genes and GAPDH are shown in Appendix A.

Immunoblotting was performed to confirm increased expression of these transcripts at protein level. Trichloroacetate (TCA) precipitation (described in Appendix B) of protein in polysome lysates from EV-NTAP+IFN and vIRF4-NTAP+IFN cell lines was performed prior to immunoblotting. Figure 4-10 shows that there is an increased expression of each of these proteins which is consistent with the abundance of the mRNA of the selected transcripts in the polysome fraction.
4.6.1. Radical SAM domain-containing 2 (RSAD2)

RSAD2 is a cluster-binding antiviral protein induced by type I and type II interferon and plays a major role in the cell antiviral state (Helbig & Beard, 2014). RSAD2 antiviral activity extends across a diverse range of DNA and RNA viruses, including CMV, HCV, WNV, dengue virus, and HIV-1 (Chan et al., 2008; Helbig et al., 2013; Jiang et al., 2010). Overexpression of RSAD2 has been observed in ovarian, colorectal carcinoma, lung cancer, and hepatocellular carcinoma (Cheng et al., 2013; Rachidi et al., 2014).

4.6.2. Epithelial Stromal Interaction 1 (EPSTI1)

EPSTI1, an interferon response gene, was first identified from heterotypic recombinant cultures of human breast cancer cells and activated breast myofibroblasts (Bezdenezhnykh et al., 2014; De Neergaard et al., 2010). The EPSTI1 gene is upregulated in tissues characterised by extensive epithelial–stromal interaction, and plays a crucial role in invasion and metastasis of cancer (Nielsen et al., 2002).

EPSTI1 is one of the ISGs induced by IFNλ2, and plays a key role in IFNλ2-mediated antiviral activity (Meng et al., 2015). Specifically, overexpression of EPSTI1 successfully inhibited HCV replication in the absence of interferon treatment, conversely EPSTI1 knockdown enhanced its replication (Meng et al., 2015).

4.6.3. CELF4: CUGBP Elav-like family member 4 (CELF4) also known as bruno-like protein 4 (BRUNOL4)

CELF4 is a member of the CELF/BRUNOL protein family, made up of six members and containing two N-terminal RNA recognition motif (RRM) domains, one C-terminal RRM domain, and a divergent segment of 160-230 amino acids. CELF4 is an RNA binding protein and binds to approximately 15%–20% of the transcriptome with specificity for the mRNA 3’ untranslated region (Wagnon et al., 2012). The CELF
protein family members including CELF4 are involved in C to U RNA editing, pre-mRNA alternative splicing, mRNA decay, and translation (Dasgupta & Ladd, 2012).

The mechanism behind the effect of CELF4 on translation is poorly understood. However, CELF2 competes with an activator of translation, HuR, for binding to A/U-rich elements in the COX2 3’ UTR while CELF1 is believed to play a role in translational silencing (Sureban et al., 2007). CELF4 is over expressed in cervical, prostate, breast cancer, neuroblastoma and medulloblastoma (Fujimura et al., 2008).

**Figure 4-10**: Validation of microarray by qPCR of selected genes upregulated by vIRF4 expression.
A subset of 3 genes was chosen to confirm the microarray results by real-time PCR. Expression of the genes was normalised to GAPDH and further normalised in the presence of vIRF4 expression relative to EV, in total and polysomal mRNAs. The results show that overall the genes are upregulated in the total as well as polysomal samples.
Figure 4-11: Western blot analysis of mRNAs identified by the translational profiling (CELF4, EPSTI1 and RSAD2)

Protein extracts were generated from polysome lysates from EV-NTAP+IFN and vIRF4-NTAP+IFN cell lines following TCA treatment. These were subjected to SDS-PAGE, immunoblotted and probed with the antibodies indicated to assess differences in expression in the proteins from CELF4, EPSTI1 and RSAD2 genes. The data show that there is an increase in the expression of each of these proteins in the presence of vIRF4, consistent with their abundance in the mRNA transcripts. GAPDH was probed to confirm equal loading.
4.7. DISCUSSION

Polysome profiling provides global analysis of translation and facilitates better understanding of viral gene regulation (Stern-Ginossar & Ingolia, 2015). It allows for isolation of translating ribosomes and polysomes in order to study changes in mRNA translation activities in the cell (Gandin et al., 2014). In the present study, polysome profiling of vIRF4-expressing cells was compared with that of the EV-NTAP in the presence of IFN. The monosome and polysome fractions for both cell lines were compared as shown in Figure 4-1A. Further analysis confirmed a significant reduction of polysome-to-monosome ratio as a result of vIRF4 expression (Figure 4-1A). The reduction in the actively translated mRNA in the presence of vIRF4 suggests a reduction in the overall level of translation. This finding ascribes a new role to KSHV vIRF4 as a negative regulator of translation.

Viral modulation of the translation process is not novel and has been discussed in detail in section 1.9. Viruses have evolved to customise the host translational apparatus and to ensure viral mRNA translation competes more efficiently with cellular translation (Mohr & Sonenberg, 2012a). Viruses and other microbial agents are capable of modifying the translational capacity of the host by limiting translation of host mRNA that mediate innate responses (Arnold et al., 2013). This is achieved by downregulation of the expression of specific host proteins with antiviral activity, alongside selective upregulation of translation of viral mRNAs (Firth & Brierley, 2012; Reineke & Lloyd, 2011).

Viruses utilise varying mechanisms for modulating host translation. The common molecular mechanisms are often tailored to the biological needs of each virus (Mohr & Sonenberg, 2012b). For example the picornaviruses induce hypophosphorylation of 4E-BP through cleavage of eIF4G, or interference with eIF4F assembly (Walsh et al., 2013a). Specifically, poliovirus, coxsakie virus, rhinovirus inhibit host protein synthesis by suppressing cap-dependent translation. The X-ORF gene encoded
within RNA segment 3 of influenza A virus accelerates host mRNA decay via global host shutoff (Jagger et al., 2012). The effect of X-ORF gene expression is to reduce the host response to infection, particularly inflammatory, apoptotic, and T-lymphocyte signalling (Jagger et al., 2012). The SARS coronavirus nsp1 protein binds to and inactivates 40S ribosome subunits, as well as promoting selective host mRNA degradation. One consequence is to suppress host type I IFN production (Kamitani et al., 2009).

DNA viruses have evolved different mechanisms to inhibit efficient viral translation, often at the expense of host protein synthesis. CMV does not inhibit host protein synthesis as it depends on host mRNA translation factors for its effective replication (Stinski, 1977). CMV increases the intracellular concentration of eIF4E, eIF4G, eIF2, and PABP by inducing host mRNA translation (Isler et al., 2005; Perez et al., 2011a). PABP accumulation is required for viral replication and spread. A CMV protein, UL38, which activates mammalian target of rapamycin complex 1 (mTORC1), increases levels of PABP to facilitate the assembly of the cap-recognition complex eIF4F (McKinney et al., 2012).

Lytic reactivation of KSHV facilitates eIF4F complex formation as well as p38 MAPK- and ERK-mediated eIF4E phosphorylation in PEL-derived B-cells (Arias et al., 2009). KSHV reactivation suppresses host translation, by inhibiting the recruitment of PABP to eIF4F complexes. This results in nuclear export of PABP, an action mediated by KSHV proteins K10/10.1 and SOX (Arias et al., 2009). This further illustrates the important role of virus-mediated translational control in determination of the fate of cellular translation (Arias et al., 2009).

To further understand the putative effect of the vIRF4 protein on the extent of translational control of the host cell, RNA extracted from pooled monosome and polysome fractions was subjected to microarray analysis in order to assess variations in mRNAs distribution pattern across the gradient between the vIRF4 expressing and
the EV cell lines. Northern Blotting or qPCR are used to determine mRNAs levels in pooled polysome fractions. Alternatively, mRNA levels may be determined by deep-sequencing.

The key biological processes downregulated as a consequence of vIRF4 expression, were muscle contraction and cell adhesion (attraction and repulsion receptors). Cytoskeletal intermediate filaments and IFN signalling were the leading upregulated processes. The reason behind these vIRF4-regulation processes are not clearly understood. However this might suggest that vIRF4 possibly plays a role in KSHV entry as successful entry and migration of herpesviruses into the nucleus requires migration through intracellular organelles and cytoskeletal membrane. An adaptor protein c-Cbl reportedly plays a major role in the recruitment of contractile elements, myosin II A, during KSHV entry via macropinocytosis. The myosin II associates with actin to coordinate signalling pathways, resulting in bleb formation and bleb retraction (Veettil et al., 2014; Veettil et al., 2010)

In the polysome sample, a total of 122 genes were significantly upregulated while 331 were downregulated. The genes of interest selected for validation were among the upregulated genes. Both CELF4 and Ribosomal RNA binding protein 1 (RRBP1) are associated with translation. Other genes of interest in this category included genes found to be indicated in antiviral immunity such as RSAD2, EPSTI1 and OAS3 (Figure 4-8).

The downregulated genes at translation level were mainly genes associated with cytoskeletal membrane reorganization such as ANK1, while the others included genes associated with lipid metabolism such as LIPE and CYP27A1. LIPE encodes for an intracellular hormone-sensitive lipase that is capable of hydrolysing a variety of esters (Aten et al., 1995; Langin et al., 1993) while CYP27A1 gene encodes for cytochrome P450 oxidase, an enzyme which is located in many different tissues and participates in cholesterol metabolism (Cali & Russell, 1991). vIRF4 regulation of
genes involved in lipid metabolism is surprising but might also be a consequence of vIRF4 playing an indirect role in KSHV entry or egress. KSHV entry is facilitated by cholesterol lipid rafts (LR) which are sphingolipid enriched microdomains of plasma membranes and the LR play a vital role in KSHV de novo infection (Raghu et al., 2007). KSHV tegument protein, KSHV ORF45 targets LR and plays a vital role in egress and production of this virus (Wang et al., 2015). ORF45 facilitates co-localisation of viral particles with trans-Golgi and endosome vesicles thereby allowing maturation and release of virion particles (Wang et al., 2015).

A subset of genes translationally regulated by vIRF4 expression was chosen for validation by qPCR based on their degree of expression change, p-value, and relevance of the genes to the objective of the thesis. These three genes summarised in table 4-3 below, share in common prior detection in various cancers thus raising the possibility of their use as prognostic tumour markers for Kaposi’s sarcoma.
All three genes listed in Table 4-3, were measured by qPCR in total and polysomal fractions thus suggesting that vIRF4 expression mediates the increased abundance of the mRNA of these genes. This finding is consistent with the translational upregulation of these genes observed from the microarray data (Figure 4-8). Figure 4-9 shows that, in the presence of vIRF4, all three genes tested by qPCR, were expressed by greater or equal to 5-fold in both the total and polysomal fractions. This correlates with the fold change observed in the microarray transcriptional log ratio for each of these genes.

RSAD2 is a highly species conserved, 361-amino-acid protein and was first identified as a CMV-inducible gene in fibroblasts (Helbig & Beard, 2014). RSAD2 is upregulated in the absence of IFN through an IRF1- or IRF3-dependent mechanism (Stirnweiss et al., 2010). Expression of this gene is also induced by a number of viruses, including HCV and dengue virus (Helbig et al., 2013; Helbig et al., 2011), CMV, Japanese encephalitis virus and Chikungunya virus (Chan et al., 2008; White et al., 2011). The
present study also shows an upregulation of the translation of RSAD2 gene. RSAD2 expression inhibits both HIV-1 and influenza A through blocking viral egress across the plasma membrane and lipid rafts (Nasr et al., 2012; Tan et al., 2012; Wang et al., 2007). Genome-wide cDNA expression data analysis for ovarian cancer revealed an upregulation of RSAD2 gene and correlates with high pathological grade of ovarian cancer (Rachidi et al., 2014).

A second gene found to be upregulated by vIRF4 expression and studied in more detail was EPSTI1. EPSTI1 has previously been shown to be upregulated in primary infection of Rhesus Macaques with Dengue Virus Type 1 (Sariol et al., 2007). EPSTI1 has been shown to be significantly upregulated in hepatitis E virus-infected patients compared to non-infected individuals (Moal et al., 2012). In another study, EPSTI1 was induced by HCV infection and found to sufficiently inhibit viral replication (Meng et al., 2015). EPSTI1 was also found to mediate anti-HCV activity by regulating the expression of other ISGs including PKR, OAS1 and RNaseL (Meng et al., 2015). EPSTI1 is a stromal fibroblast-induced gene identified in breast cancer and its upregulated expression correlates with breast cancer invasion and metastasis (Capdevila-Busquets et al., 2015; Li et al., 2014). There is a complex interplay between immune evasion and cancer as inflammation contributes to oncogenesis and is one of the recognised hallmarks of cancer (Diakos et al., 2014).

The CELF4 gene is an important regulator of translation and regulates pre-mRNA alternative splicing and mRNA editing (Good et al., 2000). Post-transcriptional gene regulation is a fundamental aspect of gene expression that largely ensures the accurate expression of genetic information. The upregulation of a translation regulatory protein by vIRF4 suggests a possible role for vIRF4 in translation control. Additionally, vIRF4 could be utilising CELF4 to suppress active translation as observed by the polysome profiling result.
CELF4 has been identified as a target gene for RE1 Silencing Transcription Factor (REST) (Roopra & Wagoner, 2011). Overexpression of CELF4 resulted in a dramatic shift in splicing of REST in MCF7 and HEK-293 cells (Barreau et al., 2006; Roopra & Wagoner, 2011). Prior studies suggest that REST also regulates the expression of multiple CELF family members, including CELF6, CELF4, and possibly CELF5 (Barreau et al., 2006). REST is believed to be a tumour suppressor gene as mutation in REST was found in several colon cancer. (Westbrook et al., 2005, *Cell*, 121:837-848). Additionally, it was observed that REST mRNA expression was lost in approximately a third of the colon cancer, small cell lung cancer and breast cancer (Westbrook et al., 2005). CELF4 is one of the important gene signatures that serve as useful biomarkers for aggressive breast cancers (Roopra & Wagoner, 2011).

As part of the antiviral response, cellular measures are initiated to inhibit protein synthesis and halt virus production. Many viruses encode proteins that modify host translation to enhance viral replication as discussed earlier in section 1.9. Virus modulation of translation facilitates virus mediated control of cellular transcriptional and translational activities. To the best of our knowledge, translation regulation by KSHV vIRF4 is a novel concept. The findings in this chapter justifies the proposition of a possible role for vIRF4 in translation control.

Future studies would focus on further investigating the impact of the vIRF4-mediated upregulation of these genes, on KSHV replication and lytic reactivation. The following experiments could be performed:

1. Confirm by western blot a possible increased expression of CELF4, RSAD2 and EPSTI1 in KSHV infected cells.

2. Polysome profiling in KSHV infected cells to see if CELF4 binds mRNA more during infection.
3. siRNA knockdown of CELF4, RSAD2 and EPSTI1: This approach would be to reduce protein function, and thereby investigate further evidence of the importance of CELF4 in KSHV infection.

4. siRNA knockdown of CELF4, RSAD2 and EPSTI1 to determine if it would lead to increased KSHV replication.

5. Immunohistochemistry could be done to investigate the possibility of using the translationally upregulated proteins; RSAD2, CELF4 and EPSTI1 as tumour markers for Kaposi’s sarcoma.
CHAPTER 5

VALIDATING THE INTERACTIONS BETWEEN USP7 AND VIRF2 AND VIRF4 AND INVESTIGATING THEIR SIGNIFICANCE ON IFN SIGNALLING
5. CHAPTER 5

5.1. Introduction to chapter 5

Herpesviruses are known to interact with USP7, suggesting a potential role for USP7 in antiviral immunity (Boutell & Everett, 2003; Chavoshi et al., 2016a; Holowaty & Frappier, 2004; Lee et al., 2011). The structural analysis of the KSHV vIRF4-USP7 complex and its role in the regulation of p53 levels has been well characterised (Lee et al., 2011). USP7 plays a vital role in the p53-Mdm2 pathway where USP7 binds and stabilises p53 and the p53 E3 ubiquitin-ligase, Mdm2, by removing ubiquitin from p53 and also from the Mdm2 (Cummins et al., 2004b; Li et al., 2002a). The combined effect of USP7 on both Mdm2 and p53 regulates functional p53 levels (Marchenko et al., 2007; Meulmeester et al., 2005). More recently, KSHV vIRF1 was shown to interact with the N-terminal domain of USP7 through its EGPS consensus sequence (Chavoshi et al., 2016b). Previously a number of other viral proteins have been identified as binding partners of USP7 and have been discussed earlier in section 1.8.

