THE ROLE OF KSHV MICRORNAS IN BYPASS OF ONCOGENE-INDUCED SENESCENCE

by

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ABSTRACT

Kaposi's sarcoma-associated herpesvirus (KSHV) is the infectious cause of Kaposi's sarcoma (KS). KSHV has the ability to bypass a host anti-proliferative defense, oncogene-induced senescence (OIS), triggered by chronic oncogene expression. KSHV encodes 18 microRNAs (miRNAs) which are thought to fine-tune host gene expression to create a local pro-tumour environment in KS tissue. However, the functions of these miRNAs remain incompletely characterized. We identified two miRNAs, miR-K5 and miR-K11 that induced bypass of senescence. Senescence bypass coincided with marked reductions in levels of the p16 tumour suppressor, a key effector of the senescence program. miRNA-induced OIS bypass also coincided with alterations in γ-H2AX and 53BP1-positive DNA damage foci. Autophagic flux is required for efficient establishment of senescence. miR-K5 and miR-K11 inhibited flux in osteosarcoma cells. These data suggest that miR-K5 and miR-K11 may suppress OIS at multiple levels, thereby contributing to the ongoing proliferation of latently KSHV-infected cells.

LIST OF ABBREVIATIONS AND SYMBOLS USED

°C	Degrees Celsius
%	Percent
AID	Activation-induced cytidine deaminase
AIDS	Acquired immune-deficiency syndrome
AMPK	Adenosine monophosphate activated protein kinase
ATM	Ataxia telangiectasia mutated gene
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ATR	ATM and RAD3-related
Bcl-2	B cell lymphoma-2
BCLAF1	Bcl-2 associated factor 1
bFGF	Basic fibroblast growth factor
BJ	BJ Human foreskin fibrobast cells
Blast	Blacticidin
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
Cdc	Cell division cycle
CDK	Cyclin-dependent kinase
CHK	Checkpoint kinase
CKI	Cyclin-dependent kinase inhibitor
CMA	Chaperone-mediated autophagy
d	Day(s)
DAPI	4',6-diamidino-2-phenylindole
DE	Delayed-early genes
DDR	DNA-damage response
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNA-SCAR	DNA segments with chromatin alterations reinforcing senescence
DR	Direct repeat
DSB	Double-strand DNA break
dsDNA	Double-stranded DNA
EBV	Epstein-Barr Virus
ECL+	Enhanced Chemiluminescence Plus
EDTA	Ethylenediaminetetracetic acid
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
ETP	Etoposide
Ets	E26-transformation specific
FADD	Fas-associated death domain
FBS	Fetal bovine serum
GFP	Green fluorescent protein
h	Hour(s)
HA	Hemagglutinin epitope

HAART	Highly active anti-retroviral therapy
HCMV	Human cytomegalovirus
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFF	Human foreskin fibroblast
HIV	Human immunodeficiency virus
HP1	Heterochromatin protein 1
HPV	Human papilloma virus
HSV-1	Herpes simplex virus type 1
HVS	Herpesvirus saimiri
ID1	Inhibitor of DNA binding 1
IE	Immediate-early genes
IFNγ	Interferon gamma
IKKγ	Inhibitor of kappa B kinase gamma
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
IRAK1	Interleukin-1 receptor associated-kinase 1
IRES	Internal Ribosomal Entry Site
Kb	Kilobases
KS	Kaposi's Sarcoma
KSHV	Kaposi's Sarcoma-associated Herpesvirus
L	Late genes
LANA	Latency-associated nuclear antigen
LC3-I	Unconjugated LC3
LC3-II	Phosphatidylethanolamine-conjugated LC3
MAF	Musculoaponeurotic fibrosarcoma
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minute 2
MEM	Minimum essential medium
MHC	Major histocompatibility complex
min	Minute(s)
MK2	MAPK-associated protein kinase 2
mRNA	Messenger ribonucleic acid
miRNA	Micro-ribonucleic acid
MK2	Mitogen-activated protein kinase-associated protein kinase 2
mTOR	Mechanistic target of rapamycin
mTORC1	Mechanistic target-of-rapamycin complex 1
mTORC2	Mechanistic target-of-rapamycin complex 2
MyD88	Myeloid differentiation primary response gene 88
NEAA	Non-essential amino acids
NF-κB	Nuclear factor kappa B
OIS	Oncogene-induced senescence
ORF	Open-reading frame
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline

PCNA	Proliferating cellular nuclear antigen
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PEL	Primary effusion lymphoma
PI3K	Phosphatidylinositol 3-kinase
PRC	Polycomb repressor complex
PRR	Pattern-recognition receptors
Puro	Puromycin
qRT-PCR	Quantitative real-time polymerase chain reaction
Rb	Retinoblastoma
RFP	Red Fluorescent Protein
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
ROI	Region of interest
rpm	Revolutions per minute
RT	Room temperature
RTA	Replication transcription activator
SA-β-gal	Senescence-associated β-galactosidase
SAHF	Senescence-associated heterochromatin foci
SASP	Senescence-associated secretory phenotype
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
shRNA	Short hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid
SNARE	Soluble N-ethylmadeimide-sensitive factor attachment protein receptor
TBS	Tris-buffered saline
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TSC2	Tuberous sclerosis 2
ULK	unc-51-like kinase
UTR	Untranslated region
v-cyclin	Viral cyclin
VEGF	Vascular endothelial growth factor
v-FLIP	Viral FLICE-inhibitory protein
v-IL-6	Viral Intrleukin-6
VSV	Vesicular stomatitis virus
VZV	Varicella zoster virus
X-gal	5-bromo-4-chloro-indolyl-D-galactopyranoside

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CHAPTER 1: INTRODUCTION

1.1 Overview

Viruses are obligate intracellular parasites that are incapable of autonomous replication. Viruses must enter host cells and exploit their cellular biosynthetic machinery in order to coordinate viral gene expression and assemble infectious progeny virions. Viruses and their hosts have experienced significant co-evolution, with viruses evolving to maximize output of virions, while host cells evolve to promote cell survival and limit viral replication. Viral control of host cell cycle regulation has led to the emergence of oncoviruses. Oncoviruses express several genes that target specific cellular pathways to promote proliferation of infected cells, sometimes causing tumourigenesis.

Oncogene-induced senescence (OIS) is a cellular anti-cancer defence mechanism characterized by cessation of growth, cytoskeletal alterations resulting in a large, flattened phenotype, resistance to apoptosis and the ability to persist for extended periods of time *in vitro*. Because it promotes permanent arrest of cellular proliferation in response to oncogenic stress, OIS is being increasingly regarded as an important barrier to tumourigenesis. Oncoviruses, such as Kaposi's sarcoma-associated herpesvirus (KSHV) induce various cellular stresses upon infection which are known to trigger OIS. This suggests that oncoviruses have evolved mechanisms to actively subvert OIS during infection, as they retain the ability to cause cancer, despite these stringent cellular anti-tumour defence mechanisms. The role of OIS in KSHV infection remains to be elucidated, but future insight into this process may further our understanding of oncoviruses and, indeed, the process of tumourigenesis.

The following introduction provides a summary of KSHV, virus-associated pathologies, virion structure and genome organization, latent and lytic phases of infection and gene expression profiles associated with tumourigenesis. Furthermore, the mechanics of OIS are reviewed, with an emphasis on its interactions between KSHV oncogenes. Finally, the process of autophagy is discussed, illustrating the links between KSHV manipulation of autophagy and OIS and its impact on tumourigenesis.

1.2 Herpesviridae

The herpesviridae are a family of enveloped viruses with large, linear, double-stranded DNA (dsDNA) genomes. These viruses undergo both lytic and latent replication cycles, a characteristic of all herpesviruses. During lytic infection most lytic viral genes are expressed leading to the production of viral particles and spread of the virus. By contrast, latency is defined by the expression of only a small subset of genes. The viral episome is tethered to the cellular genome allowing the virus to evade detection by the host cell and persist indefinitely. The Herpesviridae are associated with multiple diseases, varying from mild to severe, and exhibit a wide spectrum of cell tropisms in a range of species, including humans (Mettenleiter *et al*, 2008).

The herpesviruses are classified into three subfamilies: the alpha-, beta- and gamma-herpesviruses. They are categorized based on their size, structure and genetic homology. The α-herpesviruses are characterized by a short replication cycle and broad tissue tropism. They are known to establish latency in neurons in particular. Several notable α-herpesviruses include herpes simplex virus 1 (HSV-1) and varicella zoster virus (VSV), the causative agent of chickenpox and shingles. The β-herpesviruses are known for their long replication cycles and leukocyte specificity. The most notable member in this family is human cytomegalovirus (HCMV), a serious infection in immunocompromized individuals. The γ-herpesviruses primarily target B and T lymphocytes and are characterized by this limited cell tropism and a preference for entering latency upon infection. This subfamily includes Epstein-Barr Virus (EBV) which causes mononucleosis and is associated with nasopharyngeal carcinoma and lymphoma, in addition to Kaposi's Sarcoma-Associated Herpesvirus (KSHV), which is linked to the endothelial neoplasm Kaposi's sarcoma as well as other AIDS-related lymphoproliferative disorders (Roizman, 1996; Davidson , 2010).

1.3 Kaposi's Sarcoma-Associated Herpesvirus

KSHV is the most recently discovered human herpesvirus and acts as the etiological agent for a number of human cancers, including Kaposi's sarcoma (KS), Multicentric Castleman's Disease and Primary Effusion Lymphoma (Figure 1.1). This virus encodes several

oncogenes which act in unison to regulate transformation and tumourigenesis during both lytic and latent infection.

1.3.1 Disease Associations

1.3.1.1 Kaposi's Sarcoma

In 1872 a Hungarian dermatologist, Moritz Kaposi, published a study that described an "idiopathic multiple pigmented sarcoma of the skin" that afflicted five of his patients (Figure 1.1). One of these patients later developed lesions in the lungs and gastrointestinal tract (Kaposi, 1872; Antman and Chang, 2000). Heinrich Koebner subsequently named this disease Kaposi's sarcoma, despite the fact that it was later found to be an endothelial neoplasm, rather than a sarcoma (Mesri *et al*, 2010).

KS occurs in two distinct forms: classical KS, which is not affiliated with HIV infection, and AIDS-related KS. These forms can be further classified into four epidemiological categories (1Schwartz *et al*, 2008). Classic KS primarily affects elderly men of Mediterranean or Ashkenazi Jewish descent. It is characterized by relatively mild disease with the majority of lesions found on the legs and lower extremities. Endemic KS is commonly observed in children and young adults in sub-Saharan Africa and presents as aggressive disease in the soft tissue, bones and viscera. Iatrogenic, or transplant-associated, KS occurs only within individuals undergoing immunosuppressive therapy, such as that received following organ transplantation. Although this form of KS is very aggressive, patients typically undergo spontaneous remission following the discontinuation of the immunosuppressive therapy. Epidemic, or AIDS-associated, KS is the most common and severe manifestation of the disease and remains the most common AIDS-associated malignancy, contributing significantly to the mortality and morbidity of AIDS patients (Antman and Chang, 2000; Boshoff and Weiss, 2002; Ganem, 2010; Mesri et al., 2010). Epidemic KS presents as cutaneous lesions on the head, neck, torso, hard palate and gums and later advances to the gastrointestinal tract, lungs and lymph nodes. The incidence of AIDS-associated KS has decreased dramatically since the implementation of highly active anti-retroviral therapy (HAART), highlighting the dependence of this disease on the human

immunodeficiency virus (HIV) infection and immunosuppression. However KS remains an extensive problem in developing third-world countries that lack adequate access to HAART (Krown *et al*, 2008; Dittmer, 2010).

Regardless of the clinical manifestation, KS is a malignant neoplasm of the endothelium. It presents as multifocal dark purple patch lesions on the dermis which progress morphologically into hardened red plaques and subsequently to raised nodules, aggressive forms of which can lead to systemic illness within visceral tissues (Mesri *et al*, 2010). In contrast to classical cancers, KS tumours become highly vascularised prior to the establishment of a mass and these tumours are infiltrated with inflammatory cells (i.e. macrophages and mast cells), immune cells (i.e dendritic cells and lymphocytes) as well as vascular and lymphatic endothelial cells (Boshoff and Weiss, 2002). The primary proliferative agents within KS lesions are the unique "spindle cells," abnormally elongated endothelial cells with a spindle-like morphology (Browning *et al*, 1994; Boshoff *et al*, 1995; Boshoff *et al,* 1997; Boshoff, 1998). The origin of spindle cells remains enigmatic, as these cells express markers specific to various lineages, however accumulating evidence suggests that they are endothelial cells of lymphatic, rather than vascular, lineage. Spindle cells express high levels of vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), basic fibroblast growth factor (bFGF), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), thus contributing significantly to the pro-inflammatory and antiproliferative secretory phenotype of the tumour microenvironment (Salahuddin *et al*, 1988; Ensoli *et al*, 1989; Miles *et al*, 1990; Ensoli *et al*, 1992).

In 1994, a ground-breaking study identified herpesvirus-like DNA sequences from KS lesions, leading to the discovery of KSHV, also known as human herpesvirus-8, and its role as the etiological agent of KS (Chang *et al*, 1994). KSHV remains the only known risk factor essential for KS development and has since been linked to a number of lymphoproliferative disorders in addition to KS, emphasizing the clinical importance of this oncogenic herpesvirus (Cesarman *et al,* 1995; Moore and Chang, 1995; Moore *et al*, 1996).

1.3.1.2 Primary Effusion Lymphoma

Primary effusion lymphoma (PEL) is a rare B-cell lymphoma that presents as a lymphomatous effusion in the pleural, pericardial and peritoneal spaces, occasionally developing into solid tumour masses or lymphadenopathy (Knowles *et al*, 1989; Arvanitakis *et al*, 1996; Chadburn *et al*, 2004). PEL occurs primarily, although not exclusively, in HIV-positive patients suffering from advanced stages of immunosuppression. PEL tumour cells are characterized by latent KSHV infection and are frequently co-infected with EBV (Cesarman *et al*, 1995; Mesri *et al*, 1996; Nador *et al*, 1996; Cesarman *et al*, 1996). KSHV-positive, EBV-negative B-cell lines derived from HIV-negative PEL patients that can be induced to undergo lytic replication have been established, facilitating the generation of KSHV *in vitro*, and proving an invaluable tool for the study of the molecular biology and pathogenesis of KSHV.

1.3.1.3 Multicentric Castleman's Disease

Multicentric Castleman's disease (MCD) is another rare B-cell lymphoproliferative disorder linked to KSHV infection (Soulier *et al*, 1995; Oksenhendler *et al*, 1998). It is an aggressive systemic illness characterized by recurrent fevers and sweats, weight loss, lymphadenopathy, hepatosplenomegaly and frequent progression to non-Hodgkin lymphoma (Oksenhendler *et al*, 1996; Oksenhendler, 2002). KSHV has been identified in all HIV-seropositive cases of MCD and in half of HIV-seronegative MCD patients (Gessain *et al*, 1996; Dupin *et al*, 1999; Boivin *et al*, 2002). In particular, KSHV is associated with an aggressive subtype of MCD known as plasmablastic MCD, defined by the proliferation of KSHV-infected plasmablastic B cells (Dupin *et al*, 2000). The disease is thought to be mediated by a viral homologue of IL-6 (v-IL-6) secreted from infected cells, and the severity and course of MCD is closely correlated with KSHV viral load (Parravicini *et al*, 1997; Oksenhendler *et al*, 2000; Ahmed *et al*, 2007).

1.3.2 Molecular Biology of KSHV

1.3.2.1 Virion Structure

KSHV virions share many structural similarities to those of other herpesviruses. These particles are approximately 120 to 150 nm in diameter and consist of a 110 nm icosahedral nucleocapsid contained within a lipid bilayer envelope (Said *et al*, 1997; Wu *et al*, 2000). This envelope contains numerous different glycoproteins that aid the virus in interacting with the cell surface (Wang *et al*, 2003). Between the envelope and the capsid is a proteinaceous tegument composed of numerous KSHV proteins that play a role in viral assembly, viral entry, lytic replication and virus-host interactions, in addition to antagonizing p53-mediated apoptosis (Bechtel *et al*, 2005b; Chudasama *et al,* 2014; Full *et al*, 2014 Said *et al*, 1997; Sathish *et al*, 2012). KSHV virions assemble three distinct types of capsid, known as A, B and C type capsids. Only C type capsids contain the viral genomic DNA, whereas types A and B are empty and consequently uninfectious (Nealon *et al*, 2001). In addition to containing the full genome, mature KSHV virions also contain messenger ribonucleic acids (mRNAs) corresponding to lytic viral transcripts. These mRNAs are believed to play a role in the initiation of KSHV replication (Bechtel *et al*, 2005a).