Aside from identifying the ribosomal proteins as partners of vIRF2 and vIRF4, SILAC-MS carried out by our group on EV-NTAP, vIRF2-NTAP and vIRF4-NTAP stable cell lines (described in section 3.7) also identified USP7 and CBP as the other two common interacting partners of vIRF2 and vIRF4. The role of CBP in vIRF2-or vIRF4-mediated downregulation of JAK STAT signalling had been investigated by our group (Hindle & Blackbourn, unpublished observation). This thesis therefore focused on validating the interactions between USP7 and KSHV vIRF2 and vIRF4. The significance of such interactions on IFN signalling was also investigated.

The ubiquitin–proteasome system (UPS) has been implicated in several important regulatory roles in eukaryotic cellular processes including cell cycle progression, stress response, signal transduction, transcriptional activation, and DNA repair (Ciechanover et al., 2000; Nijman et al., 2005; Song & Rape, 2008). Ubiquitination is
one of the key post-translational modifications of proteins and has an impact on the fate of proteins vis-à-vis intracellular stability, relocalisation and activity. (Pickart & Eddins, 2004; Pickart & Fushman, 2004). Mono-ubiquitination is important in cellular processes such as endocytosis, DNA repair, protein transport and histone regulation. K48-linked ubiquitination mediates proteasomal degradation of target proteins, while K63-linked ubiquitination directs targeted proteins to lysosomes, thus regulating localisation and activity of the involved protein (Nathan et al., 2013). These signals are balanced by deubiquitinating enzymes (DUBs), which are proteases that remove the ubiquitin moiety from proteins, thus stabilising their levels or activity (Pfoh et al., 2015b). Deubiquitination is a highly regulated activity that may spare proteins from degradation, relocation to their original site or reverse conformational changes imparted by ubiquitin attachment (Hussain et al., 2009). DUBs therefore play important roles in key cellular pathways and possibly play a central role in invasion and pathogenesis of certain oncogenic viruses (Hussain et al., 2009).

The USPs form the largest and the most diverse family of DUBs. Ubiquitin conjugation of protein is an important step in regulation of cellular processes, and has been implicated in cell cycle control, protein localisation and DNA repair (Shi & Grossman, 2010). DUBs reverse the ubiquitination process thus modulating important cellular processes such as cell cycle progression, DNA damage repair pathways, immune responses and apoptosis (Jacq et al., 2013; Reyes-Turcu & Wilkinson, 2009).

USP7 has been proposed to play a critical function in a number of cellular processes; DNA replication, apoptosis and cell cycle regulation, however the role of USP7 in regulation of IFN signalling is yet to be reported (Bhattacharya & Ghosh, 2014; Chen et al., 2013; Fan et al., 2013b; Lecona et al., 2016). The structure of USP7 has been described earlier in section 1.9. It is made up of an N-terminal TRAF/Math domain and a 64 kDa C-terminal region linked in between by the catalytic domain (Faesen et al., 2011). Interestingly, the USP7 catalytic domain remains in an inactive state until
it undergoes conformational rearrangement to facilitate ubiquitin binding (Hu et al., 2002a). USP7 plays a vital role in the p53-Mdm2 pathway where USP7 binds and stabilises p53 and the p53 E3 ubiquitin-ligase, Mdm2 by removing ubiquitin from p53 and also from the MDM2 (Cummins et al., 2004b; Li et al., 2002a). The combined effect of USP7 on both Mdm2 and p53 regulates functional p53 levels (Marchenko et al., 2007; Meulmeester et al., 2005)

To confirm the interaction of USP7 with vIRF2 and vIRF4, co-immunoprecipitation studies were performed on cells that were transiently transfected to express either of these viral proteins. As we had earlier shown that vIRF2 or vIRF4 were negative regulators of IFN signalling, we hypothesised that this interaction may have relevance in the inhibition of JAK STAT signalling caused by these viral proteins.

To test this hypothesis, functional studies were then performed: the effect of the USP7 inhibitor, P22077 on JAK STAT signalling was examined and then the impact of the USP7 knockdown on ISRE-driven luciferase assay was tested.

The objectives of this chapter were to:

1) Determine the effect of USP7 knockdown on the JAK STAT pathway

2) Assess the role of USP7 knockdown on the IFN production pathway

3) Evaluate the role of P53 on USP7-mediated modulation of the interferon response

4) Determine if JAK STAT signalling is augmented by USP7 overexpression.
5.2. USP7 immunoprecipitation

To confirm USP7 interaction with vIRF2 and vIRF4 as predicted by the SILAC MS, immunoprecipitation experiments were performed in which the viral proteins were immunoprecipitated with Xpress antibody (details of IP is found in section 2.7). Previously, vIRF4 has been shown to bind to the TRAF domain of USP7 (Lee et al 2011). However, there has been no published report of an interaction between vIRF2 and USP7. HEK 293 cells were transiently transfected with pcDNA4/HisMax, pvIRF2-HisMax or pvIRF4-HisMax. The pcDNA4/HisMax vector was used as the backbone for the pvIRF2-His/Max and pvIRF4-His/Max vectors and served as a negative control in this experiment. These vectors encode an Xpress and a polyhistidine tag, contiguous with the vIRF2 and vIRF4 proteins that enables their detection with anti-Xpress and Polyhistidine antibodies, respectively.

To determine if USP7 was binding to vIRF2 or vIRF4 the immunoprecipitates were analysed by western blot using the USP7 antibody. Figure 5-2 shows that USP7 was detected in the immunoprecipitates from cells containing either pvIRF2-HisMax or pvIRF4-HisMax plasmids, respectively. (Figure 5-2 lanes 2 and 3, see USP7 output row). These results confirm that vIRF2 and vIRF4 interact with USP7. The input was probed for USP7 with USP7 antibody and the polyhistidine antibody was used to detect the His tag of vIRF2 and vIRF4.
Figure 5-1: vlRF2 and vlRF4 associate with USP7.

Co-immunoprecipitation studies were performed as described in section 2.7. The output were immunoprecipitated with Protein G Sepharose beads and analysed by western blot using the anti-His antibody to detect the vlRF2 and vlRF4 proteins and the anti-USP7 antibody to detect USP7. The input lysates on which immunoprecipitations were performed were analysed by western blot to show that USP7, vlRF2 and vlRF4 were present. β-actin was probed to confirm equal loading. The result of one experiment, representative of 3 performed separately are presented. IC- isotype control, IP-immunoprecipitate.

5.3. USP7 inhibitor studies

The USP7 inhibitor P22077 (R&D) inhibits USP7 activity. Having confirmed that USP7 interacts with vlRF2 and vlRF4, both of which have been characterised as negative regulators of IFN signalling, it was hypothesised that pharmacologic inhibition of USP7 might inhibit the JAK STAT signalling pathway. ISRE-based luciferase reporter assays were performed in the presence of the USP7 inhibitor or a DMSO control to examine its effect on ISRE activity.
Our group had earlier confirmed the activity of the USP7 inhibitor, by examining RAD18 levels. A decrease in USP7 activity leads to a decrease in the levels of RAD18 as determined by western blot (Hindle, & Blackbourn, unpublished data).

5.3.1. Examining the effect of inhibition of USP7 on ISRE-containing promoter activity

To investigate the effect of inhibiting USP7 activity on JAK STAT signalling, ISRE-reporter assays were performed. Firstly, an MTT assay was first carried out with increasing doses of the P22077 inhibitor to determine the least toxic dose (Figure 5-2). The toxicity of the inhibitor was also assessed by Trypan blue exclusion (not shown). The MTT assay and Trypan blue exclusion assay showed that the USP7 inhibitor was toxic to the cells at a dose of greater than or equal to 50µM.

The reporter assay was then performed by co-transfecting HEK 293 cells with ISRE-containing promoter and the constitutively expressing Renilla, pRLSV40 plasmid. Five hours later the cells were treated with P22077 (25µM) or DMSO. Twenty four hours later, the cells were treated with or without rIFNαB2 and DLA were performed a further 24 hours later.

The results show that following treatment with the USP7 inhibitor, ISRE-containing promoter activity was reduced by 42.6% compared to the DMSO control (Figure 5-3 compare columns 1 and 3).
Figure 5-2: Cell viability following USP7 inhibitor treatment.

HEK 293 cells were transfected with pISRE-luc (250ng) and the pRLSV40 plasmid (1ng). 5 hours post transfection, the cells were treated with USP7 inhibitor (P22077) at a concentration ranging from 0-100µM, or mock treated with DMSO, for 24 hours. rIFNαB2 (300IU/mL) was then added and cells were left for a further 24 hours. Non-transfected cells were used as positive controls while cells killed with 1% Triton X-100 were used as negative control. The percentage cell viability of each sample was derived by subtracting the negative control from each OD reading and further divided by the OD of the positive control. The data represent the mean ± the SEM of three independent experiments.
Figure 5-3: Investigating the effects of USP7 inhibitor treatment on JAK STAT signalling.
HEK 293 cells were transfected with pISRE-luc (250ng) and the pRLSV40 plasmid (1ng). 5 hours post transfection, the cells were treated with P22077 (25μM), or mock treated with DMSO, for 24 hours. rIFNαB2 (300IU/mL) was then added and the cells were left for a further 24 hours. Cells were harvested and DLA performed. The pRLSV40 plasmid was added as an internal control to which firefly luciferase levels were normalised. The results are shown as firefly activity normalised to Renilla luciferase control. The data represent the mean ± the SEM of three independent experiments ***=p<0.001 ANOVA followed by a Dunnett post hoc test.
5.4. siRNA knockdown of USP7

To assess the role of USP7 in the JAK STAT signalling pathway, the RNA interference method was employed. The Smartpool On-Target USP7 siRNA (Dharmacon) was used to knockdown USP7 levels in HEK 293. HEK 293 cells were reverse transfected with siRNA or the matched scrambled siRNA (NTC siRNA, Dharmacon) in 24-well plates. siRNA (30µM) was transfected per well using Lipofectamine 2000 transfection reagent according to the manufacturer’s protocol.

60 hours post-transfection the cells were treated with 300IU/mL of rIFNαB2 for a further 12 hours before lysis for western blot (Figure 5-4A). The efficiency of USP7 knockdown was quantified and normalised to the β-actin loading control using LICOR Odyssey software and found to be 79.6% and 84.6% in the presence and absence of IFN as shown in columns 3 and 4 (Figure 5-4B). USP7 mRNA knockdown was confirmed by extracting RNA 48 hours post siRNA transfection and then subjecting the reverse transcribed cDNA to qPCR (Figure 5-5).
Figure 5-4: siRNA knockdown of USP7 protein

HEK 293 cells were transfected with USP7 siRNA or scrambled, non-targeting control siRNA to a total concentration of 30µM using Lipofectamine™ 2000 transfection reagent for 48 hours. Cells were then lysed and cleared lysates were analysed by immunoblotting to assess USP7 knockdown efficiency. Molecular mass markers are shown on the left of the blot. A representative blot is shown in 5-4A. Knock down efficiency was calculated by quantification of the bands from three independent western blots using LI-COR Odyssey software (5-4B).
1µg of cDNA was reverse transcribed from RNA extracted from HEK 293 cells transfected with USP7 siRNA and the NTC siRNA following treatment with 300IU/mL of rIFNαβ2. The cDNA was subjected to qPCR using USP7 primers. The data represent the mean ± the SD of the three independent experiments which were each assayed in duplicate. The results are shown as normalised expression ratio (NER) of USP7 normalised to Tubulin gene and further normalised to presence of IFN. The NER of USP7 siRNA transfected cells were compared to NTC siRNA transfected cells ***=p<0.0001, ANOVA followed by a Dunnett post hoc test.
5.5. USP7 knockdown supresses ISRE activation

To investigate the effect of USP7 knockdown on JAK STAT signalling, ISRE-reporter assays were performed. HEK 293T cells were reverse transfected with USP7 siRNA or the matched scrambled siRNA (NTC siRNA) in 24-well plates. 30µM siRNA was transfected per well using Lipofectamine 2000 transfection reagent according to the manufacturer’s protocol.

48 hours later, the cells were co-transfected with the ISRE-containing promoter and the constitutively expressing Renilla pRLSV40 plasmid. 12 hours later, the cells were treated with or without 300IU/mL rIFNαB2 and DLA were performed a further 12 hours.

The results show that following USP7 knockdown, ISRE-containing promoter activity was reduced by 78.6% compared to the cells transfected with the NTC siRNA. (Figure 5-6 compare columns 1 and 3).
Figure 5-6: USP7 Knockdown suppresses ISRE activation

HEK 293 cells were transfected with USP7 siRNA or the NTC siRNA to a total concentration of 30µM for 48 hours after which the cells were transfected with pISRE-luc (250ng) and the pRLSV40 plasmids (1ng) for a further 12 hours. rIFNαB2 (300IU/mL) was then added and cells were left for a further 12 hours. Cells were harvested and DLA performed. The pRLSV40 plasmid was added as an internal control to which firefly luciferase levels were normalised. The results are shown as firefly activity normalised to Renilla luciferase control. The data represent the mean ± the SEM of the three independent experiments ****=p<0.0001, ANOVA followed by a Dunnett post hoc test.
5.6. USP7 Knockdown does not downregulate the Type II IFN pathway

The type II IFN pathway is regulated by IFNγ and the total number of IFNγ-regulated genes is estimated to be approximately 500 (Li et al., 2012). Following IFNγ treatment the STAT1 homodimers are formed and translocate into the nucleus to bind and activate the GAS-containing promoters of the IFNγ responsive genes. To investigate the effect of USP7 knockdown on the type II IFN signalling, GAS-reporter assays were performed. HEK 293 cells were reverse transfected with siRNA or the matched scrambled siRNA (NTC siRNA) as described in section 2.4.

48 hours post knockdown the cells were co-transfected with the GAS-containing promoter and the constitutively expressing Renilla pRLSV40 plasmid. 12 hours later, the cells were treated with or without 1000IU/mL IFNγ and DLA were performed a further 12 hours.

The results show that following USP7 knockdown, GAS-containing promoter activity was not significantly reduced compared to the cells transfected with the NTC siRNA. (Figure 5-6 compare columns 1 and 3). This result suggests that the USP7 effect observed in Figure 5-6 is specific to the type I IFN pathway.
HEK 293 cells were transfected with USP7 siRNA or the NTC siRNA to a total concentration of 30 µM for 48 hours after which the cells were transfected with pGAS-luc (500ng) and the pRLSV40 plasmids (1ng) for a further 12 hours. IFNγ (1000U/mL) was then added and cells were left for a further 12 hours. Cells were harvested and DLA performed. The pRLSV40 plasmid was added as an internal control to which firefly luciferase levels were normalised. The results are shown as firefly activity normalised to Renilla luciferase control. The data represent the mean ± the SEM of the three independent experiments. The result shows that USP7 knockdown did not reduce GAS activity significantly. ns, not significant.

Figure 5-7: USP7 knockdown does not interfere with the type II IFN pathway
5.7. USP7 knockdown downregulates ISG levels

To confirm the inhibitory effect of USP7 knockdown on the cellular response to IFNα, RNA was extracted from HEK 293 cells transfected with USP7 siRNA or the matched scrambled siRNA (NTC siRNA) in 24-well plates. RNA was subjected to qRT-PCR analysis to examine the expression of ISG mRNAs in these cells. USP7 knockdown significantly inhibited the IFNα-induced expression of the three ISGs tested; ISG15 (45.0%), OAS1 (90.9%) and IFIT1 (82.2%) (Figure 5-8).

**Figure 5-8: USP7 knockdown downregulates ISGs levels**

1µg of cDNA was reverse transcribed from RNA extracted from HEK 293 cells transfected with USP7 siRNA and the NTC siRNA following treatment with 300IU/mL of rIFNαB2. The cDNA was subjected to qPCR using ISG15, OAS1 and IFIT1 primers. The data represents the mean ± the SEM of the three independent experiments which were each assayed in duplicate. The results are shown as normalized expression ratio (NER) of the ISGs normalised to Tubulin gene and further normalised to presence of IFN. The NER of USP7 siRNA transfected cells were compared to NTC siRNA transfected cells *=p<0.05. **p<0.01 ANOVA followed by a Dunnett post hoc test.
5.7. USP7 Knockdown does not significantly reduce IFN production

USP7 binds and deubiquitinates RTA-associated ubiquitin ligase (RAUL), a known negative regulator of IRF7 and IRF3-mediated type I IFN production (Yu & Hayward, 2010). Having shown that USP7 positively regulates IFN signalling, we sought to investigate the effect of USP7 knockdown on the IFNβ production pathway.

ISRE-reporter assays were performed in HEK 293 cells that were reverse transfected with USP7 siRNA or the matched scrambled siRNA (NTC siRNA) as described in section 5.4. 48 hours later, the cells were co-transfected with the IFNβ-containing promoter (p125-luc) and the constitutively expressing Renilla, pRLSV40 plasmid. 5 hours later, the cells were transfected with or without 10µg/mL poly I: C to activate the IFNβ promoter. Poly (I:C) is a synthetic dsRNA that triggers the IFN signalling pathway by binding to TLR. Downstream, IRF3 is phosphorylated and translocates into the nucleus leading to activation of the IFNβ promoter (Aresté & Blackbourn, 2009; Fuld et al., 2006). The cells were lysed 20 hours post poly (I:C) treatment and subjected to DLA. The results showed no significant difference in the level of IFNβ promoter induction in the USP7 knockdown cells compared to the cells transfected with the NTC siRNA (Figure 5-9, compare columns 3 and 4).
Figure 5-9: USP7 does not inhibit poly (I:C)-driven activation of the IFNβ promoter in HEK 293 cells.

HEK 293 cells were transfected with USP7 siRNA or scrambled, non-targeting control siRNA. 24 hours later the cells were transfected with p125-luc (250ng) and 1ng of pRLSV40 plasmid. 24 hours post-transfection, the cells were transfected with 10μg/mL poly (I:C) and harvested 20 hours later. These results were normalised using the pRLSV40 plasmid. The data represent the mean ± the SEM of the three independent experiments which were each assayed in duplicate. ns= non-significant followed by a Dunnett post hoc test.
5.8. USP7 effect on interferon signalling is independent of p53

USP7 binds and deubiquitinates both Mdm2 and p53, and plays an important role in regulating the level and activity of p53 (Li et al., 2002b). The positive role of P53 in the IFN response has earlier been discussed in section 1.8.6.1. To ascertain if the positive role of USP7 in the type I IFN signalling described in Figure 5-6 is dependent on P53, USP7 knockdown was carried out in P53-deficient cell lines (SaoS2) and ISRE activities were measured by DLA.

The P53 status of the SaoS2 cell line was confirmed by immunoblotting for P53 protein (Figure 5-10A), and showed that the SaoS2 cells were deficient for P53 compared with HeLa cells used as a control. The SaoS2 cells were then reverse transfected with USP7 siRNA or the matched scrambled siRNA (NTC siRNA) as described in section 5.4. The efficiency of the knockdown was also assessed and compared with a NTC. β-actin was used to assess equal loading (Figure 5-10B). The knockdown efficiency was 63.3% and 66.9% respectively, in the presence and absence of IFN (Figure 5-10B).

For the reporter gene assay, 48 hours after siRNA transfection, the cells were co-transfected with the ISRE-containing promoter and the constitutively expressing Renilla, pRLSV40 plasmids. 12 hours later, the cells were treated with or without 300IU/mL rIFNαB2 and DLA was performed a further 12 hours. ISRE-containing promoter activity was reduced by 43.7% in USP7 knockdown states compared to the cells transfected with the NTC siRNA (Figure 5-6, compare columns 1 and 3).
**Figure 5-10: USP7 can be knocked down in SaoS2 cells**

SaoS2 cells were transfected with USP7 siRNA or scrambled, non-targeting control siRNA to a total concentration of 30\(\mu\)M using Lipofectamine 3000 transfection reagent for 48 hours. Cells were then lysed and cleared lysates were analysed by immunoblotting to detect P53 (panel A) and to assess knock down efficiency of the USP7 protein (panel B). Molecular mass markers are shown on the left of the blots. Data shows one blot representative of 3 independent experiments.
SaoS2 cells were transfected with USP7 siRNA or the NTC siRNA to a total concentration of 30µM for 48 hours after which the cells were transfected with pISRE-luc (250ng) and the pRLSV40 plasmids (1ng) for a further 12 hours. rIFNαB2 (300IU/mL) was then added and cells were left for a further 12 hours. Cells were harvested and DLA performed. The pRLSV40 plasmid was added as an internal control to which firefly luciferase levels were normalised. The results are shown as firefly activity normalised to Renilla luciferase control. The data represent the mean ± the SEM of three independent experiments **=p<0.01.
5.9. USP7 knockdown downregulates ISG levels in P53-deficient Saos2 cells

To confirm the inhibitory effect of USP7 knockdown on the cellular response to IFNα, RNA was extracted from SaoS2 cells transfected with USP7 siRNA or the matched scrambled siRNA (NTC siRNA) following stimulation with or without rIFNb2. RNA was subjected to qRT-PCR analysis to examine the expression of ISG mRNAs. USP7 knockdown significantly inhibited the IFNα-induced expression of the three ISGs tested; ISG15 (38.9%), OAS1 (76.6%) and IFIT1 (87.2%) and is shown in Figure 5-12A-C, where the USP7 knockdown cells were compared to the NTC.
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Figure 5-12: USP7 knockdown down regulates ISG levels in P53-deficient SaoS2 cells.

1µg of cDNA was reverse transcribed from RNA extracted from HEK 293 cells transfected with USP7 siRNA and the NTC siRNA following treatment with 300IU/mL of rIFNαB2. The cDNA was subjected to qPCR using ISG15, OAS1 and IFIT1 primers. The data represents the mean ± the SEM of three independent experiments which were each assayed in duplicate. The results are shown as normalized expression ratio (NER) of the ISGs in the stable cell lines normalised to Tubulin gene and further normalised to presence of IFN. The NER of USP7 siRNA transfected cells were compared to NTC siRNA transfected cells *=p<0.05, **=P<0.01 ANOVA followed by a Dunnett post hoc test.

5.10. USP7 overexpression augments IFN signalling

The previous section (5.10) detailed how USP7 knockdown suppressed ISRE activation and downregulate of the transcription of ISGs. The converse experiment was therefore performed to determine if overexpression of USP7 conferred any additive effect on JAK STAT signalling.

HEK 293 cells grown in 24-well plates were co-transfected when 60 to 70% confluent with increasing doses (up to 50ng) of pCI-USP7 plasmid (kindly provided by Roger Everett, University of Glasgow) alongside the reporter plasmids pISRE-luc and pRLSV40 in mixtures of 1µL of Lipofectamine 2000 per well. At 24 hours after
transfection, the cells were treated or mock treated with 300IU/mL of rIFNαB2 then harvested and subjected to DLA (Figure 5-14A) and also immunoblotted for USP7 antibody to confirm overexpression (Figure 5-13B). There was a significant stepwise increase in ISRE activation with increasing expression of USP7 (Figure 5-14A).
Figure 5-13: USP7 overexpression augments ISRE activation

HEK 293 cells were transfected with increasing amounts of the pCI-USP7 plasmid (0-250ng), pISRE-luc (250ng) and the pRLSV40 plasmids (1ng) for 24 hours. rIFNαB2 (300IU/mL) was then added and cells were left for a further 12 hours. Cells were harvested and DLA performed. The results are shown as firefly activity normalised to Renilla luciferase control. The pCDNA4HISMax was added as stuffer to equilibrate all transfectants to 50ng of DNA (panel A). The data represent the mean ± the SEM of the three independent experiments **=p<0.01, ****=P<0.0001 ANOVA followed by a Dunnett post hoc test. The cells were also immunoblotted with antibody for USP7. β-actin was used as loading control (panel B). The quantification of the bands was done using LICOR Odyssey software.
5.11. The catalytic domain of USP7 positively regulates ISRE activity

The USP7 structure was discussed in section 1.9.1 and includes a catalytic domain that spans 208 to 560 amino acids. In this section we took advantage of the catalytic mutant (C223S) shown in Figure 5-15 to investigate the role of the catalytic domain in USP7-mediated augmentation of ISRE activation. The catalytic domain regulates the deubiquitinating activity of USP7.

HEK 293 cells were co-transfected with either the wild type USP7 (pCI-USP7), the empty vector (pCI-neo) or the catalytic mutant (pCI-USP7C223S) (see Figure 5-15) alongside with the reporter plasmids; pISRE-luc (250ng) and the pRLSV40 plasmids (1ng) for 24 hours. rIFNα (300IU/mL) was then added and cells were left for a further 12 hours. Cells were harvested and DLA performed. The pRLSV40 plasmid was added as an internal control to which firefly luciferase levels were normalised. The results are shown as fold change of firefly luciferase activity normalised to Renilla luciferase and further normalised to the presence of IFN.

A significant increase in ISRE activation was observed with overexpression of the wild type USP7 (Figure 5-15), consistent with earlier studies (Figure 5-13). However, there was no significant difference between the levels of activation in the presence of the empty vector and the catalytic mutant (C223S) in Figure 5-15. These data suggests that the catalytic site of USP7 is required for its positive IFN-regulatory effect.
Figure 5-14: The USP7 protein used in this study

The regions of USP7 including, the N-terminal domain (yellow), the C-terminal domain (blue), the catalytic domain (red), and the point mutation (C233S) that inhibits catalytic activity are indicated relative to amino acids numbers.
Figure 5-15: The catalytic domain of USP7 is required for its activity

HEK 293 cells were co-transfected with USP7-wt, its empty vector (EV) or its catalytic mutant USP7 (C223S) alongside with the reporter plasmids; pISRE-luc (250ng) and the pRLSV40 plasmids (1ng) for 24 hours. rIFNb2 (300IU/mL) was then added and cells were left for a further 12 hours. Cells were harvested and DLA performed. The pRLSV40 plasmid was added as an internal control to which firefly luciferase levels were normalised. The results are shown as firefly activity normalised to Renilla luciferase control. The results are shown as fold change in the presence of IFN related to its absence. The data represent the mean ± the SEM of three independent experiments which were each assayed in duplicate. ** = p<0.01 ANOVA followed by a Dunnett post hoc test.
5.12. DISCUSSION

In this chapter we have confirmed USP7 interaction with vIRF2 and vIRF4. The SILAC-MS interaction between USP7 and both vIRF2 and vIRF4 has been validated by pull down assays for USP7 with both vIRF2 and vIRF4 (Figure 5-1). Both KSHV vIRFs 1 and 4 have been previously identified as binding partners of USP7 (Chavoshi et al., 2016a; Lee et al., 2011). However, in the case of vIRF2, this interaction has not been previously reported. This data add to the evidence that USP7 is being targeted by a number of herpesviruses, including KSHV, in order to subvert IFN-mediated or p53-mediated surveillance and enhance viral replication (Lee et al., 2009b).

The USP7-vIRF1 interaction leads to deregulation of USP7 deubiquitinating effect on p53 with consequent inhibition of the p53-mediated antiviral response (Chavoshi et al., 2016a). vIRF4 binds and stabilises Mdm2, a known E3 ubiquitin ligase that targets p53 thus facilitating proteasomal degradation of p53 (Lee et al., 2009b).

A number of herpesviruses also target USP7 and this interaction has been reviewed in section 1.9.3. EBNA1 protein of EBV competes with USP7 for binding to the TRAF domain of USP7 and also sequesters USP7 from p53, thus destabilising p53 (Holowaty & Frappier, 2004). Conversely, ICP0-USP7 interaction has no effect on p53 as and ICP0 is the only viral protein that binds to the terminal c-UBL domain of USP7. HSV I ICP0 ubiquitinates USP7 through its ring finger domain, while USP7 deubiquitinates ICP0, protecting it from auto ubiquitination (Boutell & Everett, 2003).

The role of vIRF2 and vIRF4 as negative regulators of IFN signalling has been discussed in Chapter 3, hence the work in this present chapter sought to investigate the possible contribution of USP7 to the regulation of type I IFN signalling pathway and might explain the mechanistic reason behind vIRF2 and vIRF4 targeting USP7.

ISRE-containing reporter gene assays were carried out in the presence of the USP7 inhibitor, P22077. The data in Figure 5-2 showed that the highest concentration of
inhibitor that was least toxic to the cells was of 25µM. Treatment with this optimised P22077 inhibitor resulted in significant inhibition of IFNα-induced ISRE activity (Figure 5-3). The small molecule USP7 inhibitor, P22077 has a limited inhibitory profile compared with the broad based effect of PR-619 hence P22077 is preferable for pharmacologic inhibition of USP7 in vitro (Fan et al., 2013b).

The next approach was to silence USP7 in HEK 293 cells to verify the role of USP7 in JAK STAT signalling. The knockdown efficiency confirmed with qPCR and western blot analysis (Figures 5-4A and 5-5). The suppression of ISRE activation following USP7 knockdown confirmed the USP7 inhibitor study and also suggested that USP7 positively regulates type I IFN signalling (Figure 5-6). This role of USP7 in the type I IFN response has not been reported in the literature previously.

USP13 has been reported to play a positive role in the regulation of both type I and type II IFN signalling via deubiquitination and stabilisation of STAT1 protein (Yeh et al., 2013). The type I IFN signalling pathway is regulated by several posttranslational modifications including ubiquitination, SUMOylation, acetylation and phosphorylation (Yeh et al., 2013). Components of the IFN signalling pathway including the STAT factors are regulated specifically by ubiquitination (Jiang et al., 2012; Shuai & Liu, 2003; Yeh et al., 2013). For example, USP13 overexpression reduced ubiquitination of STAT1, thus protecting it from degradation, while the reverse was observed with USP13 silencing (Yeh et al., 2013). Furthermore, the effects of USP13 were observed on the type II IFN signalling pathway as USP13 knockdown reduced GAS-luc activity while USP13 overexpression augmented the reporter activity of IFNγ-mediated GAS-luc. In this chapter, the role of USP7 on the type II IFN pathway was examined and indicated that USP7 activity is specific to the type I IFN pathway (Figure 5-7).

Given that USP7 knockdown downregulates ISRE activation, the effect of USP7 knockdown on transcription of ISRE-dependent ISGs was investigated. USP7 knockdown was associated with reduced transactivation of IFIT1 (83.2%), OAS1
(91.7%) and ISG15 (57.8%) (Figure 5-8). These findings suggest that basal USP7 might be important for ISG expression and establishment of an IFN-dependent antiviral state.

RTA-associated ubiquitin ligase (RAUL) is regarded as a negative regulator of IFN signalling as it inhibits type I IFN production by targeting both IRF7 and IRF3 for K48-linked proteasome degradation (Yu & Hayward, 2010). USP7 reportedly binds and stabilises RAUL, hence we examined the role of USP7 in the IFN production pathway. USP7 knockdown did not significantly inhibit the poly (I:C) driven activation of the IFNβ promoter (Figure 5-9). This result confirms previous belief that USP7 does not play a positive role in the IFN production pathway (Yu & Hayward, 2010). Furthermore, this finding is similar to the activity of another deubiquitinase, USP2b, which negatively regulates IFNβ production by targeting TANK-binding kinase (TBK1) (Zhang et al., 2014). USP2b mediates K63-linked deubiquitination of TBK1 thereby preventing its activation, TBK1 is essential for IRF3 phosphorylation and activation hence it plays a positive role in IFN-β production pathway (Zhang et al., 2014).

A study of human and mouse p53 genes revealed the presence of ISRE sequences within their promoter or first-intron regions. Hence p53 is considered a downstream target of the IFN pathway (Takaoka et al., 2003). Additionally, p53 enhances the expression of ISGs by activating the transcription of IRF9 thus contributing to innate immunity through IFN-dependent antiviral activity (Munoz-Fontela et al., 2008). USP7 is one of the first identified and best studied DUBs and a number of its functions are linked to its deubiquitinating activity in the p53 pathway (Cummins et al., 2004b; Holowaty et al., 2003; Wrigley et al., 2011b).

To ascertain that USP7-mediated positive regulation of the IFN signalling pathway was independent of p53, siRNA knockdown of USP7 was performed in the P53-deficient cell line SaoS2. The cells were confirmed to be p53 deficient by immunoblotting in comparison with lysates from HeLa cell lines (Figure 5-10). ISRE
activation was reduced by 43.7% and transcription of ISG15, OAS1 and IFIT1 were
downregulated by 38.8%, 38.9% and 76.6%, respectively when quantified by qPCR.
The level of ISRE downregulation observed in the SaoS2 cells was less compared
with HEK293 cells (43.7% vs 78.6% respectively). This finding could be a result of
the lower transfection efficiency achieved in SaoS2. Unlike HEK293, SaoS2 cells are
difficult to transfect partly due to the transfection toxicity related cell death. As the
downregulation of transcription of the ISGs also varied from 38.9%-76.6% in Saos2
compared with 45%-90.9% in HEK 293, there is the possibility that USP7-mediated
regulation of IFN response might be augmented by P53 activity.

The overexpression of USP7 augmented the reporter activity of IFN-α–triggered
ISRE-Luc in a dose dependent manner (Figure 5-13). This effect is similar to that
observed with USP13 and the type I IFN pathway and further confirms the positive
role of USP7 on IFN signalling (Yeh et al., 2013). Given the ability of overexpressed
USP7 to augment ISRE activity, we investigated the ability of a catalytic mutant of
USP7 to augment ISRE activity. The catalytic domain of USP7 are responsible for
deubiquitination activities and consists of highly conserved Cys (216-234), Asp (I),
which spans aa 285-304, His (448-464), and Asn/Asp (II) domains which lie between
residues 475-489. The USP7 catalytic mutant, with cysteine 223 replaced by alanine
is designated C223S (see Figure 5-14 for description). This mutant was expressed
and compared with wild type USP7 and an empty vector control. The augmentation
effect of USP7 on ISRE activation was absent with the catalytic mutant as there was
no significant difference in ISRE activation from endogenously expressed USP7
(empty vector-transfected cells) and the expression of the catalytic mutant (Figure 5-
15). In another study, the full length USP7 showed enhanced catalytic activity relative
to the catalytic domain suggesting that that the catalytic domain is inert and requires
the regulatory activity of a region of the USP7 protein outside the catalytic domain
(Wrigley et al., 2011a). Taken together, this present work suggests that USP7
augments ISRE activation by deubiquitinating key regulatory factors in the JAK STAT pathway.