1.3.2.2 Genome Organization

KSHV has a linear dsDNA genome, approximately 170kb in length that is synthesized and packaged into viral particles during lytic replication (Renne *et al*, 1996a). Similar to other γ-herpesviruses, the KSHV genome is maintained as a circular dsDNA multicopy episome during latency (Figure 1.2). This circularization is facilitated by a series of 801-bp G/C rich direct terminal repeats that are non-coding and flank the remainder of the KSHV genome, which is approximately 145kb in length and contains all 87 of the viral open reading frames (ORFs) (Lagunoff and Ganem, 1997; Mesri *et al,* 2010; Neipel *et al*, 1997; Russo *et al*, 1996). The KSHV genome contains marked similarities to other γ-herpesviruses, particularly in four major regions of highly conserved genes. Indeed, KSHV shares 68 conserved genes with Herpesvirus saimiri (HVS), a closely related γ-herpesvirus relative. Within the genome these conserved genes

are designated with consecutive numbers from the 5' to 3' ends of the genome and indicated with the prefix "ORF" (Russo *et al*, 1996). More recently, conserved genetic similarities have been discovered between KSHV and the murine γ-herpesvirus 68, in addition to several other non-human γ-herpesviruses (Alexander *et al*, 2000; Searles *et al*, 1999; Virgin *et al*, 1997). Those viral genes that are unique to KSHV are appointed K1 to K15 (Wen and Damania, 2010). In addition to similarities with other γ-herpesviruses, multiple KSHV genes also share homology to cellular genes (Boshoff and Weiss, 1998). This is likely a result of viral coevolution with host cells and capture of genes from ancestral primates. These viral gene products appear to have evolved distinct functions from their cellular counterparts, and have been strongly implicated in viral infection and pathogenesis (Boshoff and Weiss, 1998). More recently, reports of a functional genomics approach studying both the transcription and translation profiles of lytic KSHV infection has provided comprehensive new insight into gene expression and regulation for this virus. This group identified 49 viral transcripts and 67 new open reading frames (ORFs), suggesting that the coding capacity of this virus has been dramatically underestimated, as well as non-coding RNAs and multiple polyadenylation signals and splice junctions (Arias *et al*, 2014).

1.3.2.3 Overview of the KSHV Lytic Replication Cycle

KSHV infection begins when glycoproteins on the virion envelope bind to appropriate receptors on the cell surface that have been targeted by the virus for the purpose of mediating entry. Once bound, the KSHV virion either fuses directly with the plasma membrane of the infected cell, or is endocytosed and fuses instead with endosomal membranes. This allows for the delivery of the tegument and capsid, encompassing the viral genome, into the cytoplasm (Chandran, 2010). The capsid is subsequently trafficked to the nuclear envelope, allowing the virion to be uncoated and the viral genome to be imported through nuclear pores into the nucleus. Within the nucleus, the genome becomes circularized and is subsequently bound by histones, forming viral chromatin and facilitating transcription of the viral genes by RNA polymerase II.

As is the case with most herpesviruses, KSHV can express its genes in one of two distinct programs, depending on the conditions of infection: lytic replication or latency. Lytic replication consists of expression of the vast majority of viral genes, leading to the establishment of a

productive infection, the assembly of viral progeny and subsequent release by cell lysis (Fakhari and Dittmer, 2002; Paulose-Murphy *et al*. 2001; Sun *et al*, 1999). For all herpesviruses, this replication cycle occurs in a temporally regulated transcription cascade (Deng *et al*. 2007). The first group of genes to be expressed during lytic replication are the immediate early (IE) genes. This group includes the crucial viral gene product ORF50 encoding the replication transcription activator (RTA). This protein is required to trigger lytic reactivation by regulating the activation of later gene classes within the lytic transcriptional cascade (Jenner *et al*, 2010; Lukac *et al*, 1998; Lukac *et al*, 1999; Sun *et al*, 1998; Sun *et al*, 1999). The next transcripts, activated by RTA expression, are the delayed early (DE) genes. This class is involved in preparing the cell for viral replication and protein synthesis and includes genes linked to controlling the abundance of nucleotide precursors, host RNA turnover, enzymes and cofactors that play a role in viral DNA replication, in addition to genes involved in evasion of the immune system (Deng *et al*, 2007). The accumulation of these DE transcripts stimulates lytic DNA replication, resulting in many copies of the KSHV genome being produced within the infected cell. Finally, the last subset of genes to be expressed during lytic replication are the late (L) genes, which primarily encode structural proteins (Deng *et al*, 2007). Once transcribed, L genes stimulate the assembly and release of new viral particles through the incorporation of newly replicated genomes into viral capsids, acquisition of the tegument layer and subsequent budding from host cell membranes which contain KSHV glycoproteins.

Lytically infected cells are known to be critical for viral replication and spread, but their contribution to KSHV-induced carcinogenesis remains a mystery, as lytically infected cells are short-lived and die soon after infection. Evidence has emerged suggesting that several lytic gene products, such as viral G-protein coupled receptor (v-GPCR) may contribute to tumourigenesis by initiating inflammation and angiogenesis. Furthermore, these genes may act in a paracrine fashion to induce the proliferation of adjacent latently infected cells (Montaner *et al*, 2001; Montanger *et al*, 2003; Sodhi *et al*, 2004a; Sodhi *et al*, 2004b; Sodhi *et al*, 2006).

1.3.2.4 Latent Gene Expression

Consistent with the other γ-herpesviruses, latency is predominantly established in KSHV infection, rather than lytic replication. Indeed, approximately 85 to 99% of KSHV-infected cells in a KS lesion are latently infected (Dittmer *et al*, 1998; Miller *et al*, 1997; Sun *et al*, 1999; Zhong *et al*, 1996). During latency replication of the circularized viral genome, or episome, is entirely dependent on host cell DNA replication machinery. This episome is replicated with each cell division and is therefore able to persist as it is passed to each new daughter cell although there is experimental evidence that KSHV latency is unstable and episomes can be rapidly lost over multiple generations *in vitro* (Grundhoff *et al,* 2003). Latently infected cells often harbour multiple (1-20 copies) of the viral genome (Renne *et al*, 1996a; Renne *et al*, 1996b). The KSHV latency-associated transcripts are all expressed from the same small genomic cassette (Figure 1.2B) (Boshoff, 1998; Dittmer *et al*, 1998). These latent gene products represent the viral effectors for KS tumourigenesis and consist of the latency-associated nuclear antigen (LANA), viral (v-cyclin), viral FLICE-inhibitory protein (v-FLIP), Kaposins A, B and C and eighteen microRNAs (miRNAs).

The strong causal relationship between latent KSHV infection and cancer suggests that these latent gene products are the primary drivers of KS tumourigenesis. Indeed, characterization of these gene products has revealed roles in promoting cell proliferation and growth, inducing inflammation and angiogenesis and suppressing apoptosis (Mesri *et al*, 2010; Speck and Ganem, 2010). These latent gene products represent many putative oncogenes, each of the KSHV latent gene products, expressed independently or in concert, do not possess transforming capabilities. However, transgenic mice expressing KSHV LANA, v-cyclin or v-FLIP proteins do develop phenotypes characteristic of KSHV malignancies (Chugh *et al*, 2005; Fakhari *et al*, 2006; Verschuren *et al*, 2004a). In accordance, the group of ORFs encoding LANA, v-cyclin and v-FLIP has been designated the “oncogenic cluster” of the KSHV genome. These three ORFs are regulated as one transcription unit, suggesting that the coordinated expression of these proteins is crucial for their functions (Dittmer *et al*, 1998). Effort continues to elucidate the exact functions of each of the KSHV latent genes, in order to better comprehend the mechanisms involved in KSHV-induced tumourigenesis.

1.3.2.4.1 LANA

LANA, encoded by ORF73, is a large (222-232kDa) multifunctional nuclear protein that is highly expressed in all KSHV-associated malignancies. As such, LANA is commonly used as a serological marker for KSHV latent infection (Gao *et al*, 1996; Kedes *et al*, 1996; Kedes *et al*, 1997; Simpson *et al*, 1996). LANA is responsible for physically tethering the KSHV viral episome to host DNA (Ballestas *et al*, 1999; Ballestas and Kaye, 2001; Cotter and Robinson, 1999). Specifically, the carboxyl terminal domain of LANA directly binds to KSHV genomic terminal repeats. This interaction facilitates the formation of the viral origin of replication during latency (Ballestas and Kaye, 2001; Garber *et al*, 2001). The amino terminal domain of LANA interacts with histones H2A and H2B and chromosomal linker protein histone H1 (Cotter *et al*, 2001; Shinohara *et al*, 2002; Verma *et al*, 2013). The phosphorylated DNA damage response protein, γ-H2AX, as well as cellular replication fork proteins Timeless and Tipin are known to assist LANA in maintaining the KSHV episome (Ballestas and Kaye, 2011; Dheekollu *et al*, 2013; Giffin and Damania, 2014; Jha *et al*, 2013). Taken together, these interactions facilitate segregation of the newly replicated KSHV genome into daughter cells during mitosis.

In addition to its role in maintenance of the viral episome during cell division, LANA also displays important functions in transcriptional regulation and proliferation of latently infected cells. LANA has been shown to affect the transcription of a number of host genes, presumably through its interaction with many transcription factors. When ectopically expressed, LANA is able to bind and inhibit p53 and Retinoblastoma (Rb) tumour-suppressor proteins, leading to activation of E2F-dependent gene expression (Friborg *et al*, 1999; Radkov *et al*, 2000). The ability of LANA to regulate these pathways in cells latently infected with KSHV remains controversial (Chen *et al*, 2010; Sarek *et al*, 2007). LANA also stimulates the accumulation of β-catenin by binding to, and sequestering, GSK-3β (Fujimuro *et al*, 2003). This interaction allows for entry into the synthesis (S) phase of the cell cycle. LANA has also been implicated in increasing the lifespan of KSHV infected cells by increasing telomerase expression (Verma *et al*, 2004). Finally, LANA has been linked to transcription regulation, a role believed to be important in repressing KSHV lytic gene expression during latency and in the deregulating of host cell gene expression (Knight *et al*, 2001; Schwan *et al*, 2000).

1.3.2.4.2 v-cyclin

The KSHV latency program encodes a viral homolog of human D-type cyclins, v-cyclin (ORF72), which is involved in deregulating cellular proliferation (Chang *et al*, 1996). v-cyclin shares both sequence and functional homolog to cellular cyclin D2 (Chang *et al*, 1996; Li *et al*, 1997) and forms an active holoenzyme with cyclin-dependent kinase 6 (CDK6), independent of CDK-activating kinase phosphorylation (Godden-Kent *et al*, 1997; Kaldis *et al*, 2001; Li *et al*, 1997). v-cyclin also weakly associates with CDK2, CDK4 and CDK9, although the significance of these interactions remains as yet undetermined (Chang and Li, 2008; Platt *et al*, 2000). When complexed to CDK6, v-cyclin can phosphorylate various different cellular effectors including the Rb protein, p21, INK4 and p27 cell cycle inhibitors, the antiapoptotic Bcl-2 protein and Cdc6 and Orc1 of the origin recognition complex (Child and Mann, 2001; Ellis *et al*, 1999; Laman *et al*, 2001; Li *et al*, 1997; Ojala *et al*, 2000; Swanton *et al*, 1997). Collectively, these interactions enable ectopically expressed v-cyclin to drive DNA synthesis in various different cancer cell lines. By contrast, immortalized cells expressing v-cyclin tend to undergo apoptosis and primary cells exhibit a p53-dependent form of irreversible cell cycle arrest which bears striking similarity to oncogene-induced senescence (OIS) (Koopal *et al*, 2007; Ojala *et al*, 1999; Ojala *et al*, 2000; Verschuren *et al*, 2002). Although the precise contribution of v-cyclin to KSHV latency remains to be elucidated, Leidal and colleagues demonstrated a pro-autophagic response to v-cyclin in endothelial cells. They also demonstrated that, when co-expressed with another KSHV latency protein, v-FLIP, cells gain the ability to bypass OIS (Leidal *et al*, 2011). This coordinated latency program may, therefore, play a role in subverting cellular anti-cancer defences to facilitate proliferation and growth of KSHV latently-infected cells.

1.3.2.4.3 v-FLIP

KSHV encodes a viral homolog of cellular Fas-associated death domain (FADD) interleukin-1β-converting enzyme (FLICE, now called caspase-8) inhibitory protein, v-FLIP (K13; ORF72). Given its similarities to cellular FLIP proteins, v-FLIP was originally believed to be an inhibitor of caspase-8, thus protecting cells from Fas-mediated apoptosis (Djerbi *et al*, 1999; Irmler *et al*, 1997). It appears, however, that this viral protein has lost its function in

regulating caspase-8 over the course of KSHV evolution (Chugh *et al*, 2005). v-FLIP retains two death effector domains that can mediate interactions with the autophagy regulatory protein, Atg3, an E2 enzyme for LC3 lipidation (Lee *et al*, 2009). This interaction effectively inhibits lipid modification of LC3 and elongation of the autophagosomal membrane, thus preventing cellular autophagic degradation (see Section 1.5.1). Although the importance of this blocking of autophagy to KSHV latency and pathogenesis remains unclear, Leidal and colleagues demonstrated that v-FLIP is able to subvert v-cyclin-induced autophagy and senescence during latency, thereby suggesting a coordinated viral latency program that facilitates the proliferation of KSHV infected cells by blocking autophagy and senescence (Leidal *et al*, 2012).

v-FLIP is also able to potently activate the host cell nuclear factor (NF)-κB pathway. To trigger this signal transduction, v-FLIP binds and activates the inhibitor of κB kinase-γ (IKKγ), thus inactivating the IκB complex and leading to derepression of the NF-κB pathway (Chaudhary *et al*, 1999; Sun *et al*, 2003). Activation of the NF-κB pathway by v-FLIP has two important implications for KSHV latency. Firstly, it preserves latency by antagonizing entry into the lytic replication cycle (Brown *et al*, 2003; Grossmann and Ganem, 2008). In addition, it renders latently infected cells resistant to apoptosis by inducing anti-apoptotic factors Bcl-2 and Bcl-XL. Constitutive NF-κB activation further triggers widespread secretion of cytokines and chemokines, which contribute to the pro-inflammatory microenvironment of KS lesions (Guasparri *et al*, 2004; Sakakibara *et al*, 2009; Sun *et al*, 2009). Furthermore, the spindled shape of KSHV latently infected endothelial cells has been attributed to NF-κB activation by v-FLIP, although the relevance of this morphological change remains unknown (Grossman *et al*, 2006; Matta *et al*, 2007). However the NF-κB-dependent transforming potential of v-FLIP has been demonstrated in rodent fibroblast cells (Sun *et al*, 2003).

1.3.2.4.4 Kaposins

ORF K12 encodes three kaposins A, B and C, as a result of complex translation initiation (Sadler *et al*, 1999). Kaposin A is a small transmembrane protein that has been reported to interact with the ARF guanine nucleotide exchange factor cytohesin-1 to induce activation of the ERK/MAPK pathway and subsequent cellular transformation. Kaposin A has also been linked to

the induction of several features characteristic of transformation in rodent fibroblast cells and the subsequent injection of these cells into athymic nude mice produces small angiogenic tumours (Muralidhar *et al*, 1998). The major protein product from this locus is kaposin B, a protein composed of two sets of direct repeats (DR1 and DR2) that shares no homology to kaposin A (Sadler *et al*, 1999). Kaposin B prevents the decay of cytokine and growth factor mRNAs by binding and activating mitogen-activated protein kinase (MAPK)-associated protein kinase 2 (MK2), thus effectively blocking the degradation of normally unstable AU-rich-element mRNAs (McCormick and Ganem, 2005; McCormick and Ganem, 2006). The stabilization of these transcripts ensures their translation, increasing levels of IL-6 and VEGF, as well as other cytokines, and driving KS development and tumourigenesis (Yoo *et al*, 2010). The function(s) of Kaposin C remain unknown.