In summary, these results obtained by investigating the mechanism of KSHV modulation of interferon responses identifies a novel role for USP7 as a positive regulator of the type I IFN pathway. USP7 knockdown resulted in inhibition of ISRE activation and consequent downregulation of ISGs expression at mRNA level. This effect is sustained in the absence of P53. A catalytic site of USP7 is required for its ISRE-augmentation effect and might suggest that USP7 probably deubiquitinates some of the key components of the IFN signalling pathway. The next chapter will focus on achieving a better understanding of the mechanism behind USP7-mediated regulation of JAK STAT signalling as well as investigating the rationale behind vIHF2 and vIHF4 interaction with USP7 as earlier confirmed in this chapter.
CHAPTER 6

INVESTIGATING THE ROLE OF USP7 AND ITS INTERACTION WITH KSHV vIRF2 AND vIRF4 PROTEINS
6. CHAPTER 6

6.1. Introduction to chapter 6

One of the aims of chapter 5 was to determine whether USP7 played a role in the type I interferon signalling pathway. It was found that USP7 knockdown inhibits ISRE activation, while its overexpression augmented ISRE activation. Concomitantly, USP7 knockdown correlated with a reduction in the relative expression of selected ISGs. Hence, we proposed that USP7 is a positive regulator of IFN signalling and possibly acts by targeting and stabilising some components of the JAK STAT pathway.

The JAK STAT pathway is regulated by a number of posttranslational modifications. Phosphorylation is the most studied and easily detectable of these, however, ubiquitination has recently gained more prominence (Hunter, 2007). A number of deubiquitinases, notably USP13 and USP2a, have been found to recently regulate the IFN signalling pathways by stabilising effector molecules; STAT1 and pSTAT1 factors respectively (Ren et al., 2016; Yeh et al., 2013). In this chapter we sought to identify the specific target of USP7 in the IFN signalling pathway.

Kraus et al. have shown that constitutively active IRF9-S2C and IRF9-S1C constructs recapitulate the antiviral state in an IFN-independent manner (Kraus et al., 2003a). The IRF9 in the hybrid construct ensures nuclear translocation and ensures transcriptional activation of ISRE by targeting endogenous ISGF3 target loci. We hereby took advantage of these constructs to provide additional evidence in the identification of a probable target for USP7.

Experiments in this chapter aim to gain knowledge on the mechanism behind USP7-mediated positive regulation of IFN signalling. Additionally, experiments with IFN sensitive viruses were performed to assess the biological significance of the reduced IFN state induced by USP7 knockdown.
Given that USP7 binds vIRF2 and vIRF4 (chapter 5), a number of experiments in the present chapter were performed to better understand the rationale behind vIRF2 and vIRF4 interaction with USP7.

In summary the objectives of this chapter are to:

1) Identify the target of USP7 in the type I IFN signalling pathway.

2) Examine the anti-IFN effect of USP7 knockdown in a biological context by assessing the effect on replication of the IFN sensitive Chandipura virus.

3) Determine whether USP7 knockdown alters vIRF2 or vIRF4 levels.
6.2. The localisation of USP7 following IFNα stimulation.

IFN induces phosphorylation of STAT1 and STAT2, both of which dimerise in the cytoplasm then bind IRF9 to form the ISGF3 complex (Blaszczyk et al., 2016). The ISGF3 complex translocates into the nucleus to activate ISRE-containing promoters, thus inducing an antiviral state (Hoffmann et al., 2015). USP7 predominantly localises to the nucleus hence, to determine the possible target of USP7 in IFN signalling, IFA was carried out to assess the localisation of USP7 over a period of time following IFNα treatment. HEK 293T cells were stimulated with human rIFNαB2 (300IU/mL) over a timed interval that ranged from 0.5 to 12 hours. Cells were fixed, permeabilised and stained for endogenous USP7 protein (further detail of antibody in Table 2-5 and 2-6). Confocal microscopy was performed to analyse the localisation of USP7. The 0 hour image was obtained prior to IFN stimulation to confirm the original location of USP7. Figure 6-1A shows that IFNα treatment does not trigger nuclear exit of USP7 thus suggesting that this protein acts within the nucleus to regulate the JAK STAT pathway.
Figure 6-1A: Immunofluorescence staining of the USP7 protein.

The HEK 293T cells were plated on 16mm coverslips at the bottom of a 12 well plate. Following treatment with 300IU/mL IFNαB2 over a period of 0.5-12 hours, cells were fixed in 4% parafomaldehyde and permeabilised with 0.1% Triton X (10 minutes). Staining was achieved using USP7 antibody (Bethyl Lab A300-033A) and Goat anti-Rabbit IgG (H+L) secondary Antibody (Alexa Fluor® 568 conjugate) which detected endogenous USP7 protein. Nuclear staining was achieved with DAPI. Results were visualised by confocal microscopy. Images were obtained with a Zeiss LSM 510 META confocal microscope using 60X oil immersion objective lens. Images were scaled to 100µm. The experiment was repeated twice and a representative image is shown.
Figure 6-2B: Controls for the Immunofluorescence staining.

The HEK 293T cells were plated on 16mm coverslips at the bottom of a 12 well plate. The cells were treated with 300IU/mL of IFNαB2, for a period of 20 minutes to facilitate translocation of STAT 1 from the cytoplasm to the nucleus following which they were fixed in 4% paraformaldehyde and permeabilised with 0.1% Triton X (10 minutes). Staining was achieved using STAT1 antibody (Santa cruz, sc-346) and Goat anti-Rabbit IgG (H+L) secondary antibody (Alexa Fluor® 568 conjugate) which detected endogenous STAT1 protein. Staining was also done with the Mouse IgG isotype control (R&D, MAB002) to confirm specificity of the primary antibody. Nuclear staining was achieved with DAPI. Results were visualised by confocal microscopy and images were obtained with a Zeiss LSM 510 META confocal microscope using 60X oil immersion objective lens. Images were scaled to 10µm. The experiment was repeated twice and a representative image is shown.
6.3. Examining the effect of USP7 knockdown on ISGF3 components

Having confirmed that USP7 remains in the nucleus following IFNα stimulation, we sought to identify its possible target(s) by focusing on the ISGF3 components. Following IFNα-induced activation of the JAK STAT pathway, the ISGF3 complex is formed from IRF9, phosphorylated STAT1 and STAT2 (Hoffmann et al., 2015). The complex translocates into the nucleus and binds to ISRE sequences of ISGs, with their resultant activation (Hoffmann et al., 2015). Since USP7 knockdown inhibits this pathway, it was hypothesised that firstly, USP7 knockdown reduces levels of functional ISGF3 available to bind ISRE sequences or secondly, inhibits IFNα-induced translocation of the ISGF3 components.

To test the first hypothesis that inhibition of ISRE-containing promoters following USP7 knockdown is due to modulation of levels of ISGF3 components, the relative levels of these components were measured via western blotting following USP7 knockdown and compared with cells treated with the non-targeting control. As phosphorylation of STAT1 and STAT2 is required for their translocation, immunoblotting for levels of phosphorylated STAT1 and STAT2 was also performed.

HEK 293 cells were reverse transfected with USP7 siRNA or the NTC siRNA to a total concentration of 30µM for 48 hours after which the cells were treated with rIFNαB2 to activate the JAK STAT signalling pathway. Lysates were analysed for the relative levels of IRF9 (Figure 6-2A), STAT1, pSTAT1 (Figure 6-2B) and STAT2, pSTAT2 (Figure 6-2C). The levels of IRF9, STAT1, pSTAT1 and STAT2, pSTAT2 were similar among all samples irrespective of USP7 knockdown levels. USP7 knockdown was confirmed by immunoblotting for USP7 in all conditions. The conclusion from this experiment was that USP7 knockdown does not reduce the stability of IRF9 or the levels of total and phosphorylated STAT1 and STAT2.
Following phosphorylation, STAT1 and STAT2 bind IRF9 to form the ISGF3 complex that translocates to the nucleus (Blaszczyk et al., 2016). To ascertain whether nuclear translocation of the ISGF3 components is inhibited by USP7 knockdown, HEK 293 cells were reverse transfected with USP7 siRNA or the NTC at 30µM for 48 hours following which they were stimulated with rIFNαB2 (1000IU/mL) for 20 minutes. A higher dose of IFN over a shorter period is required for translocation assays (Ren et al., 2016). The cells were then fixed, permeabilised and stained with IRF9, STAT1 and STAT2 antibodies to detect endogenous IRF9, STAT1 and STAT2 respectively (details of antibody used can be found in section 2.7). Confocal microscopy images showed that USP7 knockdown did not alter the subcellular distribution of IRF9, STAT1 and STAT2 before or after USP7 knockdown (Figure 6-3).

The positive control for this experiment was carried out by staining for unstimulated cytoplasmic STAT1 (Figure 6-1B). Specificity of the staining was verified by using mouse IgG isotype control as substitute for the primary antibody (Figure 6-1B). These controls confirmed the efficiency of the IFNα used as well as the specificity of the primary antibody respectively. Altogether these experiments show that USP7 knockdown does not interfere with the stability of the ISGF3 complex neither does it inhibit IFNα-induced nuclear translocation of all 3 ISGF3 components.
HEK 293 cell lines were reverse transfected with USP7 siRNA or the NTC siRNA to a total concentration of 30µM for 48 hours after which the cells were stimulated with IFNαβ2 (300IU/mL), or left un-stimulated, before being harvested 12 hours later. Lysates (20µg) were analysed by western blot to detect components of the ISGF3 complex (IRF9, STAT1, pSTAT1 and STAT2, pSTAT2). For primary antibody concentrations and conditions see Table 2.7. USP7 was probed to confirm efficient knockdown. Probing for β-actin indicated equal loading and the results show no difference in the levels of IRF9 proteins (A), STAT1, pSTAT1 (B) and STAT2, pSTAT2 (C). This experiment was repeated twice and representative blots are shown here.
Figure 6-4: USP7 knockdown does not inhibit IFNα-induced translocation of the ISGF3 components.
HEK 293T cells seeded on 16mm cover slips were reverse transfected with USP7 siRNA or the NTC at 30µM for 48 hours following which they were stimulated with rIFNαB2 (500IU/mL) for 20 minutes. The cells were then fixed, permeabilised and stained with IRF9, STAT1 and STAT2 antibodies to detect endogenous IRF9, STAT1 and STAT2 respectively. Confocal microscopy images showed that all 3 ISGF3 components translocate to the nucleus in USP7 knockdown cells. All images are scaled to 10μm. The experiment was performed twice and representative data is shown.
6.4. USP7 knockdown suppresses induction of ISRE-luciferase activity by IRF9-S2C, a constitutively active ISGF3 mimic

To test the hypothesis that USP7 targets the ISGF3 complex formation, the ability of USP7 to inhibit a constitutively active ISGF3 mimic, IRF9-S2C was assessed. IRF9-S2C is a chimeric protein made of IRF9 fused with the transactivating domain (TAD) of STAT2 (amino acids 747-851) (Poat et al., 2010). IRF9 is the DNA binding component of ISGF3 and is able to translocate to the nucleus in an IFN independent manner thus directing the IRF9-S2C construct to the ISRE promoter sites of ISGs (Horvath et al., 1996; Martinez-Moczygemba et al., 1997).

The intracellular expression of the fusion protein sufficiently drives the activation of ISRE-dependent genes independent of type I IFN stimulation. This is because the IRF9 confers nuclear localisation and DNA binding to the ISRE while the TAD of STAT2 allows transcriptional activation of the associated genes (Kraus et al., 2003a).

HEK 293 cells were reverse transfected with USP7 siRNA or the NTC siRNA to a total concentration of 30µM siRNA for 48 hours after which the cells were transfected with the IRF9-S2C plasmid along with the ISRE-luciferase reporter gene (pISRE-luc) and Renilla plasmid (pRLSV40). The result is presented as fold ISRE activation with the firefly-luciferase normalised to the Renilla-luciferase, then further normalised against the empty vector. Co-expression of the IRF9-S2C fusion protein with the reporter plasmids showed >400-fold activation of ISRE in the NTC while significant suppression of this activation was observed in the presence of USP7 knockdown. This result suggests that USP7 acts downstream of the JAK-STAT signalling pathway and possibly targets the ISGF3 complex.
Figure 6-5: USP7 knockdown inhibits IRF9-S2C-driven type I IFN signalling.

HEK 293 cells were transfected with USP7 siRNA or the NTC siRNA to a total concentration of 30µM for 48 hours after which the cells were transfected with plasmids expressing IRF9-S2C (6-3A) or the empty vector pCDNA3.1 alongside the reporter plasmids pISRE-luc (250ng) or pRLSV40 (1ng) for 24 hours. Cells were harvested and DLA performed (6-3B). The pRLSV40 plasmid was added as an internal control to which firefly luciferase levels were normalised. The data represent the mean ± the SEM of the three independent experiments. ****=p<0.0001. USP7 knockdown significantly downregulates ISRE activation.
6.5. **USP7 does not require IR9-S1C to exert its effect on the IFN signalling pathway**

In the previous section, IR9-S2C recapitulated the IFN response as shown in Figure 6-4A and our data also suggests that USP7 possibly targets the IRF9-S2C chimera for its positive effect on JAK STAT signalling. As we had earlier investigated the role of USP7 in the STAT1-driven type II IFN pathway, we sought to firmly establish or rule out any contributory effect of STAT1. We therefore took advantage of the IRF9-S1C construct (Figure 6-5A) consisting of the full length IRF9 and the TAD of STAT1, to ascertain the importance of STAT1 for the USP7 effect.

HEK 293 cells were transfected with USP7 siRNA or the NTC siRNA to a total concentration of 30µM for 48 hours after which the cells were transfected with plasmids expressing IRF9-S1C (Figure 6-5A) or the empty vector pCDNA3.1 alongside the reporter plasmids pISRE-luc (250ng) or pRLSV40 (1ng) for 24 hours. The cells were harvested and DLA performed. The pRLSV40 plasmid was added as an internal control to which firefly luciferase levels were normalised. The result is presented as fold induction following normalisation of the firefly-luciferase activity to the *Renilla*-luciferase activity then further normalisation against the empty vector. These data suggest that STAT1 is not required by USP7 to exert its effect on the IFN signalling pathway, consistent with results in Figure 5-7.
HEK 293 cells were transfected with USP7 siRNA or the NTC siRNA to a total concentration of 30µM for 48 hours after which the cells were transfected with plasmids expressing IRF9-S1C or the empty vector, pCDNA3.1 alongside the reporter plasmids pISRE-luc (250ng) or pRLSV40 (1ng) for 24 hours. Cells were harvested and DLA performed. The pRLSV40 plasmid was added as an internal control to which firefly luciferase levels were normalised. The results are shown as fold ISRE activity. The data represent the mean ± the SEM of the three independent experiments. USP7 knockdown had no significant effect on STAT1-driven ISRE activation. The ‘ns’ means data showed no significant difference.

**Figure 6-6: USP7 does not require IR9-S1C for type I IFN signalling**
6.6. USP7 associates with STAT2 but not with STAT1 or IRF9.

Having shown in section 6.4 that USP7 is required for IRF9-S2C-driven ISRE activation. We sought to understand further how USP7 regulates the IFN signalling pathway at such a late stage. Therefore, the possibility of USP7 associating with components of the ISGF3 complex was assessed.

HEK 293 cells were co-transfected with pEGFP-USP7 and one of FLAG tagged IRF9, STAT1, or STAT2 for 24 hours, then treated with rIFNαB2 (1000IU/mL) for 30 minutes. Lysates from these cells were then subjected to immunoprecipitation with GFP-Trap® beads (ChromoTek, Germany) to detect the ISGF3 components that bind USP7. The GFP-Trap® is a high quality GFP binding protein coupled to magnetic agarose beads, for biochemical analysis of GFP fusion proteins. The immunoprecipitated proteins were then analysed by western blot with anti-USP7 or anti-Flag for detecting IRF9, STAT1 and STAT2. The whole cell lysates were also probed with anti-Flag and anti-USP7 antibodies. Figure 6-5 shows that USP7 binds to STAT2 but not IRF9 and STAT1.
Figure 6-7: USP7 interacts with STAT2 but not IRF9 or STAT1

HEK 293 cells co-transfected with plasmids expressing GFP tagged USP7 and Flag tagged IRF9, STAT1 and STAT2 for 24 hours and then treated with or without rIFNαB2 (1000IU/mL) for 30 minutes. GFP-Trap® beads (ChromoTek) were used to IP binding partners of GFP-USP7. The interaction of USP7 was analysed by western blotting of the immunoprecipitate and the whole cell lysates with anti-USP7 to detect USP7 and anti-Flag to detect IRF9, STAT1 and STAT2. The experiment was repeated and a representative blot is shown. WCL= whole cell lysates, IB=immunoblotting and IP= immunoprecipitation.
6.7. Examining the effect of USP7 knockdown on Chandipura titre

Since USP7 knockdown inhibits ISRE promoter activation (chapter 5), thus inhibiting induction of the antiviral state, the hypothesis that USP7 knockdown rescues IFN-sensitive virus replication from the effects of IFN was assessed to determine the biological significance of the role of USP7 in the IFN response. The experiment was performed with Chandipura virus.