1.3.2.5 KSHV microRNAs

microRNAs (miRNAs) are short, 21-23 nucleotide non-coding RNAs that have the ability to post-transcriptionally regulate gene expression by either targeting complementary mRNAs for degradation or suppressing their translation by inhibiting ribosome binding (Cullen, 2006). These RNAs originate from RNA polymerase II transcripts as part of a 70 to 80 nucleotide long RNA stem-loop, the primary (pri)-miRNA (Figure 1.4). The pri-miRNA is cleaved in the nucleus by Drosha, an RNAse-III-like enzyme, freeing the pre-miRNA, which is subsequently exported from the nucleus by Exportin 5/Ran-GTP. In the cytoplasm it is cleaved by a cytoplasmic RNAse-III-like enzyme, Dicer, resulting in the miRNA duplex. The two strands are then separated and the guide strand is incorporated into the RNA-induced silencing complex (RISC) while the passenger strand is degraded in the cytoplasm. The RISC then targets complementary mRNA for degradation or translational repression, resulting in the suppression of gene expression (Zhu *et al*, 2013). In the context of latent viral infection, these miRNAs represent an attractive solution to viral manipulation of host gene expression as they take up little room in the genome, avoid generation of latent viral gene products and are indistinguishable from host miRNAs, thus reducing the potential that they will be selectively targeted upon infection (Ramalingam, 2012).

Many members of the herpesvirus family have adapted miRNAs to aid in establishing latency and evading host immune recognition (Cullen, 2006). KSHV is no exception and encodes 12 viral pre-miRNAs that are processed by the host RNA silencing machinery to generate 18 mature miRNAs (Cai *et al*, 2005; Cai and Cullen, 2006; Pfeffer *et al*, 2005; Samols *et al*, 2005; Umbach and Cullen, 2010). Ten of these pre-miRNAs are located within the kaposin intron, while the remaining two are found within the kaposin protein-coding region and the kaposin 3' untranslated region (UTR) (Cai and Cullen, 2006). Despite sharing a common promoter, the mature KSHV miRNAs exist at variable levels in latently infected cells, and miR-K10 and miR-K12 have been detected at even higher levels during lytic replication. This is because there of a lytic promoter which gives rise to an unspliced 1.3 Kb transcript which expresses Kaposin B at minimal levels in addition to the miR-K10 and miR-K12 stem loops, thus increasing miR-K10 and miR-K12 expression during lytic replication. As the remainder of the miRNAs are located upstream of this promoter within the kaposin intron, the same effect is not observed (Gottwein, 2012; Lin *et al*, 2010; Samols *et al*, 2005; Umbach and Cullen, 2010). Functional KSHV miRNAs can be found within the virion (Lin *et al*, 2012).

Although the functions and cellular targets of many of these KSHV miRNAs remain unknown, multiple validated host and viral mRNA targets have been identified. These targets are linked to several viral and cellular processes including cell cycle progression, cell survival and apoptosis, proliferation and viral latency and immune evasion. miR-K9* represents the most well-characterized miRNA that directly suppresses viral protein expression. miR-K9* targets the 3' UTR of the RTA (ORF50) mRNA to inhibit lytic reactivation, although miR-K5 has also been shown to indirectly supress RTA mRNAs as well (Bellare and Ganem, 2009; Lu *et al*, 2010). miR-K1 has also been linked to promoting viral latency by targeting the 3' UTR of the NF-κB inhibitor IKKβ, which enhances NF-κB signalling and suppresses lytic gene expression, thus maintaining and promoting viral latency (Brown *et al*, 2003; Lei *et al*, 2010). miR-K1 is also implicated in preventing cell cycle arrest by targeting the CDK inhibiting p21, thus inducing cell proliferation and division (Gottwein and Cullen, 2010). Several components of the toll-like receptor (TLR) and interleukin (IL)-1 receptor (IL-1R) signalling cascade also represent KSHV miRNA targets. miR-K9 and miR-K5 suppress interleukin-1 receptor associated-kinase 1 (IRAK1) and myeloid differentiation primary response gene 88 (MyD88) respectively, resulting

in reduced IL-6 and IL-8 inflammatory cytokine production (Abend *et al*, 2012). In addition, the KSHV miRNAs have demonstrated various different mechanisms for preventing apoptosis of host cells. Both miR-K10 variants target the 3' UTR of the TGF-β type II receptor, thus inhibiting TGF-β signalling (Lei *et al*, 2012) and miR-K10a suppresses tumour necrosis factor (TNF)-like weak inducer of apoptosis receptor (TWEAKR). This in turn prevents TWEAK-mediated caspase activation and subsequent apoptosis and the production of proinflammatory cytokines (Abend *et al*, 2010). Additionally, miR-K1, miR-K3 and miR-K4-3 target the 3' UTR of caspase 3, thus inhibiting apoptosis (Suffert *et al*, 2011). A number of KSHV miRNAs have also been implicated in KS tumourigenesis. Several viral miRNAs, including miR-K1, miR-K3*, miR-K6-3 and miR-K11 target thrombospondin 1, an important tumour suppressor and anti-angiogenic factor (Samols *et al*, 2007). Furthermore, miR-K1, miR-K6-5 and miR-K11 target and silence cellular oncogene musculoaponeurotic fibrosarcoma (MAF), a transcription factor, which induces the reprogramming of lymphatic endothelial cells (Hansen *et al*, 2010). miR-K5 is also known to target Bcl-2 associated factor 1 (BCLAF1), an important tumour suppressor (Ziegelbauer *et al*, 2009). Collectively, therefore, these viral miRNAs may be involved in maintaining KSHV latency and contributing to KS development and tumourigenesis.

1.4 Oncogene-Induced Senescence

Primary cells are continuously exposed to various stress- and damage-inducing agents that invoke a broad range of cellular responses from apoptosis to complete recovery. One such response is cellular senescence, a form of permanent cell-cycle arrest characterized by growth cessation, alterations in the cytoskeleton resulting in a large and flattened phenotype, resistance to apoptosis and the ability to persist for extended periods of time *in vitro* (Campisi and d'Adda di Fagana, 2007). The phenomenon of senescence was first described by Leonard Hayflick and colleagues, who observed that normal cells have a limited ability to proliferate in culture. Their classic experiment demonstrated that human fibroblasts, while initially undergoing robust cell division, gradually cease proliferating until eventually all cells lose the ability to divide, but persist indefinitely in culture (Hayflick, 1965). Although Hayflick's observations were initially attributed to an inability of scientists to properly mimic a cell's natural environment *in vivo*, the process of cellular senescence is now accepted to occur in a wide variety of cell types and has

been linked to important roles in tumour suppression, aging and a number of other pathologies, underlying its crucial importance in human health.

1.4.1 Inducers of Cellular Senescence

Senescence can be triggered by a variety of stimuli. Classical or replicative senescence, as observed by Hayflick, is induced by the erosion of telomeres, stretches of repetitive DNA present at the ends of chromosomes which protect them from degradation. With each DNA replication, cells lose 50-200 base pairs of DNA due to the fact that DNA polymerases are unable to completely replicate the DNA ends. This phenomenon, known as the end-replication problem, is abrogated by telomeres, which are approximately 10-15 kilobase pairs (kb) in length and therefore afford the cell many cell divisions. Eventually, however, telomeres become critically short, which triggers the activation of the DNA-damage response (DDR) and causes the cell to senesce (d'Adda di Fagana, 2004).

Severe or irreparable DNA damage elsewhere in the genome can also induce senescence (DiLeonardo *et al*, 1994). This form of senescence is dependent on p53 and p21 expression and has also been linked to the induction of p16 (Beausejour *et al*, 2003). Several pharmaceutical agents that damage DNA, such as chemotherapeutic drugs, are potent inducers of senescence. Furthermore, chemical histone deacetylase inhibitors, which promote the formation euchromatin, initiate a similar p53-dependent DDR leading to the onset of senescence. Finally oncogenes, mutant versions of normal genes which have the ability to transform cells, can induce senescence by initiating aberrant firing of replication origins, leading to replication fork collapse, extensive DNA damage and persistent DDR signalling. Senescence in response to these deregulated mitogenic signals is referred to as oncogene-induced senescence (OIS) (Di Micco *et al*, 2006).

1.4.2 Hallmarks of the Senescent Phenotype

Senescent cells display a unique set of molecular traits that enable them to be distinguished from other forms of proliferation arrest, notably quiescence, differentiation or transient cell cycle checkpoint arrest (Adams, 2009) (Figure 1.5). Despite this characteristic

phenotype, however, to date no one feature of senescence is exclusive to the senescent state. Instead, senescence is defined by the collection of several phenotypes, each of which is induced by potent changes in cellular signalling pathways which occur during the transition to the senescent state. These include persistent DDR signalling, permanent cell cycle arrest, the accumulation of unique chromatin structures known as senescence-associated heterochromatin foci (SAHF), the activation of a distinct secretory pathway known as the senescence-associated secretory phenotype (SASP) which encompasses the secretion of various factors including cytokines, growth factors and proteases, and the expression of a β-galactosidase variant known as senescence-associated β-galactosidase (Campisi and d'Adda di Fagana, 2007).

1.4.2.1 DNA Damage Response Signalling

A common characteristic of most senescence-inducing stimuli is their ability to invoke profound DNA damage. Thus, the initiation of senescence most often begins with the detection of irreparable damage to the genome (Figure 1.5B). Oncogenes in particular induce this damage through aberrant DNA synthesis, leading to the recruitment and activation of large multiprotein complexes that sense and amplify the DDR signal (Di Micco *et al*, 2008; d'Adda di Fagagna, 2008). When DNA damage cannot be repaired, signalling through these complexes becomes persistent and senescence is induced. Surprisingly, the nature of the DNA damage affects the complexes through which it signals (d'Adda di Fagagna, 2008). Damage occurs in two variants: double-stranded DNA breaks (DSBs) or single-stranded DNA breaks (SSBs). DSBs are bound by histone variant H2AX which, together with the MRN complex, recruits and activates the Ataxia telangiectasia mutated (ATM) kinase. ATM then phosphorylates H2AX, forming γ-H2AX, which facilitates the recruitment of DDR signalling mediators, including 53BP1. These mediators amplify and transmit the DDR signal to effector proteins, notably Checkpoint kinase 2 (CHK2) and the important tumour suppressor protein, p53. In contrast at SSBs, replication protein A (RPA) binds and coats single-stranded DNA and recruits ATM-related kinase, ATR (d'Adda di Fagagna, 2008). In conjunction with the 9-1-1 complex, ATR recruits and phosphorylates H2AX and 53BP1, facilitating the transmission and amplification of the DDR signal to effector proteins, including Checkpoint kinase 1 (CHK1) and p53. This persistent activation of DDRs is essential for senescence, as signalling by ATM and ATR promotes the

formation of SAHF, the induction of the distinct SASP and proliferation arrest via activation of both the p53 and Retinoblastoma (Rb)/p16 tumour suppressor pathways (Campisi and d'Adda di Fagagna, 2007).

1.4.2.2 Control of Proliferation Arrest by Tumour Suppressors

The hallmark of cellular senescence is an inability to progress through the cell cycle. Growth arrest is dependent on the activation of p53 and Retinoblastoma (Rb)/p16 tumour suppressor pathways by DDR signalling (Figure 1.6). These pathways represent critical regulators of various aspects of the senescent phenotype. Indeed, the majority of genes involved in senescence encode proteins that feed into either of these pathways or represent downstream effectors. Reduction in p53 or p21 levels prevents the onset of damage or telomere induced senescence and can even reverse senescence growth arrest (Beausejour *et al*, 2003; Brown *et al*, 1997). Interestingly, loss of p53 or intact DDR usually results in mitotic catastrophe, which may represent a second barrier to tumour formation (Shay and Wright, 2005). The p16 tumour suppressor pathway often functions secondary to the p53 pathway (Stein *et al*, 1999). p16 is crucial to SAHF formation and cannot form with loss of p16 activity. Once formed, however, they no longer require p16 for maintenance (Narita *et al*, 2003). Unsurprisingly, many of these genes regulate cell-cycle arrest, the cytoskeleton, chromatin structure or the secretory pathway.

Senescence stimuli that generate a DDR primarily induce senescence through the p53 tumour suppressor pathway. p53 is a transcription factor critical to the integrated cellular stress response. It is regulated at various levels by proteins such as the ubiquitin ligase HDM2, which results in the degradation of p53, and the alternate-reading-frame protein (ARF) which in turn inhibits HDM2 activity (Campisi and d'Adda di Fagagna, 2007). DNA damage results in phosphorylation of p53, on serine 15, by ATM or ATR, thus stabilizing p53 and facilitating its regulation of transcription. Despite affecting the expression of up to 1500 distinct genes (Veprintsev and Fersht, 2008), the most important function of p53 in senescence is its transactivation of the gene encoding p21. p21 is a cyclin-dependent kinase (CDK) inhibitor (CKI) and, through its attenuation of CDKs, most notably CDK2, it blocks loading and firing of replication origins and impairs cell-cycle progression and proliferation. p21-mediated inhibition

of CDK2 also activates another group of master regulators of gene expression: the Rb family of tumour suppressors (Campisi and d'Adda di Fagagna, 2007). The Rb family functions primarily as repressors of gene expression, particularly targeting the expression of genes necessary for proliferation and cell-cycle progression (Campisi and d'Adda di Fagagna, 2007). Together, the p21 and Rb family proteins block the cell cycle in response to oncogene-induced DNA damage and thus reinforce proliferation arrest and senescence.

Stimuli that induce the DDR also engage another important inhibitor of proliferation, the p16 protein. p16, similar to p21, arrests the cell cycle by inhibiting CDK activity and activating Rb family proteins (Stein *et al*, 1999). Interestingly, however, p16 and p21 are not equivalent in the cellular outcomes they induce. (Sherr and Roberts, 1999). Cells that senesce due to activation of p21 via p53 are able to resume growth following inactivation of the p53 pathway (Beausejour, 2003). By contrast, cells entering senescence as a result of the p16/Rb pathway cannot resume growth, even following inhibition of p53, Rb or p16 (Bates, 1998; Zhang *et al*, 2006). p16 is expressed independently of p53 and is primarily regulated by oncogenic stress through pathways involved in chromatin remodelling (Ferbeyre *et al*, 2000; Gil and Peters, 2006; Lin *et al*, 1998; Serrano *et al*, 1997). In proliferating cells, expression from the *INK4* locus is tightly regulated by the polycomb group of transcriptional repressor complexes, a family of proteins that remodel chromatin to promote epigenetic silencing of genes (Bracken *et al*, 2007; Gil *et al*, 2004; Jacobs *et al*, 1999). By contrast, deregulated proliferation results in the disassembly of *INK4*-polycomb complexes because of the loss of histone-lysine N-methyltransferase and the specific histone methylation pattern with which it is associated (Bracken *et al*, 2007). Interestingly, the mechanisms leading to p16 expression, Rb activation and proliferation arrest can trigger senescence even in the absence of DNA damage. It is also important to note that although most cells senesce owing to the activation of the p53 or p16/Rb pathways, there exist examples of senescence which occur independently of these pathways (Michaloglou *et al*, 2005; Olsen *et al*, 2002).

1.4.2.3 Senescence-Associated Heterochromatin Foci

The role of the polycomb complexes in p16 expression highlights the importance of chromatin in regulating senescence. Transition to senescence is accompanied by profound changes in cellular chromatin structure. Within senescent cells, large sections of chromatin are condensed into distinct punctate domains throughout the nucleus, termed senescence-associated heterochromatin foci (SAHF) (Narita *et al*, 2003). These foci do not form arbitrarily on the DNA but rather incorporate, and strongly silence, genes involved in proliferation and DNA damage signalling (Di Micco *et al*, 2011; Narita *et al*, 2003). Thus, SAHF represent another mechanism by which senescent cells reinforce proliferation and growth arrest.

Predictably, the proteins that make up SAHF include multiple common heterochromatin markers. SAHF are enriched for histones that are methylated on lysine 9 of histone 3 (H3K9Me) and bound by Heterochromatin Protein 1 (HP1) (Adams, 2009). However, other components of SAHF render these structures unique, notably the presence of high mobility group AT-hook proteins 1 and 2 (HMGA1; HMGA2), macroH2A and histone 2 variant H3.3 (Narita *et al*, 2006, Zhang *et al*, 2005). Additionally, histone H1, a linker histone that plays an important role in maintaining chromatin structure, is not found within SAHF (Funayama *et al*, 2006). Regulators of SAHF formation include members of the Rb tumour suppressor family, components of the HUCA histone-chaperone complex, the Wnt signalling pathway and PML bodies (Banumathy *et al*, 2009, Narita *et al*, 2003; Ye *et al*, 2007a; Ye *et al*, 2007b). More work is needed to further elucidate the role these unique chromatin structures play in within the senescence phenotype.

1.4.2.4 The Senescence-Associated Secretory Phenotype

Cells undergoing senescence demonstrate profound changes in gene expression, particularly in those genes involved in growth and cell cycle progression, such as p16 or p21. However these changes also reflect robust activation of inflammatory responses. Senescent cells secrete high levels of cytokines, chemokines, growth factors and proteases, a secretome collectively known as the SASP. Components of the SASP include factors involved in

remodelling the extracellular matrix, such as extracellular matrix degrading enzymes, proliferation and the modulation of immune cells (Kuilman and Peeper, 2009).