6.7.1 Chandipura

Chandipura is a positive strand RNA virus and belongs to the *Rhabdoviridae* family, a diverse group of viruses that cause encephalitic illness in humans (Ramamurthy *et al.*, 2013). The virus was first identified among two patients from Chandipura village in Maharashtra state, India hence the origin of its name (Bhatt & Rodrigues, 1967).

6.7.2. Using Chandipura to examine the biological effect of proteins which inhibit JAK-STAT signalling

The use of Chandipura virus replication as a measure of IFN antiviral activity has been previously reported by other groups (Easton *et al.*, 2011; Rao *et al.*, 2004). Easton and colleagues discovered a novel approach to vaccine protection based on the use of defective interfering (DI) viruses. DI viruses are deletion mutants that are deficient in replication, and arise spontaneously from the genome of infectious viruses but only multiply in co-infection with a genetically compatible infectious virus (Easton *et al.*, 2011). Additionally, these authors reported that protection against influenza A virus also conferred protection *in vivo* against genetically unrelated pneumovirus. This level of protection was achieved through activation of the type I interferon pathway in mice. The readout was a reduction in Chandipura virus replication in the presence of IFNα (Marriott & Dimmock, 2010).

Chandipura virus can be considered IFN sensitive virus and Chandipura virus titre can therefore provide a biological read out of the effectiveness of a protein that is
believed to inhibit JAK STAT signalling. This rationale is based on the fact that if a protein inhibits JAK STAT signalling, it will downregulate the antiviral IFN response, consequently resulting in an increase in the Chandipura virus titre.

6.7.3. USP7 knockdown rescues Chandipura virus titre from the effects of IFN

To test the hypothesis that the inhibitory effect of USP7 knockdown on JAK STAT signalling can rescue Chandipura virus following rIFNαB2 treatment, plaque assays were performed.

HEK 293 cells were reverse transfected with USP7 siRNA for 48 hours and infected with Chandipura virus at a MOI of 0.1 (see section 2.13.1 for details). The cells were then treated with IFNα (300IU/mL) for 12 hours after which supernatant was harvested a further 24 hours later for quantification of the viral titre by plaque assay (see section 2.13.2 for plaque assay details and section 2.13.3 for how the plaques were counted).

Pictures of a plaque assay from one representative experiment are shown in Figure 6-7A. The number of plaques in the USP7 knockdown samples can be seen to be higher than the number of plaques seen in the presence of NTC siRNA. As expected, in USP7 knockdown states, there was an increased Chandipura virus titre that gave an average of 7.7 x 10^7 PFU/mL, compared with the NTC, which yielded a titre of 1.7 x 10^6 PFU/mL (Figure 6-7B). This result confirms that USP7 knockdown suppresses the IFN-induced antiviral state, thus verifying the hypothesis from chapter 5 that USP7 positively regulates IFN signalling.

The effect of USP7 knockdown on induction of an antiviral state was investigated using another IFN sensitive virus, Encephalomyocarditis virus (EMCV). Plaque assay methods are same as described for Chandipura virus in section 2.15. EMCV titres were determined following USP7 knockdown in HEK 293 infected with EMCV at a MOI of 0.1. The cells were then treated with IFNα (300IU/mL) for 12 hours after which
supernatant was harvested a further 24 hours later for quantification of the viral titre by plaque assay. Supernatants were titrated for plaque quantification in L929 cell lines. There was an increased EMCV titre which gave an average of $2.1 \times 10^7$ PFU/mL, compared with the NTC which yielded a titre of $13.7 \times 10^5$ PFU/mL. This data was found to be consistent with the Chandipura virus assay results as EMCV titres were increased by over 2-Log fold during USP7 knockdown thus further validating the Chandipura virus bioassay (Figure 6-7C),
Figure 6-8A and B: USP7 knockdown rescues Chandipura virus replication from the IFNα pathway.

HEK 293 cells were reverse transfected with USP7 siRNA or the NTC siRNA for 48 hours then infected with Chandipura virus (MOI = 0.1). The infected cells were pretreated with 300IU/mL rIFNαB2 for 12 hours then incubated for a further 24 hours. The culture supernatant was collected and virus titres determined by limiting dilution plaque assay on Vero cells. After 24 hours of incubation, plaques were identified by crystal violet staining and counted (A). Data are presented as the mean Chandipura virus titre +/- SEM in three independent experiments (B). Chandipura virus titre was statistically significant (**p <0.001, Student’s t-test) between the USP7 knockdown and the NTC.
Figure 6-7C: USP7 knockdown rescues EMCV replication from the IFNα pathway.

HEK 293 cells were reverse transfected with USP7 siRNA or the NTC siRNA for 48 hours then infected with EMCV (MOI = 0.1). The infected cells were pre-treated with 300IU/mL rIFNαB2 for 12 hours then incubated for a further 24 hours. The culture supernatant was collected and virus titres determined by limiting dilution plaque assay on L929 cells. After 24 hours of incubation, plaques were identified by crystal violet staining and counted. Data are presented as the mean EMCV titre +/- SEM in three independent experiments. EMCV titre was statistically significant (**p <0.001, Student’s t-test) between the USP7 knockdown and the NTC.
6.8. The co-localisation of USP7 with vIRF2 and vIRF4.

As we have confirmed by immunoprecipitation assays in chapter 5, USP7 binds to both vIRF2 and vIRF4. With this result in mind, we examined the localisation of vIRF2 and vIRF4 in the presence of USP7 by indirect immunofluorescence microscopy (Figure 6-8). All three proteins are known to independently localise predominantly to the nucleus. Hence, this experiment was performed to determine if USP7 induces a change in location for both viral proteins, or vice versa.

IFA was performed on HEK 293T cells transfected with plasmids expressing pEGFP-USP7 (1µg) and His-tagged vIRF2 or vIRF4 proteins (pcDNA4/vIRF2HisMax or pcDNA4/vIRF2HisMax) at 1µg each. The pcDNA4/HisMax plasmid was also co-expressed with GFP-USP7 to serve as a control for both viral proteins. 24 hours post transfection, the cells were stimulated or mock treated with 300IU/mL rIFNαB2. After fixation, coverslips were probed with a mixture of anti-USP7 (rabbit polyclonal) and anti-His (mouse monoclonal) antibodies followed by a mixture of anti-mouse and anti-rabbit fluorescence-coupled secondary antibodies (section 2.7).

Both vIRF2 and vIRF4 showed a diffuse distribution throughout the nucleoplasm and were found throughout the nucleus, while USP7 was found throughout most of the nucleus, with the exception of the nucleolus. Overall, there is a considerable overlap between the two viral proteins and USP7. There was no difference between co-localisation in IFN stimulated and non-stimulated states (Figure 6-8). This finding is consistent with the interaction between USP7 and the two viral proteins observed from the SILAC and Co-IP data (Figure 5-1).
Figure 6-8: vIRF2 and vIRF4 co-localise with USP7.

HEK 293T cells were co-transfected pEGFP-USP7 with either pcDNA/HisMax, pcDNAvIRF2/HisMax or pcDNAvIRF4/HisMax plasmids for 24 hours then stimulated with or without IFNαB2 (300IU/mL) for 12 hours. The cells were then fixed, permeabilised and stained for GFP to detect USP7 and anti-His to detect both vIRF2 and vIRF4. Confocal microscopy was used to analyse the localisation of USP7, vIRF2 and vIRF4 in these cells. All images are scaled to 1000μm. The experiment was performed twice and representative images are shown.
6.9. The effect of vIRF2 and vIRF4 expression on USP7 stability

Both vIRF2 and vIRF4 are negative regulators of IFN signalling (chapter 3). As both viral proteins have been confirmed as interacting partners of USP7, a known positive regulator of type I IFN signalling, we hypothesise that vIRF2 and vIRF4 counteract the USP7 positive regulatory effects either by causing its degradation or by inhibiting its function. Viral proteins have reportedly shown ubiquitinating effect on selected positive targets regulating type I IFN pathway (Precious et al., 2005). For example, ICP0 of HSV 1 ubiquitinates USP7, thus targeting it for proteasome-dependent degradation (Boutell et al., 2005a). A balance of the reciprocal activities of USP7 deubiquitination of USP7 and ICP0-mediated degradation of USP7 is found to be important in productive HSV1 infection (Boutell et al., 2005a).

In the present section, the effects of both viral proteins on USP7 stability were examined by transfecting HEK 293 cell lines with increasing dose of pcDNA4vIRF2/HisMax which ranged from 0-5µg over a period of 24 hours. The cells were stimulated with rIFNαB2 (300IU/mL), or left unstimulated, before being harvested 12 hours later. Lysates (20µg) were analysed by western blot to detect endogenous USP7 protein and the His tag was probed to detect vIRF2. The results showed no difference in the levels of USP7 despite increasing levels of vIRF2 proteins (Figure 6-9). Similar results were obtained following transfection of increasing dose of pcDNA4vIRF4/HisMax which ranged from 0-5µg for a period of 24 hours (Figure 6-10).
Figure 6-9: vIRF2 expression does not reduce the stability of USP7.

HEK 293 cell lines were transfected with increasing dose of pcDNA4vIRF2/HisMax which ranged from 0-5µg for a period of 24 hours. The cells were stimulated with rIFNαB2 (300IU/mL), or left un-stimulated, before being harvested 12 hours later. Lysates (20µg) were analysed by western blot to detect endogenous USP7 protein and the His tag was probed to detect vIRF4. For primary antibody concentrations and conditions see Table 2.7. Probing for β-actin indicated equal loading and the results show no difference in the levels of USP7 despite increasing levels of vIRF2 proteins.
Figure 6-10: vIRF4 expression does not reduce the stability of USP7.

HEK 293 cell lines were transfected with increasing dose of pcDNA4vIRF4/HisMax which ranged from 0-5µg for a period of 24 hours. The cells were stimulated with IFNαB2 (300IU/mL), or left un-stimulated, before being harvested 12 hours later. Lysates (20µg) were analysed by western blot to detect endogenous USP7 protein and the His tag was probed to detect vIRF4. For primary antibody concentrations and conditions see Table 2.7. Probing for β-actin indicated equal loading and the results show no difference in the levels of USP7 despite increasing levels of vIRF4 proteins.
6.10. vIRF2 and vIRF4 expression inhibits USP7 augmentation effect

Chapter 3 had shown that both vIRF2 and vIRF4 are negative regulators of IFN signalling, while chapter 5 had confirmed the SILAC data that identified USP7 as an interacting partner of both viral proteins. The reason behind this interaction is still largely unknown. USP7 is known to be hijacked by herpesviruses in order to modify the p53 pathway or to subvert antiviral immunity. USP7 interacts with other KSHV proteins including; LANA, vIRF1, and ORF45, for diverse reasons. The LANA-USP7 interaction results in the modulation of latent viral DNA replication as increased KSHV replication was observed with LANA mutants that lack the USP7 binding site. vIRF1 binds to USP7 thus inhibiting its deubiquitinating effect on p53 (Jäger et al., 2012). USP7 interaction with KSHV ORF45 abolished productive KSHV infection through another interacting partner of both proteins, ORF33 (Gillen et al., 2015). Presently the impact of USP7 interaction with vIRF2 and vIRF4 on IFN signalling is unknown.

The present section sought to understand the functional significance of the interaction between vIRF2 and vIRF4. USP7 was overexpressed in HEK 293 cell lines for 24 hours alongside with reporter plasmids. Alternatively, USP7 was co-expressed with either vIRF2 or vIRF4 along with the reporter plasmids. Controls for this experiment included co-transfection of the reporters with either the EV for the USP7 or mock transfected cells. After 24 hours, cells were stimulated with rIFNαB2 (300IU/mL), or left unstimulated for a further 12 hours then harvested and subjected to DLA.

The results show IFNα-induced activation of the ISRE promoter activity when the reporter plasmids or the EV plasmids were transfected (columns 1 and 3 of Figure 6-11A and B). Both vIRF2 and vIRF4 significantly downregulated ISRE (P value<0.001, P value<0.01 respectively) as shown in columns 5 of Figure 6-11A and B. This finding is in keeping with the previous observation from chapter 3. Conversely, ISRE activation was significantly augmented (P values <0.0001) in the presence of USP7 overexpression (columns 7 of Figure 6-11A and B). This USP7-induced augmentation
supports the observation in Figure 5-13. The augmentation observed by USP7 overexpression was also abrogated by 67.3% and 65.2% respectively following co-expression of USP7 with either vIRF2 or vIRF4 (columns 9 of Figure 6-11A and B).

There was no significant difference (P value, 0.1090) between vIRF2 mediated suppression and the suppression observed when USP7 was overexpressed in the presence of vIRF2 (columns E and I). This suggests that USP7 augmentation does not significantly rescue vIRF2 mediated suppression of ISRE activation. This observation was similarly to the activity of vIRF4 in Figure 6-11B, where the P value was 0.2650.
Figure 6-11: vIRF2 and vIRF4 expression inhibits USP7 augmentation effect.

HEK 293 cell lines were co-transfected for 24 hours with reporter plasmids (pISRE-luc and pRLSV40) alongside, 50 ng EV USP7 (column 3), 1 µg of pcDNA4/vIRF2/HisMax (column 5A) or 1 µg of pcDNA4/vIRF4/HisMax (column 5B). In columns 7A and B, 50ng of USP7 was over expressed while in columns 9A and B, 50 ng of USP7 plasmids were overexpressed alongside 1 µg pcDNA4vIRF2/HisMax or 1 µg pcDNA4vIRF4/HisMax respectively. The cells were then stimulated with rIFNαB2 (300IU/mL), or left un-stimulated for a further 12 hours later then harvested and subjected to DLA. Column 1 had reporter plasmids alone. The results show an induction of ISRE activity with reporter plasmids and EV alone while vIRF2 and vIRF4 downregulated ISRE activation. ISRE activity was augmented by USP7 over expression but downregulated by vIRF2 (67.3%) and vIRF4 (65.2%) expression. **P<0.01, ***P<0.001, ****P<0.0001.
6.11. viRF2 and viRF4 do not require P53 for ISRE downregulation

Both viRF2 and viRF4 downregulate the type I IFN signalling (chapter 3). The interaction of viRF2 and viRF4 with USP7 has been confirmed in chapter 5. Given that we had also shown that P53 is not required for USP7-mediated positive regulation of the IFN signalling pathway, we proceeded further in this section, to examine the importance of P53 in viRF2- and viRF4-mediated negative regulation of IFN signalling. viRF4, but not viRF2, is known to bind P53 (Lee et al., 2009b), however the impact of viRF4-P53 binding on IFN signalling has not yet been reported.

Saos2 cell lines known to be deficient for P53, were transfected with either pcDNA/HisMax, pcDNAviRF2/HisMax or pcDNAviRF4/HisMax plasmids for 24 hours then stimulated or left unstimulated with rIFNaB2 (300IU/mL) for 12 hours after which the cells were harvested and DLA performed. The pRLSV40 plasmid was added as an internal control to which firefly luciferase levels were normalised. The results are shown as fold ISRE activity with firefly luciferase activity normalised to Renilla luciferase control and further normalised against the presence or absence of IFN. The data represent the mean ± the SEM of the three independent experiments. Both viRF2 and viRF4 downregulated ISRE activity significantly (**=p<0.001) in the absence of P53 (Figure 6-12). In chapter 3, we had observed that viRF2 and viRF4 expression from the stable cells downregulate ISRE activation by 74.1 and 78.3% respectively. However, in the absence of P53, the data in Figure 6-12 show that viRF2 and viRF4 downregulate ISRE activation by 58.5% and 39.5% respectively. Interestingly, USP7 knockdown as shown in P53-deficient cell lines also showed a significant downregulation of ISRE activation (Figure 5-11).
Figure 6-12: vIRF2 and vIRF4 do not require p53 to exert their effect on the IFN signalling pathway.