The most abundant factors secreted within the SASP are interleukins, a group of cytokines that function in modulating inflammation. Senescence primarily promotes the secretion of interleukin-6 (IL-6) and interleukin-8 (IL-8) (Acosta *et al*, 2008; Kuilman *et al*, 2008; Xue *et al*, 2007), however additional interleukins, including interleukin-1α (IL-1α), interleukin-1β (IL-1β) and interleukin-7 (IL-7) are also found at low levels within senescent cells. The secretion of interleukins stimulates clearance of senescent cells by immune cells, involved in both the innate and adaptive components of the immune system (Kang *et al*, 2011; Rakhra *et al*, 2010l Xue *et al*, 2007), and suggests a role for the SASP in coordinating the removal of senescent cells. Cells deficient for IL-6 or the IL-8 receptor CXCR2 do not senesce in response to oncogenic stress, indicating that IL-6 and IL-8 secretion is necessary for growth arrest. IL-6 and IL-8 bind to their receptors on the surface of cells, resulting in the activation of the INK4 family of CKIs and p53 respectively, thus enforcing proliferation arrest. However, although the SASP helps reinforce senescence, it is not sufficient to induce senescence in proliferating cells (Acosta *et al*, 2008; Kuilman *et al*, 2008). Surprisingly, the SASP can also induce growth and angiogenic activity in adjacent cells, both in culture and *in vivo* (Coppe *et al*, 2008; Krtolica *et al*, 2001). Thus, although the SASP contributes primarily to immune cell recruitment and tissue repair, it may inadvertently induce the progression of nearby pre-malignant cells and thus create a pro-oncogenic microenvironment.

One important regulator of IL-6 and IL-8 expression, in addition to many other factors secreted within the SASP, is the nuclear factor-κB (NF-κB) signalling pathway (Hoffmann and Baltimore, 2006). NF-κB complexes are dimers composed of five key proteins: p65, p50, p52, RelB and c-Rel. Within the cell, NF-κB complexes are repressed by inhibitor of κB (IκB) proteins, which sequester them in the cytoplasm. When a stress stimulus occurs, the inhibitor of κB kinase γ (IKKγ) is activated and phosphorylates IκB, promoting its degradation and resulting in the activation of NF-κB and its translocation to the nucleus (Hoffmann and Baltimore, 2006). Within the nucleus, NF-κB complexes transactivate genes that encode many pro-inflammatory factors, including IL-6 and IL-8. The contribution of NF-κB to senescence remains controversial.

Although some reports imply that NF-κB promotes senescence bypass (Batsi *et al*, 2009; Guerra *et al*, 2011), others suggest a critical role for this protein in senescence, given the accumulation of NF-κB in senescent cells and impaired proliferation arrest upon its repression (Chien *et al*, 2011). The exact mechanism by which NF-κB is activated during senescence remains unknown, although reports suggest that this may be via activation of IKKγ by the DDR, epigenetic changes to or the activation of signalling pathways of p38 mitogen-activated protein kinases and Retinoic acid-inducible gene 1 (Salminen *et al*, 2012). Despite this controversy, regulation of the NF-κB pathway appears to play a crucial role in controlling SASP gene expression.

1.4.2.5 Senescence-Associated β-Galactosidase

DNA replication is stalled in senescent cells which can be measured by the incorporation of 5-bromodeoxyuridine or ^{3}H-thymidine or immunostaining for proteins such as proliferating cell nuclear antigen (PCNA). These markers, however, do not distinguish between senescence and other forms of growth arrest. Although all cells express β-galactosidase, senescence-associated β-galactosidase (SA-β-Gal) is the only lysosomal β-D-galactosidase that becomes strongly upregulated during senescence, primarily due to the massive expansion of the lysosomal compartment (Lee *et al*, 2006). Senescent cells activate autophagy, an important cellular pathway responsible for degrading and recycling organelles and other cytoplasmic components via lysosomal acidification (Young *et al*, 2009). Autophagy is known to be necessary for senescence and also appears to play a role in regulating the SASP, by coupling catabolic degradation of cytoplasmic components with remodelling and secretion during the transition to the senescent state (Narita *et al*, 2011). Thus, owing to its presence uniquely in senescent cells, SA-β-Gal became the first marker to be used for the specific identification of senescent cells (Dimri *et al*, 1995). Lysosomal β-D-galactosidase evolved to be maximally active at low pH, an unsurprising phenomenon given the acidic environment of the lysosome. Despite this preference, however, the enzyme remains moderately active under more neutral pH conditions (Debacq-Chainiaux *et al*, 2009). SA-β-Gal assays, which detect the presence of lysosomal β-D-galactosidase through cleavage of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) are thus conducted at pH 6.0 to measure only increased SA-β-Gal activity, thus reducing basal

background activity. SA-β-Gal remains the most commonly employed senescence marker and is detected by histochemical staining of senescent cells.

1.4.2.6 Senescence and Cancer

Cancer is defined as a disease caused by cells that have the ability to proliferate indefinitely, despite the presence of extensive DNA damage. It is a logical step, therefore, to presume that senescence, which represents an anti-proliferative process, should be tumour-suppressive. As all senescence-inducing stimuli are potentially oncogenic, cancer cells must acquire mutations that allow them to avoid this senescence. Indeed many mutations within cancer cells occur in the p53 and p16 tumour suppressor pathways (Hanahan and Weinberg, 2000). For example, cells derived from genetically engineered mice deficient in p53 fail to senesce in response to oncogenic stimuli and these mice experience higher rates of cancer compared to wild type mice (Braig *et al*, 2005; Donehower *et al*, 1992). Additionally p53 deficient cells derived from patients with Li-Fraumeni syndrome, who are notably cancer-prone, bypass senescence more readily than normal cells (Shay *et al*, 1995). Pre-malignant lesions contain large numbers of senescent cells which points to a role for senescence in halting tumourigenesis.

Studies of tumourigenesis in mice have further indicated that senescence does indeed represent a critical anticancer mechanism that prevents malignant transformation of cells through growth arrest. The most striking example of senescence in humans is melanocytic nevi (moles), which are comprised almost entirely of senescent cells (Michaloglou *et al*, 2005). Human melanocytes frequently incur mutations in the *Ras* or *BRAF* oncogenes, resulting in rapid proliferation, activation of oncogenic stress responses and the onset of OIS. Tissues from human moles have markedly upregulated markers of senescence, including profound activation of SA-β-Gal (Michaloglou *et al*, 2005). Furthermore, human melanomas often contain mutations in p53 and Rb, leading to inactivation of these respective pathway and progression to cancerous melanocytes (Ha *et al*, 2008).

Intriguingly, recent studies have indicated that, despite the beneficial effects of senescence, OIS may also play a role in tumour progression, consistent with the theory of antagonistic pleiotropy (Campisi *et al*, 2007). In particular, the senescence-associated secretory phenotype releases interleukins, chemokines, growth factors and proteases which promote proliferation of epithelial cells, stimulate angiogenesis, trigger the epithelial to mesenchymal transition and increase the growth of tumours *in vivo* (Krotlica *et al*, 2001; Liu *et al*, 2007; Coppe *et al*, 2006; Coppe *et al*, 2008). Furthermore, senescent cells are known to release nitric oxide and reactive oxygen species (ROS) that enhance cancer cell aggressiveness and promote age-related degeneration (Finkel *et al*, 2000; Finkel *et al*, 2007). The SASP releases IL-1, IL-6 and IL-8, which are responsible for increasing the invasiveness of multiple forms of cancer (Coppe *et al*, 2008), in addition to vascular endothelial growth factor (VEGF) that promotes endothelial cell migration (Strieter *et al*, 2006) and hence cancer metastasis.

1.5 Autophagy

Autophagy, derived from the Greek meaning "eating of self", is a cellular homeostatic process wherein cells engulf portions of their own cytoplasm within membranous sacs and degrade the contents (Klionsky & Emr 2000). This process, conserved from yeast to humans, represents an important means of controlling the quantity and quality of intracellular biomass. Although initially known for its role in the degradation of mitochondria and other intracellular structures, roles for autophagy have since been identified in cell development and differentiation (Mizushima & Levine, 2010), homeostasis and metabolism (Rabinowitz and White, 2010), the integrated stress response (Kroemer *et al*, 2010) and immunity (Deretic and Levine, 2009; Levine *et al*, 2011). Given its critical role in diverse biological processes, it is unsurprising that dysfunctional autophagic activity has been implicated in the pathogenesis of multiple human diseases, including aging (Madeo *et al*, 2010), neurodegenerative disorders (Nixon, 2006), microbial infections (Deretic and Levine, 2009; Deretic, 2010), various metabolic diseases (Rabinowitz and White, 2010) and cancer (Jin and White, 2007; Levine, 2007; Liang and Jung, 2010; Maiuri *et al,* 2009b; Mathew *et al*, 2007a). Consequently, autophagic pathways are subject to intense scrutiny, with the hope that the development of new therapeutic applications targeting

these pathways will emerge. As such, the characterization of the molecular basis for autophagy and its link to disease remains at the forefront of medical research.

1.5.1 The Autophagic Process

At least three distinct mechanisms of cellular autophagy have been extensively characterized in eukaryotic cells: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). In microautophagy cytosolic components are directly taken up and internalized by the lysosome via invaginations of the lysosomal membrane. CMA selectively targets cytoplasmic proteins via chaperone-mediated recognition. These substrates are subsequently translocated to the lysosome and degraded (Cuervo, 2010a; Glick *et al*, 2010; Klinosky *et al*, 2007; Mizushima *et al*, 2008). In contrast, macroautophagy (hereafter referred to as autophagy) is a major catabolic process involved in the large-scale degradation and recycling of proteins, damaged organelles, large aggregates or other intracellular biomass (Figure 1.7).

In most cell types autophagy is always active at basal levels where it acts in a constitutive fashion, removing damaged or non-functional organelles and proteins and maintaining the integrity of the cytoplasm. However, a variety of different stresses are further able to induce a strong autophagic response. These include starvation, energy deficiencies, unfolded or misfolded proteins, infection, hypoxia, mitochondrial damage and DNA damage. Under these stressful conditions, autophagy plays a role in maintaining cellular homeostasis by facilitating the clearance of damaged organelles, protein aggregates or pathogens which represent dangers to normal cellular function, and by recycling the components of these degradations back into the cytoplasm to facilitate energy production and the synthesis of new macromolecules to mitigate stress.

Autophagy begins with the formation of a flattened membranous sac known as the isolation membrane or phagophore (Figure 1.7). Although the exact origin of the isolation membrane remains elusive, recent evidence suggests that it is likely derived from the lipid bilayer (Ravikumar *et al*, 2010), the endoplasmic reticulum (Axe *et al*, 2008), the trans-Golgi network (Young *et al*, 2006) or the mitochondria (Hailey *et al*, 2010). The phagophore expands

to sequester a portion of the cytosol or organelle for degradation and eventually engulfs the cargo by forming a double-membrane bound autophagosome around it. The autophagosome matures through fusion with the lysosome, forming the autolysosome and prompting the degradation of the inner autolysosomal membrane and its contents by lysosomal hydrolytic enzymes. Lysosomal permeases and transporter molecules then mediate the export of by-products of the degradation, such as amino acids, back out to the cytoplasm where they can be reused in metabolism and the building of macromolecules (Glick *et al*, 2010; Klionsky and Emr, 2000; Yorimitsu and Klionsky, 2005; Mizushima *et al*, 2008). As such, autophagy is often viewed as a cellular recycling process that generates energy through ATP formation and protects the cell by eliminating damaged proteins and organelles (Glick *et al*, 2010).

1.5.2 Molecular Mechanism of Cellular Autophagy

The process of autophagy is initiated in response to a wide variety of stresses (Figure 1.8). This pathway requires multifaceted interplay between a group of mediators known as the autophagy-related gene (Atg) proteins, which are conserved from yeast all the way to high order eukaryotes (Mizushima *et al*, 1998). Several cellular signalling pathways converge on the Atg proteins to facilitate the induction and regulation of autophagy (Levine and Klionsky, 2004; Yorimitsu and Klionsky, 2005; Klionsky, 2007; Rubinsztein *et al*, 2007; Mairui *et al*, 2007; Levine and Kroemer, 2008; Maiuri *et al*, 2009a).

The primary integration point for stress-signalling pathways that trigger autophagy is the unc-51-like kinase (ULK) complex (Figure 1.8). ULK-complex activity is inhibited by the mechanistic target-of-rapamycin (mTOR), an important regulator of cell growth and metabolism, and activated by adenosine-monophosphate-activated protein kinase (AMPK) (Egan *et al*, 2011; Hosokawa *et al*, 2009; Kim *et al*, 2011). AMPK functions as a master regulator of energy homeostasis, sensing cellular energy levels to regulate mTOR activity. When stress is absent, mTOR complex 1 (mTORC1) is activated by Akt (also known as protein kinase B) via signalling through the class I phosphatidylinositol 3-kinase (PI3K) complex. mTORC1 phosphorylates the ULK complex, inhibiting its interaction with, and activation by, AMPK. When nutrients are limiting, or upon stress stimuli, AMPK is activated by phosphorylation on threonine 172 via

allosteric activation or phosphorylation by calcium/calmodulin-dependent protein kinase 2 (CCDPK2) in response to intracellular calcium levels. Activated AMPK phosphorylates mTORC1 repressor Tuberous sclerosis 2 (TSC2) which inhibits mTORC1 signalling to all downstream effectors and relieves its repression of the ULK complex, allowing it to interact with AMPK and resulting in ULK activation (Egan *et al*, 2011; Gao *et al*, 2002, Hosokawa *et al*, 2009; Inoki *et al*, 2002; Kim *et al*, 2011). The relief of mTOR-mediated suppression of the ULK-complex permits ULK to phosphorylate Beclin 1, an essential autophagy protein which, in conjunction with the class III PI3K and other molecules, stimulates a cascade of Atg proteins to initiate the autophagic process (Levine and Kroemer, 2008). Phosphorylation of the Beclin 1/class III PI3K complex promotes its relocalization to the endoplasmic reticulum where it functions in nucleation of the phagophore membrane (Axe *et al*, 2008). Additional regulatory proteins may also influence this complex to either promote or inhibit autophagy. One such group of regulators is the anti-apoptotic protein B cell lymphoma-2 (Bcl-2) family of proteins. Bcl-2 interacts with Beclin 1, inhibiting its interaction with class III PI3K and thus preventing the initiation of autophagy (Pattingre *et al*, 2005; Mairui *et al*, 2007). Bcl-2 is phosphorylated, and thus inhibited, by c-Jun N-terminal kinase (JNK) on serines 69,70 and 87, relieving repression of the Beclin1/class III PI3K complex and facilitating downstream activation of the autophagy pathway (Wei *et al*, 2008a; 2008b; Sinha and Levine, 2008). Autophagy is further regulated by the tumour suppressor protein, p53. In response to DNA damage, p53 transactivates a number of p53-dependent target genes, including the phosphatase and tensin homolog (PTEN), TSC2, AMPKβ, the damage-regulator autophagy modulator (DRAM), Sestrin 1 and Sestrin 2 (Budanov and Karin, 2008; Crighton *et al*, 2006; Feng *et al*, 2005). Sestrin 1 and Sestrin 2 activate AMPK to attenuate mTOR and induce the activation of autophagy. In contrast, PTEN, TSC2 and AMPKβ negatively regulate mTORC1 signalling. The role of DRAM in initiating autophagy remains unknown.

Following activation of the Beclin 1/class III PI3K complex, various important downstream components of the autophagy pathway are activated, including transmembrane proteins Atg9 and VMP1. These two proteins both function in elongation of the isolation membrane. Atg9 recruits lipids from various subcellular compartments (Tarakanova, 2010) and interacts with VMP1 and Beclin 1 to recruit proteins such as Bif-1/endophilin which help to

curve the elongation membrane (Kroemer *et al*, 2010). Continued membrane elongation and ultimate closure are controlled by two ubiquitin-like systems (Yang and Klionsky, 2010). In the first ubiquitin-like system, Atg7 acts as an E1 ubiquitin activating enzyme and activates Atg12. Atg12 is transferred to Atg10, an E2-like ubiquitin carrier protein, which covalently links Atg12 to Atg5. Conjugated Atg12-Atg5 complexes are subsequently bound by Atg16, forming an E3 ligase for the LC3 (LC3-I) protein. In association with the E1-like enzyme Atg7, and the E2-like enzyme Atg3, the Atg-15-Atg12-Atg16 complex catalyses the covalent modification of LC3 with phosphatidylethanolamine (LC3-II) (Yang and Klionsky, 2010). This lipid modification inserts LC3-II asymmetrically into the luminal and cytosolic sides of the autophagosomal membrane, resulting in curvature and closure of the autophagosome and recruitment of cytoplasmic cargo for digestion. LC3-II also has the capacity to bind to the actin cytoskeleton and regulate autophagosome trafficking through the cell. The increased synthesis and processing of LC3 in autophagy make it an important indicator of autophagy levels within the cell. Following autophagosomal closure, the autophagosome fuses with the lysosome in a process which remains poorly understood, although some evidence exists to suggest that the small G protein Rab7, in its GTP-bound active state, is required, in addition to Lamp-1 and Lamp-2 on the lysosome (Gutierrez *et al*, 2004; Jager *et al*, 2004). Evidence also suggests that this process is regulated through the Beclin 1/class III PI3K complex interacting with the class C Vps/HOPS complex (Kroemer *et al*, 2010). Fusion of the autophagosome and lysosome exposes autophagosome contents to the lysosomal hydrolases, leading to their degradation. Degraded material is then recycled back into the cytoplasm by permeases in the membrane of the autolysosome (Glick *et al*, 2010).

1.5.3 Autophagy as a Regulator of Cell Fate

1.5.3.1 Autophagy in Cancer

Given that multiple important tumour suppressors, notably AMPK, PTEN and p53, are involved in the activation of autophagy, a role for this cellular process as an anti-cancer mechanism seems indicated. Increasing evidence suggests that members of the autophagy pathway are intricately linked to the suppression of cancer development. Several key regulators

of autophagy are allelically deleted or repressed in various types of cancer. In particular, Beclin 1 is monoallelically deleted in breast, ovarian and prostate cancers (Aita *et al*, 1999). Other tumour suppressor genes which are commonly mutated or silenced in cancer, for example the aforementioned p53 and PTEN, and hyperactivated oncogenes, particularly those encoding class I PI3K, Akt, mTOR and Bcl-2, have been demonstrated to inhibit autophagy (Botti *et al*, 2006). Additionally, several types of tumour cells induce autophagy to maintain metabolic homeostasis during oncogenic transformation, allowing them to survive hypoxia and extracellular matrix detachment (Debnath, 2011, Mathew and White, 2011). Additionally, cellular defects in autophagy increase the incidence of necrotic cell death, which can lead to inflammation and acceleration of nascent tumour growth. Indeed, mice defective for autophagy and apoptosis display increased tumourigenesis compared to mice defective only for apoptosis, highlighting the importance of increased inflammation within the tumour microenvironment (Degenhardt *et al*, 2006). Autophagy, therefore, is able to suppress tumourigenesis by preventing inflammation associated with non-apoptotic cell death. Although autophagy is most commonly accepted as a tumour suppressive mechanism, several lines of evidence suggest a context-dependent role for autophagy in the promotion of tumourigenesis (Levine, 2007, Levine and Kroemer, 2008), due primarily to its role in dictating cell fate. The roles of autophagy in tumour suppression are described below.

1.5.3.2 Autophagy in Cell Survival

Autophagy is predominantly characterized as a pro-survival mechanism in the cell. Basal levels of autophagy maintain normal cellular homeostasis and activated autophagy functions as an adaptation to stress (Levine and Kroemer, 2008; Mizushima *et al*, 2008; Moreau *et al*, 2010). In its most basic form, autophagy acts as a catabolic recycling process during starvation stress, facilitating the degradation of non-essential cellular components through the lysosomal machinery into their most basic elements which are then reused as essential nutrients to maintain energy metabolism and cell survival (Mizushima and Klionsky, 2007). Indeed mice defective in autophagy die during the neonatal stage of development, highlighting the importance of autophagy in cell survival during starvation, especially in the neonatal period (Kuma *et al*, 2004). Autophagy also prevents the aggregation of misfolded proteins which would otherwise

accumulate in the cytoplasm and impede normal cellular functions (Rubinsztein, 2006). Furthermore, mitophagy, the selective autophagic degradation of mitochondria, is crucial in mitigating genotoxic stress and maintaining metabolic homeostasis. Mitochondria are key players in energy metabolism and apoptosis. When damaged, they produce massive amounts of reactive oxygen species, which are significant inducers of genomic DNA damage (Jin and White, 2007; Mathew *et al*, 2007). Autophagy also contributes to reducing genomic instability by regulating p62 levels. p62 recruits protein aggregates to the autophagosome for clearance and regulates the NF-κB signalling pathway. In cells defective for autophagy, p62 accumulates resulting in deregulated NF-κB expression, an increase in reactive oxygen species production and subsequently DNA damage (Mathew *et al*, 2009). Autophagy also plays an important role in microbial defence (see section 1.5.4.1), preventing microbial-induced cell death. Given its role in protecting the cell against these various stresses, it is no surprise that dysfunctional autophagy is associated with a large number of human diseases.

1.5.3.3 Autophagy in Cell Death

Although primarily considered to be a pathway that promotes cell survival, autophagy has also been implicated as a pro-death mechanism, albeit with some controversy. Cells which have undergone irreparable damage undergo apoptosis, the canonical programmed cell death and a common tumour suppressor mechanism (Kerr *et al*, 1972). Recently, a form of non-apoptotic cell death, known as autophagic cell death (ACD) has been described. In contrast to cells undergoing apoptosis, cells succumbing to ACD lack chromatin condensation and display large-scale engulfment of the cytoplasm within autophagosomes (Galluzzi *et al*, 2007). Initially, this autophagic degradation of cellular components essential for survival was thought to induce cell death. However emerging evidence suggests that these autophagosomes are not sufficient to cause cell death on their own and may in fact be working in collaboration with the apoptotic machinery.

1.5.3.4 Autophagy and Senescence

Owing to the fact that autophagy and cellular senescence share many characteristics, namely the ability to protect the cell from external stresses, research in the area of a possible link between these two processes is very active. Conceptually, both represent stress responses with cytoprotective functions, allowing prolonged cell survival under conditions of stress (Ouyang *et al*, 2012). The link between these processes, however, remains controversial. A number of groups have provided indirect evidence for the concurrent induction of autophagy and senescence. Recently, Young *et al.* implicated autophagy in the execution of oncogene-induced senescence (OIS). Expression of oncogenic *Ras* triggered an mTOR-dependent increase in autophagy as cells transitioned from hyperproliferation to the senescent state. Furthermore, cells deficient for essential autophagy proteins failed to undergo senescence (Young *et al*, 2009). However genetic silencing of autophagy delayed and attenuated the onset of senescence, rather than abrogating it, and the autophagy-compromised cells eventually achieved identical levels of senescence as their autophagy-competent counterparts. This suggests that autophagy may accelerate senescence but that, once induced, senescence is independent of autophagy (Young *et al*, 2009). Other groups have described correlative increases in autophagic vacuoles in senescent fibroblasts (Gerland *et al*, 2003), bile duct cells (Saski *et al*, 2010; Saski *et al*, 2012), dying senescent keratinocytes (Deruy *et al*, 2010; Gosselin *et al*, 2009), senescent endothelial cells (Patschan *et al*, 2008) and senescent human dental pulp cells (Li *et al*, 2012). Autophagy and senescence also appear to be regulated by overlapping signalling pathways (Goehe *et al*, 2012). Furthermore, Singh *et al*. have reported a significant reduction in senescent non-small cell lung cancer cells when autophagy was inhibited (Mosieniak *et al*, 2012; Singh *et al*, 2012). Finally, both autophagy and senescence are induced by BRAF and CDK inhibitors (Capparelli *et al*, 2012; Maddodi *et al*, 2010), however it remains to be determined whether a direct causation exists, or whether these results suggest a merely correlative link.

Several reports also exist to suggest an inverse relationship between senescence and autophagy, wherein the inhibition of autophagy promotes the onset of the senescent phenotype. Kang *et al*. demonstrated that the suppression of autophagy is actually permissive for senescence. They hypothesized that this was a result of the increased generation of reactive oxygen species

by damaged mitochondria in the absence of mitophagy (Kang *et al*, 2011). Furthermore, tumour protein p53 binding protein 2 (TP53BP2) mediates Ras-induced senescence by blocking Atg16-Atg5-Atg12 complex formation, thus suppressing autophagy. This finding was further supported by the fact that Ras-induced senescence is inhibited by Atg5 expression, but facilitated by Atg3 deletion (Wang *et al*, 2012). Taken together, current data is insufficient on which to base a conclusive link between autophagy and senescence. However this ambiguity may indicate a context-dependent regulation of senescence by autophagy.

1.5.4 Autophagy and Immunity

In addition to its role in cellular regulation, a function for autophagy as a defence mechanism against intracellular pathogens, and in various other aspects of immunity, has recently emerged. This defence mechanism occurs as a result of two distinct processes. The autophagic machinery is involved in the direct elimination of pathogens by selectively sequestering, and subsequently degrading, them in autophagosomes, a process known as xenophagy. These partially degraded microbial nucleic acids and peptide antigens are then delivered to autophagic structures where they play a role in the activation of both the innate and adaptive immune responses (Deretic and Levine, 2007; Deretic and Levine, 2009; Paludan *et al*, 2005; Wild *et al*, 2011). More recently, autophagy has been linked to the suppression of both the immune and inflammatory responses, suggesting that the role of this process in immunity is more complex than initially hypothesized (Levine *et al*, 2011).

1.5.4.1 Autophagy in Pathogen Defence: Xenophagy

Xenophagy (‘foreign-eat’) enables cell-autonomous elimination of pathogens by lysosomal degradation following sequestration of the pathogen in autophagosomes (Levine, 2005; Mizushima *et al*, 2008; Lin *et al*, 2010b). Xenophagy has been well characterized in anti-bacterial defence where it sequesters invading bacteria following entry and delivers them to the lysosome for degradation. Autophagy has also proved instrumental in anti-viral defence, sequestering and degrading newly synthesized virions assembling in the cytoplasm (Levine and Deretic, 2007). Although xenophagy resembles canonical autophagy, several subtle differences

exist between these two processes. One pronounced difference is the size of the autophagosomes that engulf bacteria, which tend to be substantially larger than regular autophagosomes involved in cytoplasmic recycling (Nakagawa *et al*, 2004). However given that autophagy is designed to engulf membrane-bound organelles, the autophagic degradation of a pathogen should not represent a sizeable difficulty (Levine and Deretic, 2007). The mechanisms underlying how xenophagy specifically targets free, cytosolic pathogens, on the other hand, remain poorly defined, although several possible theories exist. Firstly, recent evidence suggests that microbial proteins may be marked by different modifications, such as ubiquitination, already known to modify bacterial products, or other molecular tags and thus selected for autophagy (Balachandram, *et* al, Dupont *et* al, 2009; Fujita and Yoshimori, 2011; 2002; Kirkin *et* al, 2009; Levine and Deretic, 2007; Wild *et al*, 2011). Alternatively, pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) or retinoic-acid-inducible gene I (RIG-I)-like helicases, may recognize pathogen-associated molecular patterns (PAMPs) on the surface of bacteria or viruses to stimulate autophagic activity (Levine and Deretic, 2007).

Although the role of xenophagy in limiting bacterial infection is relatively well characterized (Nakagawa, 2004), evidence for the clearance of viruses by autophagy is only recently emerging and remains inconclusive (Orvedahl *et al*, 2010). Whether autophagy targets viruses during cell entry remains unknown, although this seems probable given its similarity conceptually to findings in the bacterial system. Furthermore, autophagy may be involved in directly eliminating viruses by breaking down host factors required for viral replication, inhibiting innate immune signalling and removing any toxic viral components (Levine and Deretic, 2007). Alternatively, several studies have suggested that autophagy may be manipulated by the virus as a source of intracellular membrane to support viral RNA replication complexes (Kirkegaard *et al*, 2004), although evidence on this point remains inconclusive. Multiple investigations support the importance of autophagy as an antiviral defence mechanism, the most profound of which suggests that to successfully infect a cell viruses need to counter autophagy. For example, multiple viruses, including herpes simplex virus and oncogenic gammaherpesviruses, encode proteins that inhibit beclin 1, thus shutting off host autophagy (Orvedahl *et al*, 2007). Other viruses inhibit the interferon (IFN)-inducible double-stranded-RNA-dependent protein kinase (PKR) antiviral signalling that induces the initiation of autophagy

in virally infected cells or else activate signalling pathways that inhibit autophagy, such as the mTOR pathway (Levine, 2005, Reviewed in more detail in Section 1.5.4.4) Evidently, therefore, xenophagy plays an important role in capturing and degrading newly assembled, and possibly cytoplasmic, virions in host cells (Kirkegaard *et al*, 2004; Levine, 2005; Wileman, 2007).

1.5.4.2 Innate Immunity

Recently, autophagy has emerged as a regulatory process in immune surveillance in infected cells. Activation of the innate immune response depends in part on the sensing of microbial PAMPs by PRRs. A subset of toll-like receptors (TLRs) that specifically sense viral nucleic acids has been found in the lumen of endosomes (Kawai and Akira, 2006). However this mechanism of endosomal TLR activation does not account for the fact that viral nucleic acids are frequently released into the cytoplasm. Recently, evidence was brought to light which suggests that autophagy mediates the recognition of viral single-stranded RNAs (ssRNAs) by TLR7 in endosomal compartments during infection by vesicular stomatitis virus (VSV) and Sendai virus. TLR7 activation by viral ssRNAs within these endosomal compartments triggers a type I IFN anti-viral response (Lee *et al*, 2007). Furthermore, detection of PAMPs by their respective PRRs upregulates autophagy in a positive feedback look, demonstrating that the innate immune system can also promote the activation of autophagy (Sanjuan *et al*, 2007; Delgado *et al*, 2008).

1.5.4.3 Adaptive Immunity

The role of autophagy in immunity is not restricted to the innate immune response; emerging evidence suggests a role for autophagy in adaptive immunity as well. Autophagy plays an important role in major histocompatibility complex (MHC) class II presentation of cytosolic antigens (both viral and self). Following autophagic sequestration and degradation of endogenously synthesized pathogenic antigens, the autophagic machinery delivers these foreign peptides to MHC class II loading compartments in late endosomes and facilitates the presentation of these antigens to the host cell, subsequently activating $CD4^+$ T lymphocytes and resulting in the activation of an adaptive immune response. Whereas canonical class II MHC involves the presentation of exogenous antigens, acquired by endocytosis and lysosomal

degradation, autophagy facilitates the antigen presentation of exogenous antigens (Schmidt *et* al, 2007). Effective viral antigen presentation to T cells has been demonstrated with a number of viral pathogens including influenza (Schmidt *et al*, 2007) and EBV (Paludan *et al*, 2005). The role of autophagy in the induction of both the innate and adaptive immune responses highlights the therapeutic potential of targeting autophagy to combat infectious diseases, and the importance of this process in eliminating intracellular pathogens.

1.5.4.4 Viral Adaptations to Autophagy

Given the importance of autophagy as a cellular defence in pathogen clearance, it comes as no surprise that many bacteria and viruses have, as a result of selective pressures and coevolution with the host to promote persistent infection and evade immunity, evolved sophisticated strategies to impede and subvert this process. Although too numerous to describe in detail, a tribute to the paramount importance of autophagy in host defence, many of these microbial operations have been reviewed elsewhere (Dreux and Chisari, 2009; Kirkgaard *et al*, 2004; Levine and Deretic, 2007; Lin *et* al, 2010; Orvedahl and Levine, 2009).

Although viruses target multiple different autophagic regulators, one common target of viral autophagic subversion is Beclin 1, an essential autophagy protein. Herpes simplex virus 1 (HSV-1) infected cell protein (ICP) 34.5 directly targets Beclin 1, as do viral homologs of Bcl-2 encoded by KSHV and murine γ-herpesvirus 68 (γ-HV68) (Ku *et al,* 2008; Pattingre *et al*.m 2005; Sinha *et al*, 2008). Further highlighting the importance of viral subversion of autophagy is the fact that direct interaction between HSV-1 ICP34.5 or murine γ-HV68 and Beclin 1 is critical for viral infection and pathogenesis in mouse models (Leib *et al*, 2009; Orvedahl *et al*, 2007). KSHV encodes a second inhibitor of autophagy, v-FLIP, a latent protein that binds to Atg3, thus blocking its function and preventing LC3 lipid modification and elongation of the autophagosomal membrane (Lee *et al*, 2009).