Saos2 cells were transfected with either pcDNA/HisMax (EV), pcDNAvIRF2/HisMax or pcDNAvIRF4/HisMax plasmids for 24 hours then stimulated or left unstimulated, with rIFNαB2 (300IU/mL) for 12 hours after which the cells were harvested and DLA performed. The pRLSV40 plasmid was added as an internal control to which firefly luciferase levels were normalised. The results are shown as fold ISRE activity with firefly activity normalised to Renilla luciferase control and further normalised against the presence or absence of IFN. The data represent the mean ± the SEM of the three independent experiments ***=p<0.001.
6.12. Examining the effect of vIRF2 truncated mutants on ISRE-containing promoter activity

To identify the region of vIRF2 responsible for the inhibition of the ISRE-containing promoter, vIRF2 truncated mutants were tested in the DLA. The N-terminal and C-terminal regions of the vIRF2 gene were cloned into the pcDNA4TO-HA (Figure 6-13A). The details of the cloning are found in section 2.12. Evidence of successful cloning was confirmed by restriction enzyme digests to determine the correct insert. Briefly, a plasmid miniprep was performed on selected colonies from the overnight culture using Qiaprep spin minikit. Restriction enzyme digest was done by incubating 1µg of the purified plasmid DNA with Not1 and XbaI enzymes at 37°C for 2 hours. The digested plasmid was run on a 1% agarose gel, which confirmed that the vector backbone and inserts were of the expected sizes (Figure 6-13B).

6.12.1. The effect of vIRF2 truncated mutants on the ISRE-containing promoter

Chapter 3 showed that vIRF2 significantly downregulates the ISRE promoter. To examine what region of the vIRF2 has an inhibitory effect on the transactivation of the ISRE-containing promoter, DLAs were performed by co-expressing either the full length vIRF2 or the mutants with ISRE-promoter containing plasmid.

To ensure that the mutants localise within the nucleus, IFA was performed on HEK 293T cells co-transfected with vIRF2-FL, the mutants and pEGFP-USP7. Figure 6-14 showed that the vIRF2-FL and the 2 mutants co-localise with USP7 within the nucleus.

HEK 293 were co-transfected with the full length or the different vIRF2 mutants (N-terminal, C-terminal) alongside reporter plasmids, pISRE-luc (250ng) or pRLSV40 (1ng) for 24 hours. Cells were stimulated, or left unstimulated with rIFNαB2 for 12 hours, then harvested and DLA performed. The pRLSV40 plasmid was added as an internal control to which firefly luciferase levels were normalised. Fold ISRE activation
is shown after normalising IFNα-stimulated against the unstimulated. The full length vIRF2 protein inhibited ISRE activity by 64.5% (Figure 3.14, column 4). Between the two mutants, the N-terminal mutant significantly (p<0.001) inhibited the activity of the ISRE-containing promoter by 73.7% when compared to the empty vector (Figure 6-13, columns 3 and 5). However, the C-terminal had no significant inhibitory effect on ISRE activation (Figure 6-15, row 7). The region in common between the full length and the N-terminal spans from amino acid residues 1-568. This suggests that the N-terminal domain of vIRF2 is required for its inhibitory effect on ISRE activation.
Figure 6-13: The vIRF2 mutant proteins.

Schematic representation of the HA-tagged truncated versions of the N-terminal domain aa (1 to 568) and C-terminal domain of USP7 (aa 569 to 1102) used in this study (A). A miniprep of the plasmids was resolved on a 1% Agarose gel to confirm successful cloning. The bands for vIRF2-Full length (FL), vIRF2-N-terminal (N-TER) and vIRF2-C-terminal (C-TER) are seen at 2052bp, 1080bp and 993bp respectively while the vector can be seen on bands corresponding to 5100bp (B).
Figure 6-14: Immunofluorescence staining of the vIRF2 mutant proteins.

The vIRF2-Full length (FL), vIRF2-N-terminal and vIRF2-C-terminal plasmids were each co-transfected with pEGFP-USP7 into HEK 293T cell lines plated on 16mm coverslips at the bottom of a 12 well plate. The cells were then fixed, permeabilised and stained for GFP to detect USP7 and anti-HA to detect the HA-tagged vIRF2-FL and mutants. Confocal microscopy was used to analyse the localisation of USP7, vIRF2 and mutants in these cells. All images are scaled to 1000 μM. The experiment was performed twice and representative data is shown. vIRF2-FL and the mutants co-localise with USP7 in the nucleus.
HEK 293 cells were co-transfected with each of vIRF2- Full length (FL), vIRF2-N-terminal and vIRF2-C-terminal plasmids alongside the reporter plasmids pISRE-luc (250ng) or pRLSV40 (1ng) for 24 hours. Cells were harvested after a further 12 hours stimulation with or without rIFNαB2 (300IU/mL) and DLA performed. The pRLSV40 plasmid was added as an internal control to which firefly luciferase levels were normalised. The results are shown as firefly activity normalised to Renilla luciferase control. Compared with the EV, THE vIRF2-FL suppressed ISRE activation by 64.5% while the N-terminal showed a suppression of 73.7%. The C-terminal had no significant effect on ISRE activation. The data represent the mean ± the SEM of the three independent experiments. **** P<0.0001 while ns, not significant difference.
6.13. vIRF2 and vIRF4 recapitulate the IRF9-S2C phenotype with ISRE activation

Given that USP7 knockdown inhibited IRF9-S2C induced activation of ISRE activity, the ability of vIRF2 and vIRF4 to inhibit IRF9-S2C-induced activation of JAK STAT signalling was tested in the present section to determine if vIRF2 and vIRF4 possibly act on the same target as USP7, downstream of the JAK STAT pathway. The IRF9-S2C construct has already been described in section 6.4. This would further confirm the possibility of vIRF2 and vIRF4 negative regulation of type I IFN signalling through a similar target as USP7.

EV-NTAP, vIRF2-NTAP and vIRF4-NTAP cell lines were treated with 0.125µg/mL of tetracycline to induce the expression of the proteins, after which they were co-transfected with IRF9-S2C (7ng), pISRE-luc (250ng) and Renilla plasmids, pRLSV40 (1ng), for a period of 24 hours. The cells were harvested for DLA after a further 24 hours (Figure 6-16A). Firefly activity was normalised against Renilla luciferase activity. Both vIRF2 and vIRF4 significantly (**p<0.0001) downregulated IRF9-S2C-driven ISRE activation by 90.7% and 71.7% respectively. This finding suggests that the viral proteins either share the same ISGF3 target as USP7 or act by antagonising USP7 positive effect on the ISGF3 complex.

As described in section 6.4, the same experiment was performed in the stable cell lines by overexpressing the IRF9-S1C construct alongside with pISRE-luc (250ng) and Renilla plasmids, pRLSV40 (1ng) for a period of 24 hours after which the cells were harvested for DLA (Figure 6-14B). Firefly luciferase activity was normalised against Renilla luciferase activity. Both vIRF2 and vIRF4 had no effect on IRF9-S1C driven ISRE activation. This result again suggests that USP7 as well as vIRF2 and vIRF4 possibly act on the same target. These experiments were performed three times and representative figures are shown in Figure 6-16.
Figure 6-9: vIRF2 and vIRF4 suppress IRF9-S2C-driven ISRE activation but not IRF9-S2C driven ISRE-activation.

EV-NTAP, vIRF2-NTAP and vIRF4-NTAP cell lines cells were transfected with plasmids expressing either IRF9-S2C (7ng) or IRF9-S1C (250ng) alongside the reporter plasmids pISRE-luc (250ng) or pRLSV40 (1ng) for 24 hours. Cells were harvested and DLA was performed. The empty vector pCDNA3.1 was also transfected as a control. The results are shown as ISRE activity with the firefly activity normalised to Renilla luciferase control. Both vIRF2 and vIRF4 significantly downregulated IRF9-S2C-driven ISRE activation by 90.7% and 71.7% respectively (A). Neither vIRF2 nor vIRF4 had significant effect on IRF9-S1C-driven ISRE activation (B). The data represent the mean ± the SEM of the three independent experiments ****=p<0.0001 and ns means data is not significant.
Figure 6-17 vIRF2 and vIRF4 targeting of USP7 and proposed deubiquitination of STAT2 by USP7.

A novel positive role has been identified for USP7 in the type I IFN response pathway. USP7 acts within the nucleus by targeting STAT2 to augment ISRE activation (Figure 6-17A), possibly by deubiquitination. Other hypothesised mechanisms of action involve vIRF2 and vIRF4 binding to USP7 to inhibit its association with STAT2. Alternatively, the viral proteins might bind and directly ubiquitinate STAT2 (Figure 6-17B).
6.14. DISCUSSION

This chapter has explored in detail the mechanism behind the interaction of vIRF2 and vIRF4 with USP7 in the context of the type I IFN response. As we have shown that USP7 positively regulates IFN signalling, it is plausible that either vIRF2 or vIRF4 have evolved to target USP7 in order to downregulate type I IFN signalling.

USP7 is a deubiquitinating protein and has been shown in chapter 5 to positively regulate IFN signalling. Ubiquitination of proteins impact significantly on their stability, intracellular localisation, conformation and activity as well as protein-protein interaction. Deubiquitinating enzymes, including USP7, reverse the ubiquitin modification of proteins thereby stabilising the target protein. USP7 plays an important role in virus-mediated ubiquitination pathways (Li et al., 2014a), and is known to target a number of cellular and viral proteins including KSHV vIRF1 and vIRF4. In this chapter, we sought to identify the target of USP7 in the JAK STAT pathway.

The reciprocal activity between HSV1 ICP0 and USP7 in antiviral immunity has been well characterised. ICP0-mediates nuclear to cytoplasmic translocation of USP7 in response to TLR engagement. In the cytoplasm, USP7 interacts with TRAF6 and IKKγ, with consequent inhibition of the TLR-mediated NF-κB and JNK activation (Daubeuf et al., 2009). vIRF1 and vIRF4 interact with USP7 to enhance KSHV survival and replication by deregulating the P53 cell cycle pathway (Chavoshi et al., 2016a; Lee et al., 2011).

To identify the location of USP7 activity in response to IFNα, two approaches were used, firstly the location of USP7 following IFNα treatment was investigated. USP7 knockdown was associated with a reduction in ISRE activity and reduced expression of ISGs, thus suggesting that USP7 acts downstream of the JAK STAT pathway (chapter 5). Interestingly, USP7 remained localised within the nucleus following interferon treatment over a period of 0.5-12 hours thus suggesting that USP7-
mediated regulation of IFN signalling occurs within the nucleus (Figure 6-1). USP7 is unlikely to affect the IFN receptor, or the janus or tyrosine kinases as they are cytoplasmic or membrane bound. We thus hypothesised from Figure 6-1 that USP7 acts within the nucleus by targeting and possibly deubiquitinating one or more of the ISGF3 components.

Latent STAT1 and STAT2 occasionally shuttle between the nucleus and cytoplasm prior to IFN stimulation (Banninger & Reich, 2004a; Meyer et al., 2002). However, tyrosine phosphorylation of STAT dimers is required for its nuclear accumulation (Blaszczyk et al., 2016). IRF9 plays a major role in nuclear export of the ISGF3 complex as it contains a nuclear localization signal (NLS) that confers nuclear translocation capabilities to non-phosphorylated STAT2 (Blaszczyk et al., 2016). In the present thesis, USP7 knockdown did not inhibit nuclear translocation of the ISGF3 components following IFNα stimulation thus suggesting USP7 is not important for nuclear accumulation of IRF9, activated STAT1 and STAT2 (Figure 6-3).

The constitutionally active ISGF3 mimic when expressed in cells, drives expression of ISRE-dependent genes in an IFN independent manner as the IRF9 presence allows nuclear localisation and the DNA binding sequences of IRF9 direct the IRF9-S2C construct to the ISRE, while the TAD of STAT2 confers transcriptional induction ability (Kraus et al., 2003a). USP7 knockdown was associated with a significant inhibition of IRF9-S2C-driven ISRE activation (Figure 6-4). This finding suggests that USP7 acts at the level of the ISGF3 complex and possibly targets and stabilises IRF9 or STAT2. In contrast, there was no significant inhibition of IRF9-S1C-driven ISRE activation following USP7 knockdown thus indicating that USP7 does not require STAT1 nor IRF9 for its augmentation of ISRE activity thus the most likely ISGF3 target for USP7 was STAT2 (Figure 6-5). This finding that STAT1 is not targeted by USP7 is consistent with the lack of significant reduction in GAS activity following USP7 knockdown in the Type II IFN pathway as shown in chapter 5 (Figure 5-7). Our finding
suggests that USP7 shows a specificity for the type I IFN pathway. This observation varies from the effect of another deubiquitinase, USP13, which was found to be important for positive regulation of both the type I and II IFN pathway (Yeh et al., 2013).

Given the ability of USP7 to inhibit IRF9-S2C-driven ISRE-luciferase expression, the association of USP7 with members of the ISGF3 complex was investigated. USP7 associated with STAT2 but not IRF9 or STAT1 (Figure 6-6). The plausible interpretation for this result is that USP7 possibly targets and stabilises STAT2 to protect it from proteasomal degradation. The STAT factors have long half-lives but are regulated by posttranslational modifications such as sumoylation, acetylation, neddylation and ubiquitination (Haspel et al., 1996; Steen & Gamero, 2013). Therefore, USP7 might have evolved to stabilise STAT2 and protect it from proteasome-mediated degradation effects (Figure 6-17A).

To provide a biological readout of the extent of the USP7 knockdown effect, the rescue of the IFN sensitive Chandipura virus titre was investigated. USP7 knockdown rescued Chandipura virus titre following rIFNαB2 treatment (Figure 6-7A). This approach of using IFN-sensitive virus to measure the biologic impact of IFN inhibition has previously been used in our laboratory for experiments showing vIRF2-mediated suppression of the IFN antiviral state. A similar effect was observed against a different IFN sensitive virus, EMCV by Mutocheluh et al., 2011. This result is consistent with the observation that USP7 enhances cellular antiviral ability. Given that the USP7 knockdown-mediated inhibition of the JAK STAT signalling pathway significantly rescues Chandipura virus from the effect of IFNα, it could be hypothesised that USP7 inhibition plays a role in allowing more efficient KSHV infection either during primary infection or lytic reactivation. One way to test this hypothesis would be to knockdown USP7 in permissive cell lines, then infect with KSHV and quantify levels of the virus. This experiment could be performed in stable cell lines latently infected with rKSHV.
which constitutively expressed the GFP protein (VK219). The GFP would allow for quantification of levels of this virus using flow cytometry. If USP7 knockdown increased the levels of KSHV it would provide evidence that USP7 plays a role in increasing KSHV infection. Additionally, the role of USP7 in lytic reactivation of KSHV could also be assessed using the VK219 cell lines reactivated with RTA and sodium butyrate or in Body cavity based lymphoma cell lines (BCBL), reactivated with Phorbol 12-myristate 13-acetate (PMA).

The effect of USP7 knockdown was tested on another IFN sensitive virus, EMCV. EMCV titres were determined following USP7 knockdown in HEK 293 infected with EMCV, supernatants were titrated for plaque quantification in L929 cell lines. This data was found to be consistent with the Chandipura virus assay results. EMCV titres were increased by over 2-Log fold during USP7 knockdown thus further validating the Chandipura virus bioassay (Figure 6-7B).

Both vIRF2 and vIRF4 were shown to co-localise with USP7 compared with the EV control thus further confirming the interaction observed from the SILAC-MS data (Figure 6-7). Chapter 3 had identified both vIRF2 and vIRF4 as negative regulators of IFN signalling while the interaction of both viral proteins with USP7 was confirmed in chapter 5. The next experiments that were performed were to understand the rationale behind USP7 binding to vIRF2 and vIRF4. Boutell et al., 2005, had earlier shown that HSV1 ICP0 targets USP7 for ubiquitination and proteasome-dependent degradation (Boutell et al., 2005a). However, the reciprocal event of USP7-mediated stabilisation of ICP0 is dominant over ICP0-dependent degradation of USP7 during productive HSV-1 infection. In the present study, overexpression of vIRF2 and vIRF4 had no significant effect on the levels of USP7 thus suggesting that vIRF2 and vIRF4 are unlikely to directly ubiquitinate and induce degradation of USP7.

In chapter 5, USP7 overexpression was shown to augment ISRE activation through its catalytic site. This effect was attenuated when USP7 was overexpressed with
either vIRF2 or vIRF4 (Figure 6-11A and B). This observation is not altogether surprising as vIRF2 and vIRF4 bind to USP7, possibly inhibiting it from exerting its positive effect on ISRE activation. Furthermore, there is a possibility that the attenuation is an indirect effect of vIRF2 and vIRF4 on other components of the JAK STAT pathway that are also targeted by USP7. Viruses have been shown to negatively regulate specific components of the JAK STAT pathway. Previously, the V protein of simian virus 5 (SV5) was shown to target STAT1 for proteasome-mediated degradation, while the V protein of human parainfluenza virus type 2 (hPIV2) target STAT2 (Precious et al., 2005).

As we have shown in Figure 6-6, USP7 targets STAT2, there is a possibility that vIRF2 and vIRF4 could also target and ubiquitinate STAT2 thus negating the effect of USP7 on STAT2 (Figure 6-17B). The hypothesis that vIRF2 and vIRF4 could ubiquitinate STAT2 could be explored by performing in vitro ubiquitination using programmed rabbit reticulocyte lysates to demonstrate increased ubiquitination of STAT2 in the presence of vIRF2 and vIRF4. Additionally, further experiments are required to further understand the effect of USP7 on STAT2. In vivo or in vitro ubiquitination studies might be important in this regard.

Both vIRF2 and vIRF4 downregulate ISRE activation independent of p53 (Figure 6-12). To enhance KSHV survival and replication, vIRF4 but not vIRF2, interacts with Mdm2, to reduce p53 levels via proteasomal degradation, thereby abrogating p53-mediated apoptosis (Lee et al., 2009b). Given the positive role p53 plays in IFN signalling (as discussed in chapter 5), it is surprising to find that vIRF2 and vIRF4 do not require p53 for their negative regulation of the JAK STAT pathway. The observation of p53 independence with vIRF2 and vIRF4 is similar to the finding with USP7 in chapter 5 thus suggesting that vIRF2 and vIRF4 possibly share a similar target with USP7 in the JAK STAT pathway.
In the present study, two mutants of vIRF2 were created in order to identify the binding site of vIRF2 to USP7. The N-terminal and the C-terminal of vIRF2 were created and their nuclear localisation was confirmed by IFA. The attempt to confirm the expression of these proteins by western blotting failed despite having cloned these genes into three different expression vectors. The failed expression might be due to toxicity of vIRF2 to cells. A previous study on vIRF2 have found that its constitutive expression is detrimental to cells (Fuld & Blackbourn, un-published data). This finding had also informed the generation of the vIRF2 stable cell lines described in chapter 3, as the stable cell lines allow for inducible expression of this viral protein. Regrettably a Co-IP to assess binding vIRF2 with USP7 could not be done. The synthetic genes were designed by Invitrogen and the final construct had been verified by sequencing and the sequence congruence within the insertion sites was found to be 100%. The genes were cloned into a GFP tagged vector and found to express well on fluorescent microscopy (data not shown). In a reporter gene assay, the N-terminal of vIRF2 significantly suppressed ISRE activation by 73.7% while the full length vIRF2 showed an inhibition of 64.5%. The vIRF2 inhibition observed is similar to previous observations by Mutocheluh et al.2011 thus confirming the functionality of the cloned genes (Mutocheluh et al., 2011). The N-terminal spans the first 568 amino acids and showed the highest ability to inhibit ISRE activation. Previously binding assays have shown that the USP7 TRAF domain binds specifically with a segment in the N-terminal region of vIRF4, specifically aa 153-256 (Lee et al., 2011). More recently vIRF1 of KSHV was also found to interact with the TRAF domain of USP7 through a \textsuperscript{47EGPS}\textsuperscript{50} consensus sequence located in its N-terminal region (aa 1-158) acids (Chavoshi et al., 2016a). The interaction of vIRF1 was found to be important for its deregulation of P53-regulated cell cycle control (Chavoshi et al., 2016a). To the best of our knowledge, the importance of the USP7-vIRF1 and USP7-vIRF4 binding site in downregulation of IFN signalling has not been reported previously.
The present chapter has also shown that vIRF2 and vIRF4 significantly downregulate IRF9-S2C-mediated ISRE activation but had no effect on IFR9-S1C-driven ISRE activation (Figure 6-16). This finding is similar to our observation with USP7 (Figures 6-3 and 6-4). Again this finding supports the possibility of a common target for the vIRFs and USP7.

In summary, the work described in the present chapter was designed to understand the mechanisms behind USP7-mediated regulation of JAK STAT signalling. Analysis of the IFNα-stimulated JAK STAT signalling pathway showed that USP7 does not inhibit the phosphorylation or nuclear translocation of the ISGF3 components. The finding in this chapter that USP7 binds to STAT2 led to the hypothesis that USP7 may exert its augmentation effect on type I IFN signalling by deubiquitinating STAT2. Similar to USP7, both vIRF2 and vIRF4 downregulate the type I IFN response independent of p53 and also inhibit IRF9-S2C-driven. Analysis of USP7 regulation of the type I IFN pathway did not extend beyond the results described above due to time constraints and further experiments will be required to ascertain the mechanism by which USP7 stabilises STAT2 and whether this stabilisation mechanism is being inhibited by vIRF2 and vIRF4.
CHAPTER 7

GENERAL DISCUSSION
7. **CHAPTER 7**

7.1. **Summary of findings in relation to previous studies**

The type I interferon antiviral response is the first line of defence against invading viral pathogens. To establish a productive infection, viruses must first evade the host immune system, hence a number of viruses have evolved diverse strategies to subvert innate immunity. Almost one quarter of the KSHV genes specify either demonstrated or potential immunomodulatory activity (Aresté & Blackbourn, 2009; Rezaee *et al.*, 2006). Two such genes are vIRF2 and vIRF4, encoding proteins with homology to cellular IRFs (Cunningham *et al.*, 2003a).

The study of immune modulatory mechanisms of viruses has provided critical insights into virus host cell interactions. The characterisation of cellular interacting partners of these viral immune regulatory proteins has contributed enormously to these insights. Moreover, this characterisation could provide opportunities for development of effective antiviral therapy as well as identification of rational vaccine targets. Such an understanding can be used to further inform the management of viral diseases such as Kaposi’s sarcoma. The main aim of this thesis was to identify and characterise the cellular partners of the KSHV vIRF2 and vIRF4 proteins, which in turn could help our mechanistic understanding of their action.

The initial work carried out in chapter 3 optimised all experimental tools to be used throughout the thesis. The dose of reporter plasmids as well as duration and length of IFN treatment were optimised and these results formed the biologic readout for subsequent experiments throughout this thesis. The experiments in this chapter also detailed the development and optimisation of quantitative readouts for IFNα treatment. This included the use of conventional and quantitative PCR as well as reporter gene assays. IFNα-induced expression of ISGs by qPCR was confirmed by repetition in two different cell types; HeLa and HEK 293 cell lines (Figures 3-5A and
B). Subsequent experiments focused on characterising the role of KSHV vIRF2 and vIRF4, in the regulation of the JAK STAT pathway.

The JAK STAT pathway is activated by the binding of IFNα/β to the IFNAR with resultant transactivation of ISGs downstream and induction of an antiviral state (Randall & Goodbourn, 2008). Consequently, a number of viruses have evolved counter measures to target and inhibit different effector components of the JAK STAT pathway, thus attenuating the antiviral state. For example, KSHV ORF 10-encoded regulator of IFN function (RIF) protein forms a complex with JAK1, Tyk2 and STAT2 thus preventing the activation of JAK1 and Tyk2 and also facilitating abnormal recruitment of STAT1 and 2 to the IFNAR (Bisson et al., 2009).

In this thesis, we have shown that both KSHV vIRF2 and vIRF4 act downstream of the JAK STAT pathway to inhibit ISRE activation with consequent downregulation of gene expression (Figure 3-10). Our group had previously demonstrated the pleiotropic activity of vIRF2 in inhibiting the IFN response, as vIRF2 expression resulted in the attenuation of IRF9 and STAT1 but not the STAT2 component of the ISGF3 complex thus resulting in the formation of a deficient functional ISGF3 with reduced affinity for ISRE (Mutocheluh et al., 2011). It might be that vIRF2 and vIRF4 indirectly target STAT2 via USP7 and this explains why STAT2 had previously not been proposed as a target for vIRF2 and vIRF4. The negative role of vIRF4 on IFN signaling is yet to be reported and consistent with a previous observation from our lab (Hindle & Blackbourn, unpublished observation). Other DNA viruses also strategically target the ISGF3 complex. For example, the E7 oncoprotein of HPV inhibits IRF9 translocation into the nucleus, thus abrogating IFNα-mediated immune response. Similarly, CMV reduces the expression of IRF9 thus preventing the formation of the ISGF3 complex and inhibiting transcriptional activation of ISRE (Miller et al., 1999).

Analysis of the IFNα-induced expression of multiple ISGs by qPCR demonstrated that vIRF2 and vIRF4 could inhibit the expression of specific ISGs (Figure 3-11). This
confirms earlier findings from our group that studied the effect of vIRF2 on IFNα-induced expression of a larger set of ISGs through microarray analysis of RNA from IFNα-stimulated cells (Mutocheluh & Blackbourn, unpublished observation). The microarray result showed that vIRF2 expression significantly downregulated 73% of the 78 IFNα-upregulated genes (Mutocheluh, M & Blackbourn, DJ, unpublished observation). Other herpesviruses including CMV reportedly inhibit IFNα-stimulated expression of selected ISGs (Miller et al., 1999).

Further studies in chapter 4 suggest that the vIRF4 protein supresses cellular translation. Hence the putative effect of vIRF4 on translation was explored further by microarray which confirmed a novel role for vIRF4 in host translational control. Polysome profiling was performed on vIRF4 expressing cells compared with the EV control in the presence and absence of IFN. In both instances, a similar profile with reduced polysome peaks in the presence of vIRF4 were obtained which suggested that vIRF4 expression reduces translation. Modulation of host translation by viruses is not a novel concept. More recently, Vaccinia virus protein 169 was found to suppress translation initiation (Strnadova et al., 2015). Ectopic expression of protein 169, followed by polysome profiling showed accumulation of 80S subunit of the monosome as well as a reduction in polysome peaks (Strnadova et al., 2015). Altogether, these findings suggest that viral inhibition of host protein synthesis might be an immunoevasive strategy. Several mechanisms by which viruses modulate host translation have been highlighted earlier in Table 1-3.

The role of KSHV in translational control has previously been reported. KSHV targets and alters the subcellular distribution of specific translation factors. For example, KSHV SOX and/or K8.1 interferes with Poly(A)-binding protein (PABP) recruitment and causes its redistribution into the nucleus in KSHV-infected cells (Arias et al., 2009; Covarrubias et al., 2009; Kumar & Glaunsinger, 2010). PABP is an RNA-binding protein that binds to the poly(A) tail of mRNA, thus protecting the tail from
degradation (Miller et al., 1999). A similar redistribution of PABP has been observed with HSV1 viral proteins ICP27 and UL47 in infected cells (Dobrikova et al., 2010b). Notably, both viruses induce host shut-off and, in the case of KSHV, SOX mutants that fail to redistribute PABP to the nucleus do not impair host translation (Covarrubias et al., 2009).

The data in chapter 5 validates the interaction of USP7 with both vIRF2/vIRF4 that we previously identified by SILAC-MS, and further identifies a novel role for USP7 in the type I IFN response. USP7 knockdown selectively downregulates the type I IFN signalling and reduces the antiviral state. Analysis of USP7 regulation of STAT1-mediated GAS promoter activity, a target of type II FIN pathway, was assessed and confirmed the specificity of USP7 for the type I IFN pathway (Figure 5-7). Recently, other deubiquitinases; USP13 and USP2a were reported to positively regulate the type I antiviral response by targeting STAT1 and pY701-STAT1 respectively (Ren et al., 2016; Yeh et al., 2013). USP2a translocates into the nucleus where it inhibits K48-linked degradation of pY701-STAT1, thus enhancing all three classes of IFN-mediated antiviral activity (Ren et al., 2016). USP13 positively regulates both the type I and II IFN pathway by deubiquitinating and stabilising total STAT1 (Yeh et al., 2013).

Overexpression of USP7 in the present study (Figure 5-13) had an additive effect on the type I IFN pathway, a finding that is similar to the positive effect observed following USP13 overexpression (Yeh et al., 2013). The catalytic domain of USP7 is required for its additive effect on the JAK STAT pathway (Figure 5-13). As the catalytic domain of USP7 confers its deubiquitinating effect, it is possible that USP7 deubiquitinates an effector component of the type I IFN pathway.

In the present study, USP7 had no significant effect on the IFNβ promoter and might not be essential for the IFN production pathway (Figure 5-9). This finding validates previous observations that USP7 stabilises the E3 ubiquitin ligase, RAUL, a negative regulator of IFN production which targets IRF3 and IRF7 for proteasomal degradation.
Given that the present study has shown that USP7 positively regulates the IFN response, it could be suggested that USP7 provides an autoregulatory mechanism to maintain equilibrium between IFN production and IFN response, thus preventing ‘over speeding’ of the type I IFN signalling pathway. Conversely, USP7 has been found to be critical for the NF-κB pathway. USP7 deubiquitinates NF-κB, thereby enhancing its transcriptional activation with consequent increased expression of target genes in response to TLR and TNF-receptor activation (Colleran et al., 2013).

P53 is a known positive regulator of IFN-mediated immunity (Munoz-Fontela et al., 2008). Since USP7 stabilises P53 levels, reporter gene assays in P53-deficient Saos2 cells were performed and confirmed USP7-mediated augmentation of IFN signalling was independent of P53 (Figure 5-11). A number of USP7 mediated activities including cell cycle regulation, DNA repair and apoptosis, centre on its ability to bind and stabilise P53 (as already described in detail in section 1.8). It is therefore important to observe that USP7-mediated regulation of IFN signalling was sustained even in the absence of P53, and is therefore independent of this protein.

In chapter 6, the biological significance of USP7 and the importance of its interaction with vIRF2 and vIRF4 upon JAK-STAT signalling was investigated in more detail. A search for a plausible target revealed that USP7 acts within the nucleus and the ISGF3 complex was proposed as a target. Co-immunoprecipitation experiments confirmed the association of USP7 with STAT2, but not STAT1 and IRF9 (Figure 6-6). This finding confirms USP7 specificity for the type I IFN pathway and differs from the activity of USP13 and USP2a, where the targets are STAT1 and pY701STAT (Ren et al., 2016; Yeh et al., 2013). Based on the USP7-STAT2 interaction and the established role of USP7 as a deubiquitinase, it could be hypothesised that USP7 regulates the type I IFN response by targeting STAT2 for deubiquitination. For example, USP7 may stabilise STAT2 levels thus protecting it from proteasomal
degradation. As described in section 6.14, more experiments are necessary to confirm this hypothesis.

Pre-treatment of HEK 293 cells with IFNα results in an antiviral state. However, the knockdown of USP7 inhibited the induction of IFNα-mediated antiviral state thereby increasing the titres of EMCV and Chandipura virus (Figure 6-7). This result is consistent with previous reports from our group (Mutocheluh et al., 2011), which showed that vIRF2 could rescue EMCV titre following IFN treatment. This finding further confirmed the biological relevance of USP7 on type I IFN signalling.

The co-localisation of vIRF2 and vIRF4 with USP7 supports the SILAC data and justifies the need for further studies to understand the rational of USP7 interaction with vIRF2 and vIRF4 (Figure 6-8). As vIRF2 and vIRF4 counter USP7 in the type I IFN signalling pathway, we hypothesised that these viral proteins bind and ubiquitinate USP7 to counter its positive regulation of the JAK STAT pathway. We thus examined the effect of overexpression of the viral proteins on USP7 stability and activity. A dose dependent increase in levels of both viral proteins had no significant effect on the abundance of USP7. However, the USP7 augmentation effect on the JAK STAT pathway was significantly suppressed in the presence of USP7 co-expression with vIRF2 and vIRF4 (Figure 6-11A and B). These data suggest that these viral proteins do not directly exert their negative effect on IFN signalling through targeting of USP7. A more plausible hypothesis would be that vIRF2 and vIRF4 competitively bind USP7 and inhibit its activity in the JAK STAT pathway. Given that USP7 associates with STAT2, we hypothesise that vIRF2 and vIRF4 inhibit the catalytic activity of USP7 towards STAT2. This hypothesis can be confirmed with Co-immunoprecipitation to determine possible interaction of USP7 with vIRF2 or vIRF4.

A number of viral proteins inhibit the early stages of the JAK STAT pathway by targeting specific effectors of the type I IFN signalling pathway (Schneider et al., 2014). Viral inhibition of the STAT proteins has evolved as a common mechanism for
pathogen immune evasion (Fleming, 2016). The STAT1 and STAT2 factors are critical for antiviral responses, hence they have evolved as key targets for many viruses that manipulate the JAK STAT pathway (Fleming, 2016; Schneider et al., 2014). The common mechanistic findings involve ubiquitination, degradation and dephosphorylation of the STAT factors (Severo et al., 2013).

STAT phosphorylation may also be inhibited, or the STAT factors may be sequestered or relocated (Hoffmann et al., 2015). Other examples of viral proteins modulating the JAK STAT pathway include the V proteins of PiV5 and PiV2, which induce degradation of STAT1 and STAT2, respectively (Didcock et al., 1999; Precious et al., 2005); Sendai virus C protein inhibits STAT1 phosphorylation (Oda et al., 2015); Vaccinia virus VH1 protein induces the dephosphorylation of IFNγ-activated STAT1 in epithelial cells (Koksal & Cingolani, 2011; Mann et al., 2008). Such early actions ensure pan-inhibition of downstream ISG activation and are therefore more efficient.