Surprisingly, not all viruses block autophagy as a mechanism to evade antiviral defences. In fact, several viruses actively exploit autophagy to facilitate viral replication (Kirkegaard *et al*, 2004; Lin *et al*, 2010b; Wileman, 2007). Most positive-sense RNA viruses manipulate host

endosomal and secretory compartments, believed to be autophagosomes, for membrane scaffolding during viral replication and virion assembly (Miller and Krijnse-Locker, 2008). Several RNA viruses including poliovirus (Schlegal *et al*, 1996; Suhy *et al*, 2000), hepatitis C virus (Dreux *et al*, 2009; Sir *et al*, 2008) and Dengue virus (Heaton and Randall, 2010) induce autophagy in the infected host following infection. The role of autophagy in viral infection remains unclear, however several groups have suggested that the double membranes of autophagosomes may serve as a structural support for viral genome replication (Kirkegaard *et al*, 2004; Lin *et al*, 2010).

1.6 Rationale and Objectives

At the time this study was initiated, modulation of senescence by KSHV miRNAs had yet to be examined. v-cyclin, a potent oncogene, induces a form of permanent cell-cycle arrest, known as oncogene-induced senescence (OIS), a process partially abrogated by v-FLIP. No reports existed to link viral miRNAs in aiding v-FLIP to bypass v-cyclin-induced senescence and thus allow for the development of KS.

Prior to this study, several KSHV viral miRNAs had been demonstrated to play a role in various different aspects of cancer. Reports linking KSHV miR-K9 and miR-K5 to the escape of immune recognition and clearance by the host, and thus allowing the virus to establish latency, crucial to oncogenesis, had been published (Abend, 2012). Furthermore, other miRNAs were known to inhibit apoptosis (Ziegelbauer *et al,* 2009), thus allowing for transformation. More recently, miR-K1 was demonstrated to target tumour suppressor p21, allowing cells to overcome p21-mediated cell cycle arrest (Ramalingam *et al*, 2012). Finally, other KSHV viral miRNAs were shown to interfere with TGF-β signalling, which may facilitate the development of extensive vasculature associated with KS (Samols *et al,* 2007).

Given the precedent for viral miRNAs in establishing a cellular environment conducive to tumour formation, the McCormick lab investigated whether any of these viral miRNAs may be involved in OIS bypass. To address this question, an OIS bypass screen was conducted, revealing two viral miRNAs, miR-K5 and miR-K11, with the ability to bypass v-cyclin-induced

senescence. To determine whether this effect is unique to viral infection, or whether these miRNAs are targeting more fundamental aspects of the senescence pathway, the same OIS bypass screen was conducted with Ras oncogene, a gene mutated in many different forms of cancer. Both miR-K5 and miR-K11 demonstrated the ability to bypass Ras-induced senescence, suggesting that they are targeting common elements of the senescence pathway.

Following identification of these miRNAs, further experiments were conducted to elucidate the mechanism by which they are bypassing OIS. Examination of the DDR revealed that both miR-K5 and miR-K11 altered DDR foci appearance and upregulated the phosphorylation of histone H2AX. This is consistent with data demonstrating a reduction in p16 expression as p16, a CDK inhibitor, prevents v-cyclin-induced hyperproliferation and subsequent DNA damage and the DDR. Furthermore, miR-K5 and miR-K11 inhibited autophagic flux, a key regulator of senescence, in osteosarcoma cells.

The results presented in this thesis provide compelling evidence that miR-K5 and miR-K11 are inducing bypass of OIS by suppressing p16 expression, which would be expected to result in in derepression of E2F target genes previously linked to OIS bypass (Leidal *et al*, 2012). The reduction in p16 expression may also be responsible for the observed alterations in DNA damage foci. These miRNAs appear to be contributing to senescence bypass at multiple levels, allowing the on-going proliferation of latently-infected KSHV cells despite an accumulation of DNA damage.

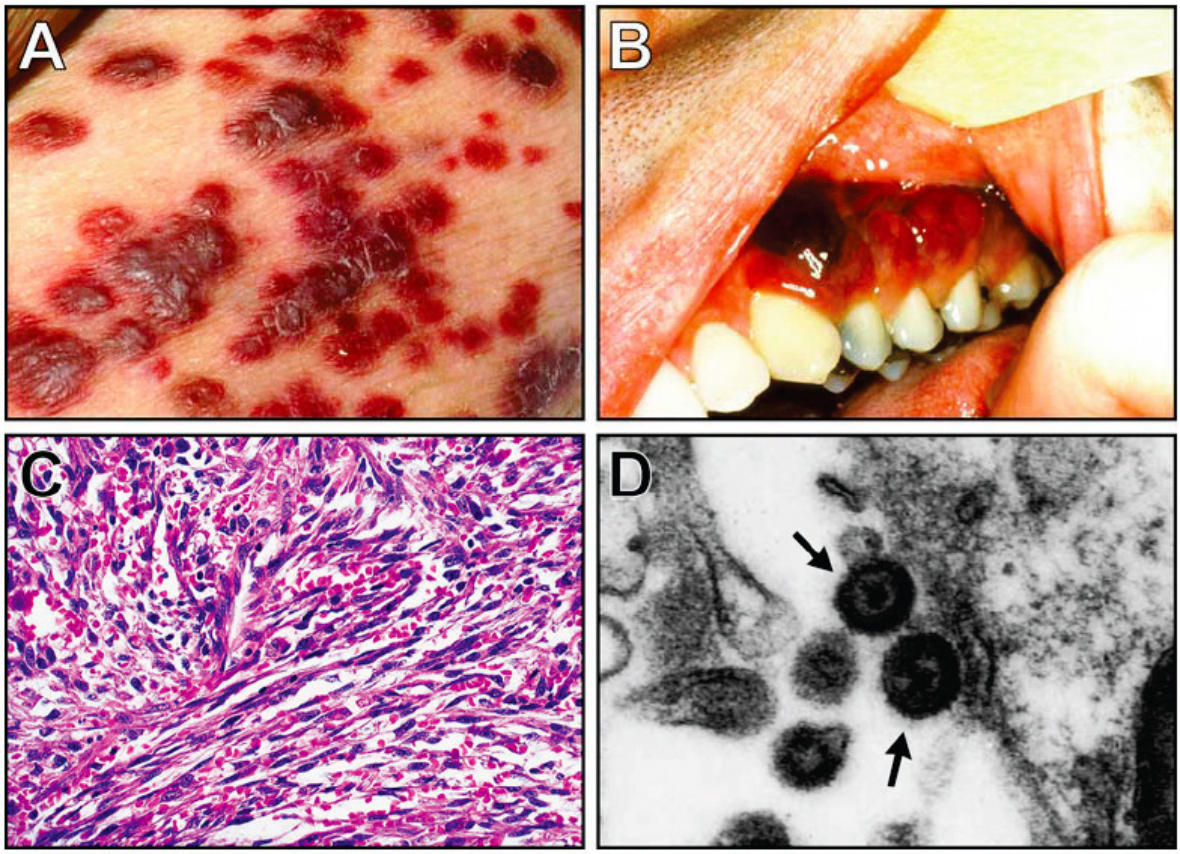
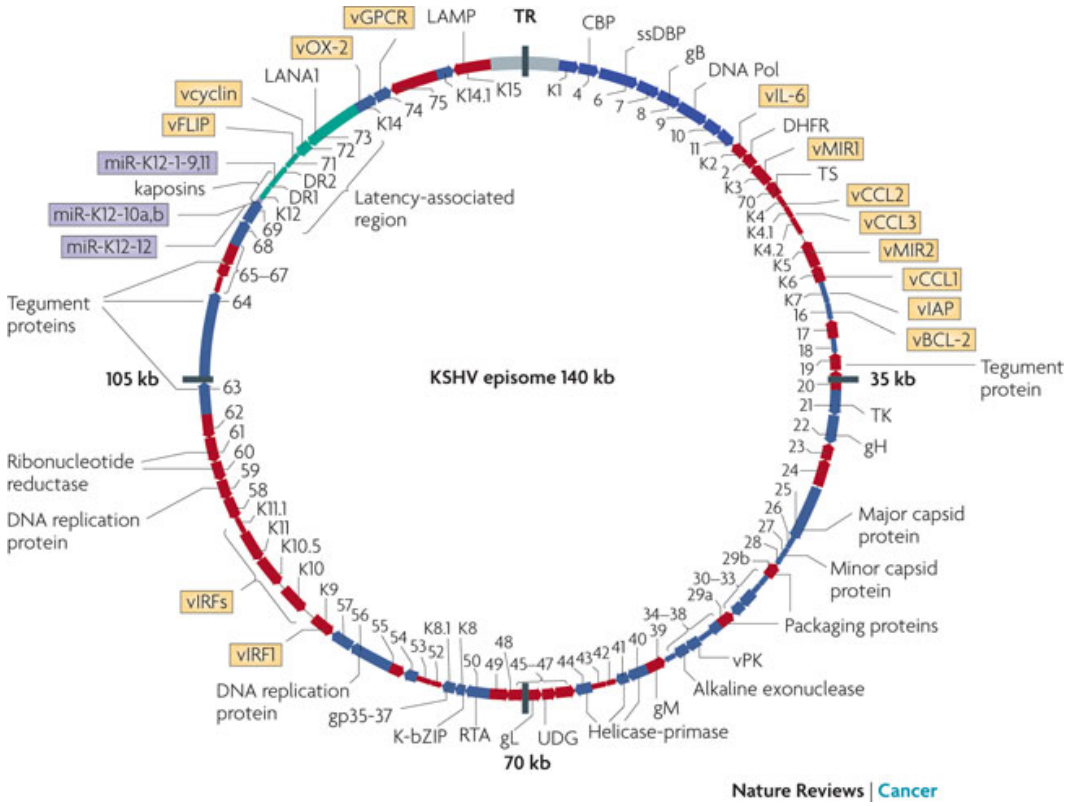


Figure 1.1 Kaposi's sarcoma. Kaposi's sarcoma (KS) often presents as dark purple or red nodular lesions. These lesions are primarily localized to the skin **(A)**, but can also be found in the oral cavity **(B)** and, in more advanced cases of KS, may spread to the lungs and gastrointestinal tract (not shown). The driving force of KS is the 'spindle cell' **(C)**, an abnormally elongated endothelial cell that is unique to KS. The etiological agent for KS development is a γ-herpesvirus **(D)** known as Kaposi's sarcoma-associated herpesvirus (KSHV; transmission electron microscopy at 36,000X magnification). Images from **(A)** and **(B)** were reproduced from the open access National Cancer Institute (NCI) Visuals Online. Image for **(C)** was reproduced from Rosai, 2004. Image for was reproduced from Antman and Chang, 2000.

A



B

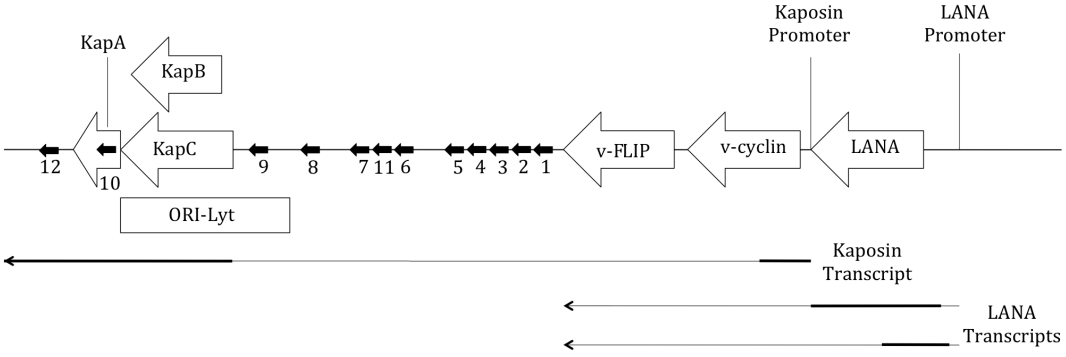


Figure 1.2 The KSHV Episome and 'Oncogenic Cluster'. (A) During latency, the KSHV genome is circularized by way of terminal repeats (TR) and maintained as an episome. Like many viral genomes, the KSHV genes are predominantly grouped based on their expression profiles. All of the latent genes are expressed from a latency-associated genomic region. Identified ORFs and various select protein products are indicated. The putative latent transcripts are in green, human orthologues are boxed in yellow, and KSHV miRNAs are boxed in purple. Refer to text for additional details. Figure was reproduced from Mesri *et al.*, 2010. **(B)** The KSHV 'oncogenic cluster' and the transcripts that are expressed from the latency locus. Alternative splicing produces three transcript variants. Refer to text for additional details.

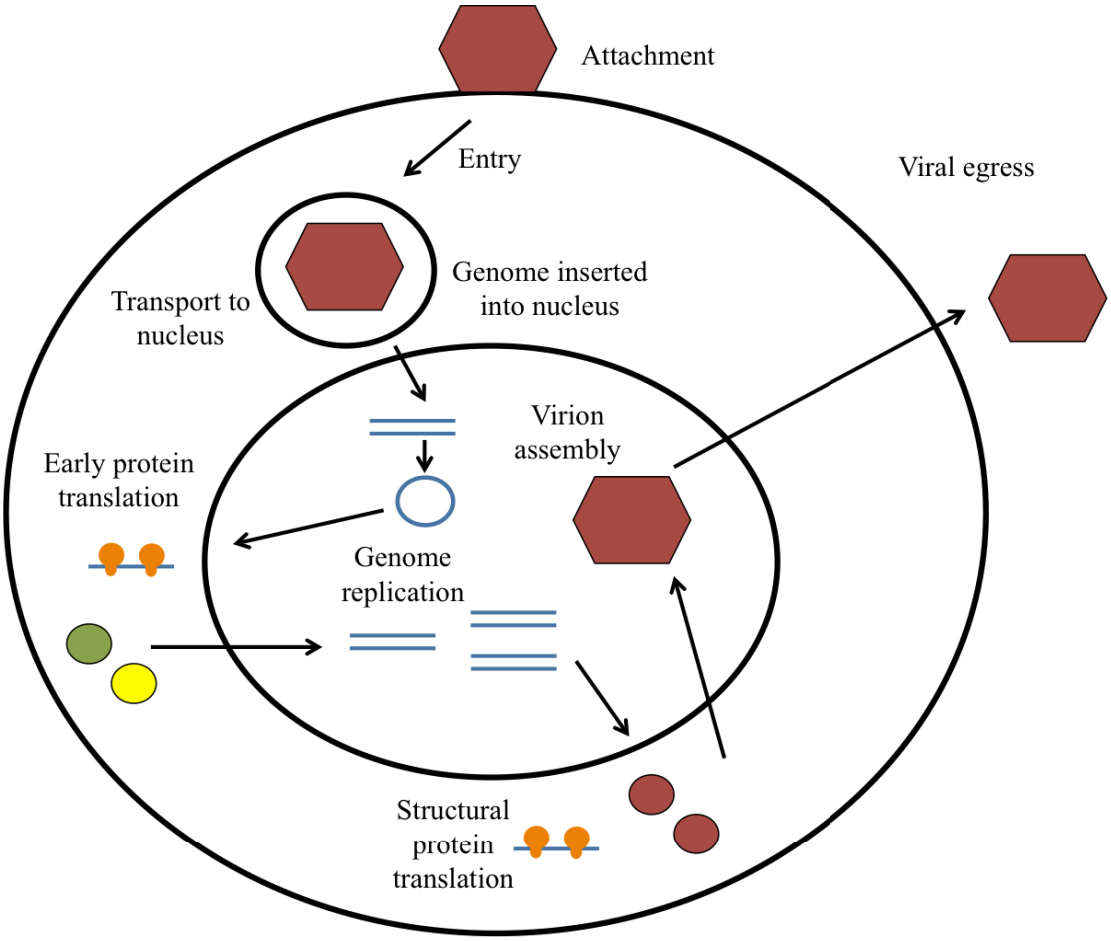


Figure 1.3 The KSHV Replication Cycle. KSHV interacts with multiple cell surface receptors allowing for viral entry via endocytosis. Tegument and capside are delivered to the nucleus and the viral genome is introduced into the nucleus via nuclear pores. Inside the nucleus the linear dsDNA genome is circularized and rapidly bound by chromatin, effectively blocking gene expression from lytic viral promoters and promoting establishment of latent infection. Expression of the viral lytic transactivator protein RTA is required to initiate lytic replication by transactivating multiple viral early gene promoters. Delayed early genes are then expressed and orchestrate genome replication. Finally, late genes are expressed, encoding structural proteins which form new viral particles into which the genome is incorporated. The virus then acquires a tegument layer and buds from host membranes containing KSHV glycoproteins.