Additionally, to maximise abrogation of IFN mediated antiviral immunity, many viruses target more than one factor in the IFN signalling pathway. Our group had earlier shown that vIRF2 downregulates the JAK STAT signal pathway through pleiotropic means, hence it could be proposed that vIRF2 and vIRF4 exert their inhibitory effect on the JAK STAT pathway by targeting STAT2. Figure 6-17 summarises the proposed mechanistic understanding of the interaction between USP7 and vIRF2/vIRF4.

In this thesis, the role of the KSHV proteins vIRF2 and vIRF4 in modulation of innate immunity has been studied by ectopically expressing these proteins. This approach is not unusual, as several previous studies have also been based on the study of individual proteins (Didcock et al., 1999; Precious et al., 2005; Unterholzner et al., 2011). The limitations for conclusive deductions is nonetheless acknowledged, considering that in KSHV infection, genes are expressed in a temporal order following lytic reactivation. Hence there is the possibility that the effects observed could either be amplified or attenuated by other viral proteins.
In conclusion, we elucidated that both KSHV vIRF2 and vIRF4 are negative regulators of type I IFN signalling and act by pleiotropic means; inhibiting ISRE activation and also downregulate the transcription of ISGs mRNA (Figure 7-2). In the present thesis, we further validated a previous SILAC data from our group which showed an interaction between vIRF2/vIRF4 and a number of ribosomal proteins as well as a cellular protein, USP7. vIRF4 was characterised as a negative regulator of IFN signalling, similarly a novel role was identified for vIRF4 in translational control of the host mRNA. Furthermore, we have identified a novel positive role for USP7 in type I IFN signalling. USP7 action was localised within the nucleus where it binds to an ISGF3 component, STAT2, hence it was hypothesised that USP7 augments ISRE activation by targeting and possibly deubiquitinating STAT2. Other hypothesised mechanisms of action involved vIRF2 and vIRF4, and their negative regulation of the JAK STAT signalling pathway possibly through USP7 (Figure 7-1).

A summary of the conclusions of this thesis are as follows:

1. The vIRF2 and vIRF4 protein inhibit the JAK-STAT signalling pathways by downregulating IFNα-mediated activation of ISRE-containing promoters (Figure 3-10) and consequently the expression of ISGs (Figure 3-11).
2. We hereby propose a novel role for vIRF4 in translational control. vIRF4 expression supressed translation (Figure 4-1) and exerts translational control of specific host proteins (Figure 4-14).
3. USP7 binds both vIRF2 and vIRF4 (Figure 5-1), and plays a positive role in the type I IFN signalling pathway independent of P53 (Figure 5-11).
4. Mechanistic studies reveal that USP7 acts within the nucleus and targets STAT2 but not IRF9 and STAT1 (Figure 6-6).
5. vIRF2 and vIRF4 co-localise with USP7 (Figure 6-8) and inhibit USP7 mediated augmentation of type I IFN signalling (Figure 6-11).
6. The biological relevance of USP7 on IFNα-mediated antiviral defences was assessed in the presence of IFN sensitive viruses; EMCV and Chandipura virus (Figure 6-7)

7. The proposed mechanism behind vIRF2 and vIRF4 association with USP7, suggests this interaction may have relevance in STAT2-mediated regulation of JAK STAT signalling (Figure 6-17).

7.2. Recommendations for future research

Future work in this area should aim to (i) investigate the effect of KSHV infection on the expression of the validated proteins from the microarray data; (ii) further validate the microarray data by investigating the expression of the upregulated proteins by Immunohistochemistry carried out on Kaposi’s sarcoma biopsy sections, to consider the possibility of utilising them as diagnostic or prognostic markers for KS (Table 4-3); (iii) verify if USP7 protects STAT2 from K48- or K63-linked ubiquitination; (iv) investigate the importance of USP7 for KSHV replication and lytic reactivation. Other studies could investigate the impact of vIRF2 and vIRF4 on STAT2 stability and activity. Further experiments would be required to map out the site of interaction of vIRF2 with USP7 and to determine if this site is important for IFN signalling.
Figure 7-1: Proposed model of vIRF2 and vIRF4 modulation of the JAK-STAT signalling pathway and the counter regulatory role of USP7.

The binding of IFNα/β to the IFNAR activates the type I IFN pathway, a signalling cascade is evoked which leads to the phosphorylation of the STATs, both of which dimerise and bind IRF9 to form the ISGF3 complex. The ISGF3 complex translocates into the nucleus where it binds to ISRE consensus sequences and activates the transcription of ISGs. In this thesis, we confirmed that vIRF2 and vIRF4 supress ISRE activation and also downregulate transcription of ISGs as shown in the figure. This thesis also identified a novel role for USP7 in IFN signalling. USP7 acts within the nucleus to augment ISRE activation by targeting STAT2, possibly for deubiquitination. Other hypothesised mechanisms of action involved vIRF2 and vIRF4 binding directly to USP7 to inhibit its association with STAT2. Alternatively, the viral proteins could bind and directly ubiquitinate STAT2.
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universally modified by conserved protein kinases of herpesviruses in mammalian cells. The Journal of general virology 82, 1457-1463.


8. APPENDIX

APPENDIX A

Table 8-1: Solutions for Agarose gel Solution Composition/Source

<table>
<thead>
<tr>
<th>Solution</th>
<th>Components/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>Sigma</td>
</tr>
<tr>
<td>TBE Buffer</td>
<td>45mM Tris base, 45mM boric acid and 1mM EDTA, pH 8.0</td>
</tr>
<tr>
<td>Loading buffer</td>
<td>25mg Bromophenol Blue, 1.5g Ficoll 400, 10ml sterile distilled water</td>
</tr>
</tbody>
</table>

Table 8-2: Buffers used for polysome profiling

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysome lysis buffer (10X)</td>
<td>3 M NaCl, 7.5 M Tris HCL, 150 mM MgCl2</td>
</tr>
<tr>
<td>Polysome lysis buffer (1X)</td>
<td>0.5mL polysome lysis buffer (10X), 0.5mL Triton (10%), 50µL Cycloheximide, 5µL RNAsen inhibitor, made up to 5ml with RNAsen-free water</td>
</tr>
<tr>
<td>10 X TMN</td>
<td>3M NaCl, 150mM MgCl2, 150mM Tris-HCl at pH 7.5, 1mg/mL Cycloheximide</td>
</tr>
<tr>
<td>1 X TMN</td>
<td>10 X TMN buffer was diluted 1 in 10 to produce 1X TMN buffer</td>
</tr>
</tbody>
</table>

Table 8-3: Composition of the Sucrose gradients

<table>
<thead>
<tr>
<th>Components</th>
<th>10%</th>
<th>18%</th>
<th>26%</th>
<th>34%</th>
<th>42%</th>
<th>50%</th>
<th>60%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>2g</td>
<td>3.6g</td>
<td>5.2g</td>
<td>6.8g</td>
<td>8.4g</td>
<td>10g</td>
<td>12g</td>
</tr>
<tr>
<td>10XTMN Buffer</td>
<td>2mL</td>
<td>2mL</td>
<td>2mL</td>
<td>2mL</td>
<td>2mL</td>
<td>2mL</td>
<td>2mL</td>
</tr>
<tr>
<td>RNAsen inhibitor</td>
<td>2µL</td>
<td>2µL</td>
<td>2µL</td>
<td>2µL</td>
<td>2µL</td>
<td>2µL</td>
<td>2µL</td>
</tr>
<tr>
<td>Cycloheximide (1mg/ml)</td>
<td>200µL</td>
<td>200µL</td>
<td>200µL</td>
<td>200µL</td>
<td>200µL</td>
<td>200µL</td>
<td>200µL</td>
</tr>
</tbody>
</table>

NB: Make each gradient up to 20ml with RNAsen free water and ensure even mix before filter sterilization with 0.22µM.
APPENDIX B

Mowiol mounting media

2.4 g of Mowiol 4-88 to was added to 6 g of glycerol and stirred to ensure even mixture. 6 mL of H2O as added and the solution left on a stirrer for several hours at room temperature. A 12 mL volume of 0.2 M Tris-Cl (pH 8.5) was then added and the solution heated to 50°C for 10 minutes with occasional mixing. The Mowiol was allowed to dissolve then clarified by centrifugation at 5000g for 15 minutes. The mixture was distributed in 1mL aliquot in airtight containers and stored at −20°C.

DAPI stock preparation

The DAPI reagent (Roche, Cat No. 10236276001) was reconstituted by dissolving 10mg of DAPI in 2mL of distilled water. Aliquots were made and stored at -20°C. The working stock was made by diluting the DAPI stock at 1:1000 dilution in the Mowiol.

Trichloracetate protein precipitation

1 volume of TCA stock was added to 4 volumes of protein sample in 1.5mL tube then left to incubate for 10 minutes at 4°C. The tube was spun in microcentrifuge at 14K rpm, 5 min. Supernatant was removed and the pellet was washed with 200µL of cold acetone. The solution was spun in microfuge at 14K rpm, 5 minutes. The acetone wash steps were repeated twice after which pellet was left to dry by placing tube in 95°C heat block for 10 minutes to drive off acetone.
## APPENDIX C

Table 8-4: PCR protocol Step Temperature Time Number of cycles

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>2 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>1 minute</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>55-60°C</td>
<td>45 seconds</td>
<td>25-35 cycles from denaturation to extension</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 minute</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>5 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>Indefinite</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>
Table 8-5: The sequences and primers used in PCR to detect interferon sensitive genes.

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SOURCE</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFIT1</td>
<td>SIGMA</td>
<td>5'GCAGAACGGCTGCTAATT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'TCAGGCAATTTTCATCGTCATC-3'</td>
</tr>
<tr>
<td>OAS1</td>
<td>SIGMA</td>
<td>5'CAAGCTCAAGAGCCTCATCC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'TGGGCTGTGTGGAAATGTGT-3'</td>
</tr>
<tr>
<td>MX1</td>
<td>SIGMA</td>
<td>5'ATTTCGGATGCTTCAGAGGTAGA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'CCCGGCGATGGCATT-3'</td>
</tr>
<tr>
<td>IRF 7</td>
<td>SIGMA</td>
<td>5'AGCGGCTGCTATGAGGGGCT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'GCCACAGCCCAGCCTGAA-3'</td>
</tr>
<tr>
<td>ISG56</td>
<td>SIGMA</td>
<td>5'CACCATTTGCTGTGTTAGC TCC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'GGCAGCCGTTCTCAGGGT-3'</td>
</tr>
<tr>
<td>IRF1</td>
<td>SIGMA</td>
<td>5'AGGCCAAGAGGAATCATGTG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'CTGTGTAGCTGCTGTTGCA-3'</td>
</tr>
<tr>
<td>ISG15</td>
<td>SIGMA</td>
<td>5'TTTGCAGTACAGGCTTG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'GGGTGATCTGCGCCT TCA-3'</td>
</tr>
<tr>
<td>OAS2</td>
<td>SIGMA</td>
<td>5'TGGCCATAGGTGGCTCCTAT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'CGAGGATGTCAGTTGGCTT-3'</td>
</tr>
<tr>
<td>PKR</td>
<td>SIGMA</td>
<td>5'GCATGGGCCAGAAGGTGA T-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'AGCGGCAATTTGCTTCCTCA-3'</td>
</tr>
<tr>
<td>ISG54</td>
<td>SIGMA</td>
<td>5'GCACTGCAACCATGAGTGAGA A3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'TTCTCCCCT CAT CAAGTTCCA G-3'</td>
</tr>
<tr>
<td>ISG15</td>
<td>PrimerDesign</td>
<td>5'GCCGAACCTCATCTTTGGCAGTA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'CAGCTCTGACACCAGATG-3'</td>
</tr>
<tr>
<td>MX1</td>
<td>PrimerDesign</td>
<td>5'CCCCAATATGTTGGACATCG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'ACCTTGTCCTCAGTTTGGT-3'</td>
</tr>
<tr>
<td>OAS1</td>
<td>PrimerDesign</td>
<td>5'TGTGTTGTCGAAAAGTTGAGG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'TGATCCTGAAAAATGTGGAG-3'</td>
</tr>
</tbody>
</table>
### Table 8-6: Reference genes used to normalize qPCR data

<table>
<thead>
<tr>
<th>Reference gene</th>
<th>Sequence/Source</th>
</tr>
</thead>
</table>
| Tubulin        | 5'-CCTGGATGGTGGTACGGAAGG-3'  
|                | 5'-TGAAGGACCACACTGAAGG-3' |
| GAPDH          | Applied Biosystems (4310884E ), VIC-labelled |

### Table 8-7: The sequences of primer and probes used to detect vIRF2 and vIRF4 cDNA gene Sequence

<table>
<thead>
<tr>
<th>GENE</th>
<th>SEQUENCE</th>
</tr>
</thead>
</table>
| VIRF2  | Forward: 5'- GCATCGCGAAGAAGAATAGG -3'  
|        | Reverse: 5'- TGGTAAAAATGGGGCAAGGTA-3' |
| VIRF4  | Forward: 5'- GGGATGGGTGGCCTCAGGGCG -3'  
|        | Reverse: 5'- CTAGCACATTGGCCGCTTTG-3' |

### Table 8-8: The sequences of primer and probes used to quantify levels of mRNA by qPCR Gene Sequence/Source.

<table>
<thead>
<tr>
<th>GENE</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIRF2</td>
<td>Forward: 5'- TCAGCTGCGGAGGATGTTG -3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'- CCATGATGACAAACACAGAGAAAAG -3'</td>
</tr>
<tr>
<td></td>
<td>FAM-labelled Probe: 5'- CCGGCTCCCTCTGGGCTTTTTC -3'</td>
</tr>
<tr>
<td>VIRF4</td>
<td>Forward: 5'- GCCCCTGCCTCCTCGTA -3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'- TGTCCCCCCCAATGCA -3'</td>
</tr>
<tr>
<td></td>
<td>FAM-labelled Probe: 5'- CTTTTGTTCTCTAGTGTCACTGCCTCGCG -3'</td>
</tr>
</tbody>
</table>
Table 8-9: The sequences of primers used in qPCR for microarray validation.

<table>
<thead>
<tr>
<th>GENE</th>
<th>SEQUENCE</th>
</tr>
</thead>
</table>
| CELF4  | 5'-CTACGAGCGGTAGGC-3'  
|        | 5'-TCGGACTGTGGCTTGAGCA-3'                                               |
| RSAD2  | 5'-CTACTGCGATGAGTCAG-3'  
|        | 5'-TCGGACTGTGGCTTGAGCA-3'                                               |
| EPST11 | 5'-CGAGCTTACGGTTCTGAAGG-3'                                               
|        | 5'-GGACTGGCTTGGAGCA-3'                                                  |
8.1. Research output from the PhD.

  - “Viruses and immunomodulatory activities: Lessons from KSHV” (*Oral presentation*).

- **Annual meeting of the Microbiology Society**, Abstract No. 275. Liverpool, March 2016
  - “Viral targeting reveals the positive role of USP7 in the interferon response.” (*Oral presentation*)

  - “Viral regulation of gene expression – Insights from KSHV” (*Poster presentation*).

- **University of Surrey, Faculty of Health and Medical Sciences Research Festival**, Abstract No.20. Guildford, Surrey. June 2015
  - “Understanding the role of USP7 in the regulation of interferon signalling” (*Poster presentation*).

- **Postgraduate research Conference of the University of Surrey**. Abstract No. 56. Guildford, Surrey. 2014
  - “Investigating ribosomal regulation of gene expression by Kaposi’s sarcoma-associated herpesvirus” (*Poster presentation*).

**Manuscripts in preparation**

1. **Adeola Fowotade**, Laura Hindle, Mohamed Mutocheluh, Carlos Maluquer de Motes, Nicolas Locker and David Blackbourn.
   
   *Viral Targeting reveals the positive role of Deubiquitinase USP7 in the interferon response. (to be submitted to mBiol)*

Persistent KSHV infection increases EBV associated PEL-like tumorigenesis in vivo (Submitted to Science journal)

Ribosome Imprinted Polymers for Sensitive Detection of Translational Responses (submitted to Nature Communication)

8.2. Awards and Grants

1. Travel Grant to attend 35th Annual meeting of the American Society for Virology. Blacksburg, Virginia, USA (June, 2016).

2. Travel Grant to attend the Annual meeting of the Microbiology Society (SCG/16/087). Liverpool, UK (March, 2016).

3. Travel Grant to attend the Annual meeting of the Society for General Microbiology (SCG/15/037). Birmingham, UK (March, 2015)

4. Travel Grant to attend the Annual meeting of the Society for General Microbiology (SCG/15/037). Birmingham, UK (March, 2014)

5. 2014 PrimerDesign Gold student sponsorship package