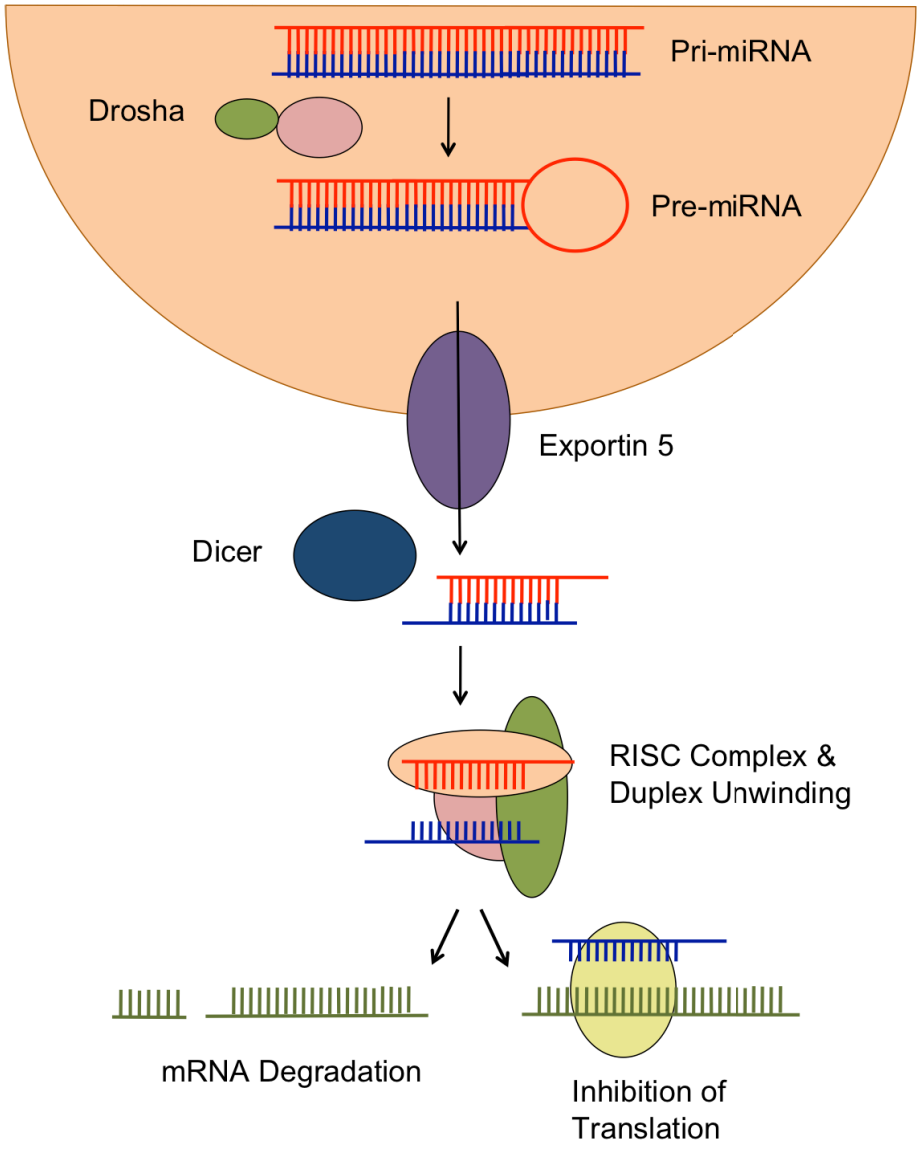


Figure 1.4 miRNA biogenesis and RNA silencing. RNA polymerase II-like primary-miRNA transcripts are cleaved by Drosha, freeing the pre-miRNA. The pre-miRNA is subsequently exported from the nucleus into the cytoplasm where the hairpin loop structure is cleaved by Dicer forming a mature miRNA. This miRNA is incorporated into the RISC wherein the strands are separated and guide strand used to target and degrade complimentary mRNAs, resulting in sequence-specific gene silencing.

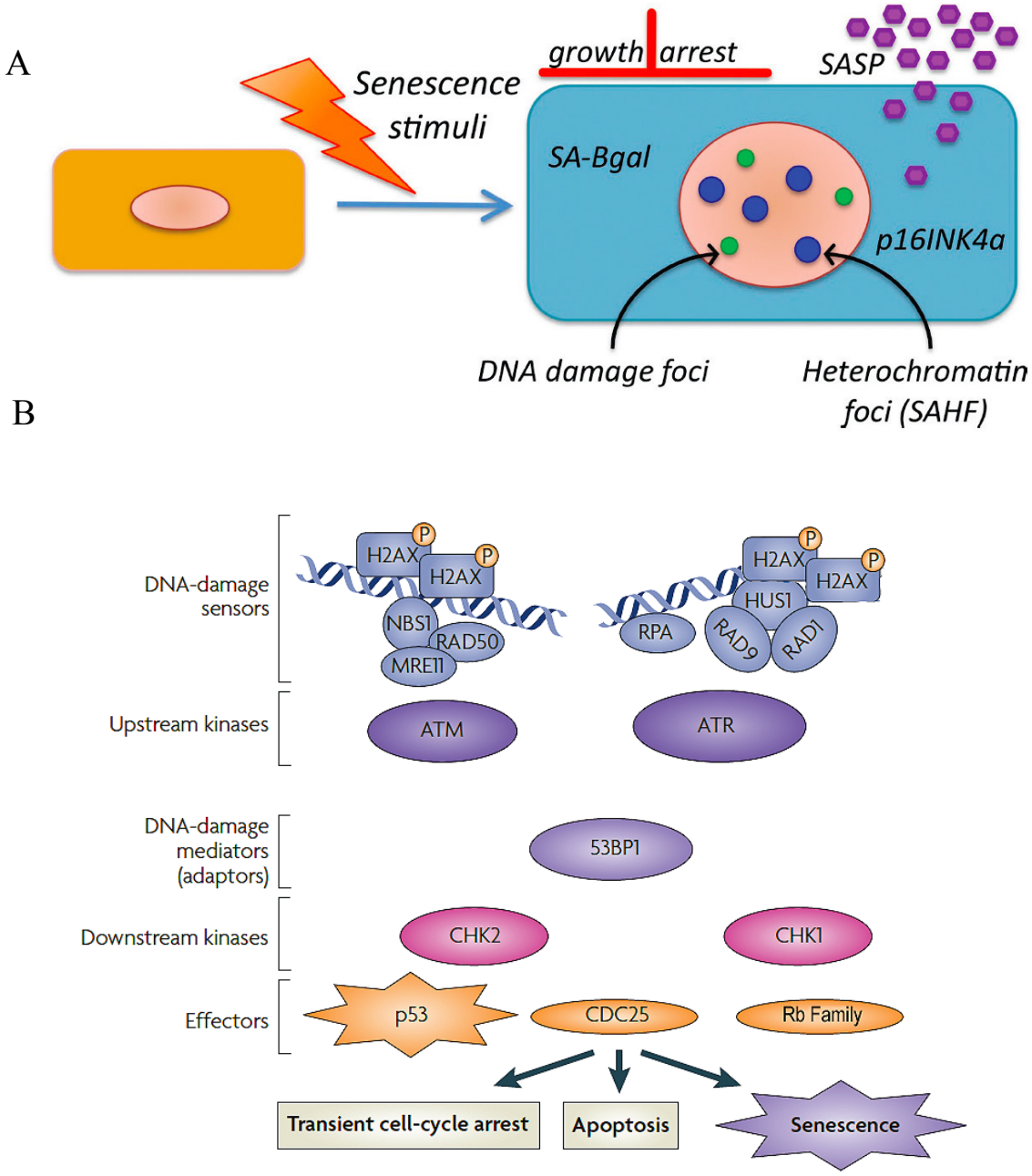


Figure 1.5 Hallmarks of Senescent Cells and DDR Activation. (A) Hallmarks of senescent cells include permanent growth arrest, DNA-damage foci, SAHF, the SASP; increased SA-β-gal activity and often an upregulation of p16. Adapted from Rodier and Campisi, 2011. **(B)** DNA damage in the form of dsDNA breaks (left) is bound by histone H2AX and the MRN complex to promote activation and recruitment of ATM, which signals to downstream effectors to amplify and transmit the DDR signal; at ssDNA breaks (right) RPA, H2AX and the 9-1-1 complex help to activate the ATR signal transduction. Ultimately, both ATM and ATR signaling cascades converge upon key effectors, which are recruited to the kinases by DNA damage mediators, such as 53BP1, and function to induce senescence or other cell fates. See text for further details. Adapted from Campisi and d'Adda di Fagagna (2007).

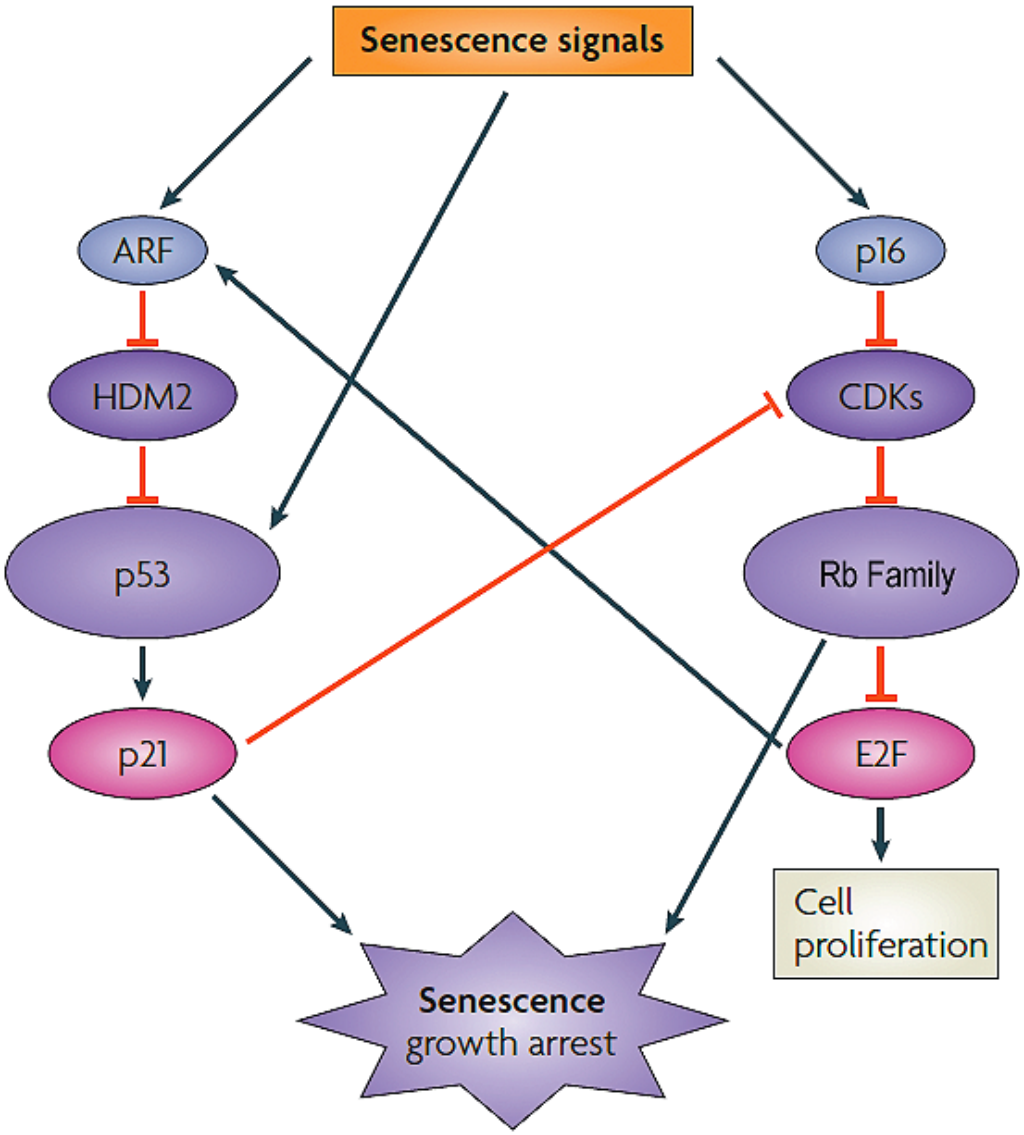


Figure 1.6 Control of Senescence by the p53 and p16 Tumour Suppressor Pathways. Senescence stimuli trigger DDRs and engage the p53 and p16 tumour suppressor pathways to induce cell cycle arrest. p53 is activated by phosphorylation and proteins, such as ARF, that disrupt its interaction with HDM2. Subsequently, p53 transactivates target genes, leading to the expression of the CKI p21. Blockade of cellular CDK2 by p21 activates the Rb family of transcription repressors that inhibit expression of many genes involved with cell-cycle progression, including those transactivated by the E2F family. Senescence stimuli, through complex mechanisms involving chromatin remodeling, upregulate p16 expression. Subsequently, p16 blocks CDK4, activating the Rb family. Together, these mechanisms enforce proliferation arrest within the senescence program. Adapted from Campisi and d'Adda di Fagagna, 2007.

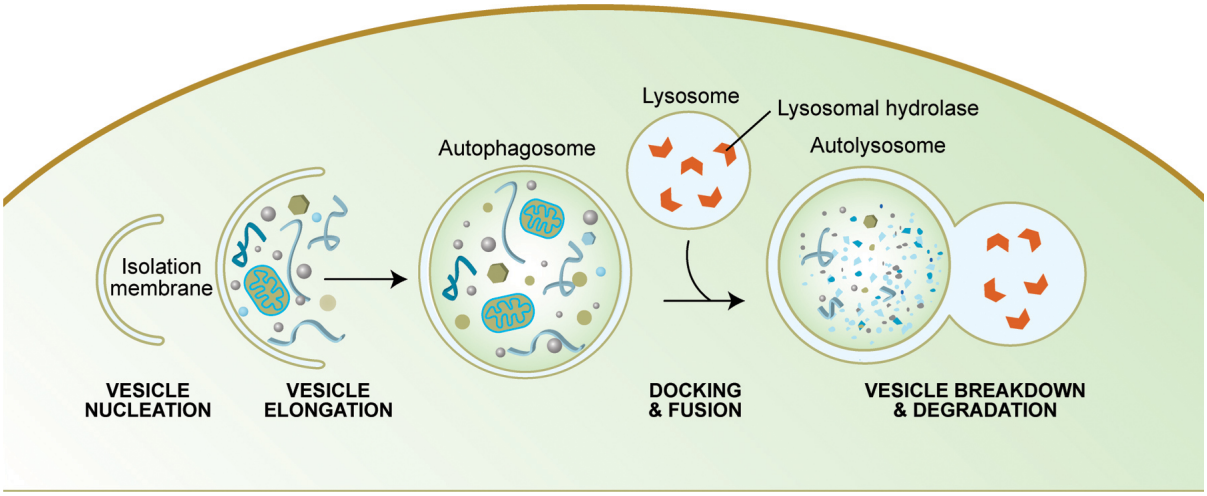


Figure 1.7. Steps Involved in the Biogenesis and Maturation of Autophagosomes. Cellular stress signaling pathways trigger the ULK complex, which subsequently promotes the activation of the Beclin 1/class III PI3K complex, inducing nucleation of the phagophore membrane. Atg9 and VMP1, in addition to LC3 processing, induce phagophore membrane elongation and, ultimately, closure of the autophagosome. The autophagosome, complete with its sequestered cytoplasmic contents, fuses with the lysosome, creating an autolysosome and triggering the degradation of autophagosomal contents via lysosomal hydrolases. The degraded contents are subsequently released back into the cytoplasm where they are reused by the cell. See text for more details Adapted from Melendez and Levine, 2009.

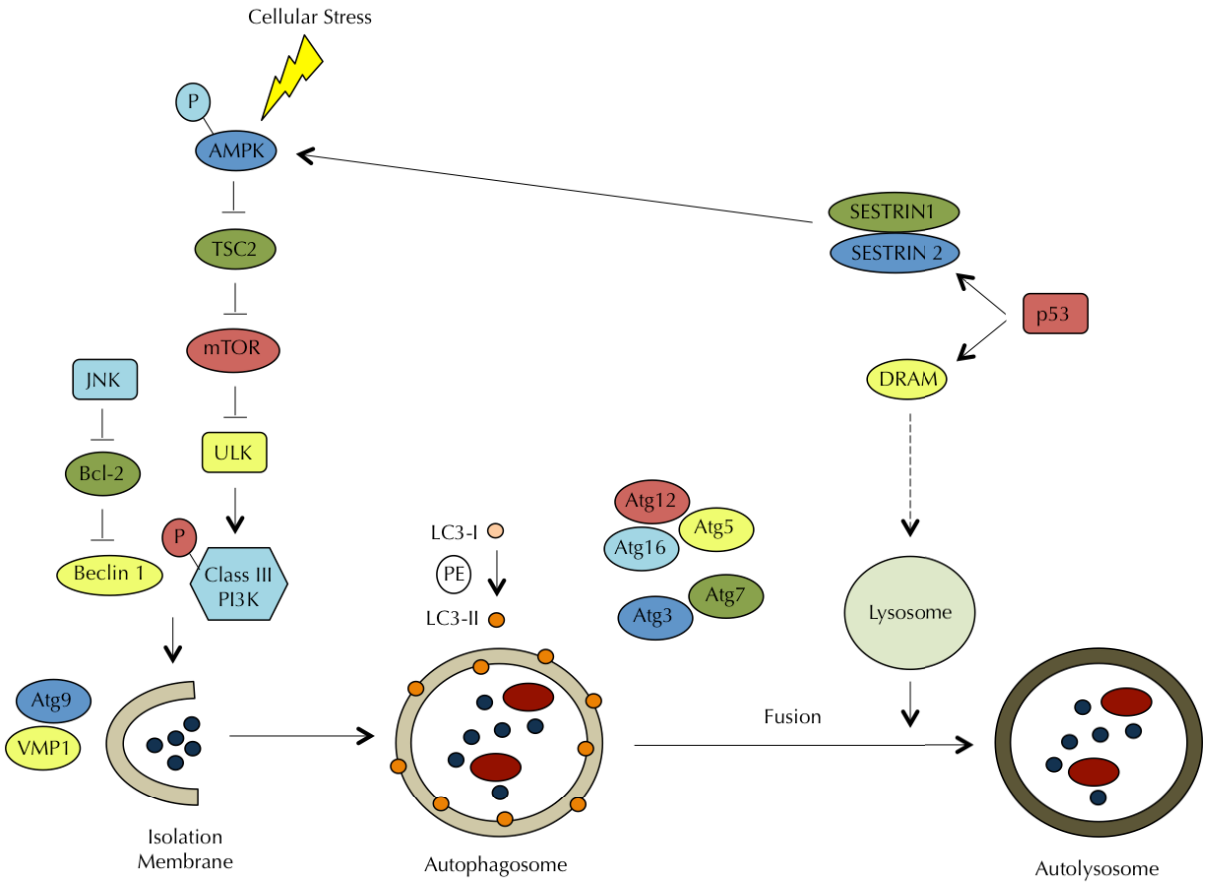


Figure 1.8 Stress Signaling and the Activation of Autophagy. A simplified schematic of the regulation of autophagy by the three different mechanisms: Regulation of the AMPK signaling axis through ULK activation, JNK-mediated repression of Bcl-2 and the modulatory role of p53 promoting the expression of Sestrin 1, Sestrin 2 and DRAM. In addition, covalent lipid modification of LC3, a well-characterized essential component of the autophagosome, and its incorporation into the autophagosomal membrane is also noted. Refer to text for additional details.

Table 1.1 Validated Targets of KSHV miRNAs

Target	miRNA	Reference
MICB	miR-K7	Nachmani *et al*, 2009
CEBPB	miR-K3, miR-K7	Qin *et al*, 2010
p21	miR-K1	Gottwein and Cullen, 2010
NFKB1A	miR-K1	Lei *et al*, 2010
RBL2	miR-K4-5	Lu *et al,* 2010
IKBKE	miR-K11	Liang *et al*, 2010
NFIB	miR-K3	Lu *et al*, 2010
THBS1	miR-K1, miR-K3*, miR-K6-3, miR-K11	Samols *et al*, 2007
BCLAF1	miR-K5, miR-K9, miR-K10a, miR-K10b	Ziegelbauer *et al*, 2009
BACH1	miR-K11	Skalsky *et al*, 2007
TWEAKR	miR-K10a	Abend *et al,* 2010
MAF1	miR-K1, miR-K6-5, miR-K11	Hansen *et al*, 2010
NHP2L1	miR-K3	Dolken *et al*, 2010
LRRC8D	miR-K3	Dolken *et al*, 2010
EXOC6	Intronic Cluster	Dolken *et al*, 2010
GEMIN8	miR-K4-3	Dolken *et al*, 2010
ZNF684	Intronic Cluster	Dolken *et al*, 2010
CDK5RAP1	Intronic Cluster	Dolken *et al*, 2010
RTA	miR-K9*, miR-K7, miR-K5	Bellare and Ganem, 2009
MyD88	miR-K5	Abend *et al*, 2012
IRAK1	miR-K9	Abend *et al*, 2012
AID	miR-K5, miR-K11	Bekerman *et al*, 2013

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Culture

Phoenix retroviral-packaging cells were provided by Garry Nolan (Stanford). U2OS osteosarcoma cells and BJ and IMR90 human foreskin fibroblasts (HFFs) were obtained from American Type Culture Collection (ATCC). All cell lines were grown at 37^{o}C in a humidified 5% CO_2 atmosphere. Phoenix and U2OS cells were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 10% heat-inactivated (HI) fetal bovine serum (FBS; Life Technologies), 100 units/mL penicillin and 100 μg/mL streptomycin (10,000 U/mL Pen/Strep; Life Technologies). BJ and IMR90 HFFs were cultured in minimum essential medium (MEM; Life Technologies) supplemented with 10% HI FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 1X MEM non-essential amino acids (NEAA; Life Technologies) and 1mM sodium pyruvate (Life Technologies). Phoenix cells were subcultured every two days and BJ and IMR90 HFFs were subcultured every four days. Unless otherwise specified, media was refreshed every second day to ensure that cellular activation of autophagy or senescence was not due to culture-associated stresses.

2.2 Vectors

The pBMN retroviral vector system was used for this study to stably express genes (Gary Nolan, Stanford University) within the cells. To ensure transduction and expression of each gene in infected cells, a variety of retroviral vectors were engineered to express puromycin (puro) or blasticidin (blast) resistance from an internal ribosomal entry site (IRES) within the vector-derived transcripts. Drew Leidal (University of California, San Francisco) generated the pBMN-IRES-puro-v-cyclin and pBMN-IRES-puro-Ras vectors.

2.3 Recombinant Retrovirus Production and Transduction

For preparation of recombinant retroviruses, Phoenix cells were seeded (4.5 x 10^6 cells/dish) into 100 mm cell culture dishes, incubated for 24 hours then transfected with 6 μg of a

retroviral plasmid using polyethylenimine (PEI; Sigma) in serum- and antibiotic-free DMEM. Transfection medium was removed 4 hours later and replaced with complete medium. Viral supernatants were collected at 48 hours post-infection, filtered through a 0.45 μm filter (Millipore) and supplemented with 4 μg/mL sequabrene (Sigma). Retroviral preparations were then aliquoted and stored at -80^{o}C until use.

For retroviral transductions, target cells were seeded into 12-well dishes 24 hours prior to retroviral infection. Culture media was replaced with virus-containing supernatants and culture plates were centrifuged at 2,000rpm for 2 hours at 30^{o}C in a JS-5.3 rotor. Following centrifugation, cells were overlaid with fresh growth medium and allowed to recover for 24 hours. Transduced cells were subsequently selected in 1 μg/mL puromycin (Sigma) for 48 hours or 5 μg/mL blasticidin (Sigma) for 96 hours. Following selection, cells were washed twice with PBS (Wisent). Cells were subsequently re-seeded into tissue culture dishes appropriate for individual assays or individual assays were performed directly.

Clonal U2OS mCherry-GFP-LC3 cells were generated by infecting U2OS cells with the pBMN-IRES-blast-mCherry-GFP-LC3 vector. Following infection, cells were selected with blasticidin for 96 hours. Selected cells were subsequently serially diluted in 96-well dishes and wells harbouring a single clone were isolated to form a stable cell line.

2.4 Antibodies

Primary antibodies used for immunoblot analysis are listed in Table 1. Primary antibodies used for indirect immunofluorescence (IF) microscopy included rabbit anti-53BP1 PAb at 1:200 (Santa Cruz Biotechnology Inc; sc-22760) and rabbit anti-γH2AX PAb at 1:50 (Cell Signalling Techonology (CST); 2577). Secondary antibodies used for immunoblot detection included Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (CST; 7076) and HRP-conjugated goat anti-rabbit IgG (CST; 7074). All secondary antibodies for immunoblotting were used at 1:2000. Secondary antibodies used for indirect detection of primary antibodies for immunofluorescence included Alexa Fluor® 488-conjugated goat anti-rabbit IgG at 1:500

(Molecular Probes; A-11008) and Alexa Fluor® 555-conjugated goat-anti-rabbit IgG at 1:500 (Molecular Probes; A-21428).

2.5 Immunoblotting

To prepare whole-cell extracts, cells were washed twice with ice-cold PBS, lysed in equal volumes of 2X Laemmli buffer (62.5 mM Tris-HCL pH 6.8, 25% glycerol, 2% SDS) then scraped and collected in microcentrifuge tubes. Lysates were passed through a 21-gauge needle then boiled for 5 minutes at 95^{o}C then vortexed briefly. Lysates were supplemented with 10 μg/mL dithiothreitol (DTT; Sigma) and 0.01% bromophenol blue (Bio-Rad) and stored at -20^{o}C. Upon thawing, sample lysates were boiled again at 95^{o}C for 5 minutes then resolved alongside pre-stained protein markers (New England Biolabs (NEB); P7708S) by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to methanol-activated Immuno-Blot PVDF Membranes (Bio-Rad) by wet transfer. Gels were transferred in transfer buffer (20% v/v Methanol, 25mM Tris, 192mM Glycine pH 8.3) for 1 hour at 100V. Membranes were blocked for 1 hour at room temperature (RT) in either milk or bovine serum albumin (BSA) blocking buffer (Tris-buffered saline [TBS; 20mM Tris pH 7.5, 150mM NaCl] containing 0.05% Tween-20 [TBS-T] and either 5% w/v non-fat dry milk powder or 5% w/v BSA [BioRad]) and incubated with the indicated primary antibodies at the specified dilutions (see section 3.5) in milk or BSA blocking buffer at 4^{o}C. Membranes were then washed thoroughly in TBS-T, incubated with the indicated HRP-conjugated secondary antibodies at specified dilutions (see section 3.5) in milk or BSA blocking buffer for 1 hour at RT. Membranes were washed thoroughly in TBS-T, developed using the enhanced chemiluminescence assay according to the manufacturer's instructions (ECL 2 Western Blotting Detection System; Pierce) and visualized with a Kodak Image Station 4000MM PRO (Carestream Health Inc.). Image capture was performed using Carestream Molecular Imaging Software (Carestream Health Inc.) and densiometric quantification of protein signals was conducted in ImageJ. Protein signals were measured as an integrated volume with correction for a defined background then normalized to pan-actin signals. Images were annotated using Microsoft PowerPoint (Microsoft).

2.6 Immunofluorescence

Cells to be processed for immunofluorescence were re-seeded at 60% confluency onto glass coverslips 24 hours prior to fixation. Cells were washed twice with PBS then fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 10 minutes at RT. Following fixation cells were washed twice with PBS and permeabilized in ice-cold 90% methanol for 10 minutes. Cells were then washed twice with PBS, blocked in filtered 3% FBS in PBS for 1 hour at RT and incubated with primary antibody (see section 3.5) in blocking buffer overnight at 4^{o}C. Cells were subsequently washed thoroughly with PBS and incubated with secondary antibodies (see section 3.5) in blocking buffer for 1 hour at RT then washed thoroughly again with PBS. Coverslips were then mounted onto microscope slides with Prolong Gold Antifade Reagent (Molecular Probes), sealed with nail varnish and examined using a Zeiss Axiovert 200M microscope equipped with a Hamamatsu Orca R2 Camera. Images were captured using Axiovision software (Carl Zeiss Inc.).

Foci number and size were quantified using a macro program in FIJI software (Schindelin *et al*., 2012) developed by Robertson Holden. This program splits the image into the red/green/blue (RGB) channels then closes the red, as there is no information of note in this channel. It then allows the user to manually threshold the images and each channel is thresholded individually. The program then clears any previous results from the region of interest (ROI) manager and sets the desired measurements to be made in the blue channel, in this case, measuring nuclear area. The two thresholded images are merged into a stack and the area of the nuclei in the blue channel are analysed. A watershed function is then performed on the foci in the green channel. This function determines the centre of each foci then calculates a distance map from object centre to the edges of the object then fills in the middle. It also creates dams where two watersheds meet to ensure they remain separate. The program then uses the ROIs determined by the analysis of the nuclei to analyse the foci within each nucleus, measuring their number and size.

2.7 microRNA Transfection

miRVANA miRNA mimics were obtained (Invitrogen) for miRNAs of interest and diluted to 5 μM stock concentrations in H_2O. BJ, IMR90 and U2OS cells were seeded at 2.5 x 10^4 cells/well into 12-well plates. 24 hours later, media was removed and cells were washed twice in antibiotic-free media. Cells were then transfected with miRNA mimics using DharmaFECT 1 Transfection Reagent (Thermo Scientific) to a final miRNA concentration of 25 nM according to manufacturer's instructions. 48 hours post-transfection, cells were washed twice with PBS and lysed or fixed, according to individual assay being conducted. miRNA sequences listed in Table 2.

2.8 Senescence-Associated β-Galactosidase Assay

Senescent cells were identified using a Senescence-Associated β-Galactosidase (SA-β-Gal) assay according to manufacturer's instructions (CST). This protocol detects SA-β-Gal activity at pH 6, a characteristic unique to senescent cells (Dimri *et al*., 1995). Cells were fixed as described above then stained for 24-36 hours at 37^{o}C in a non-humidified incubator, washed twice and overlaid with PBS. Plates were stored at 4^{o}C until analysis. The SA-β-Gal staining was analysed using a Nikon Inverted Diaphot-TMD Microscope and imaged with a Nikon D300s Camera. Images were digitally captured with Camera Control Pro 2 (Nikon Corporation). SA-β-Gal activity appears as blue staining in this assay. The number of senescent cells was calculated by evaluating SA-β-Gal staining of approximately 3000 cells at 100X magnification for each experimental condition.

2.9 Chemical Treatments

To examine autophagy in BJ and U2OS cells, culture media was supplemented with 250nM Torin 1 (Sigma). 4 hours post-treatment, cells were washed twice with PBS then fixed or lysed, according to individual assays being conducted. To investigate effects on autophagic flux, culture medium was supplemented with Bafilomycin A1 (Sigma), an inhibitor of lysosomal acidification. Bafilomycin was prepared in DMSO and diluted from frozen stocks into culture

medium for a final concentration of 100nM. Cells were treated for 4 hours prior to lysis of fixation for autophagy analysis. Alternatively, cells were treated with Chloroquine (Sigma), an inhibitor of autophagosome/lysosome fusion. Chloroquine was prepared in DMSO and diluted from frozen stocks into culture medium for a final concentration of 1mM.

To elicit a cellular DNA damage response (DDR), BJ and U2OS cells were treated with 5 μg/mL etoposide (ETP; Sigma) for 6 hours, washed twice with PBS then fixed or lysed, according to individual assays being conducted.

To induce expression of H-Ras in the inducible cell line, BJ cells stably transduced with the pLNCX-ER-H-Ras vector were treated with 1mM 4-hydroxytestosterone for 72 hours. Following treatment, cells were washed twice with PBS and cells were overlaid with complete media.

2.10. Fluorescent Microscopy of Autophagic Structures

To visualize autophagic structures by fluorescence microscopy, U2OS mCherry-GFP-LC3 cells were seeded onto coverslips then transfected with the miRNA mimics and/or treated with 250 nM Torin 1, 100 nM Bafilomycin and/or 1 mM Chloroquine for 4 hours. Cells were then washed twice with PBS and fixed with 4% paraformaldehyde as described above. Coverslips were mounted onto slides using Prolong Gold Anti-Fade Reagent and sealed with nail varnish. Slides were visualized directly for autophagic structures using a Zeiss Axiovert 200M microscope equipped with a Hamamatsu Orca R2 Camera. Images were captured using Axiovision software (Carl Zeiss Inc.).

2.11 Statistics

Data was analysed using Graphpad Prism 6 software. Data was subjected to a multiple comparison comprising a one-way ANOVA followed by a Dunnett's test.

Table 2.1 Antibodies and Dilutions

Target	Company/Catalogue #	Dilution
γH2AX	Cell Signalling/ #2577	1:1000
53BP1	Santa Cruz/ sc-22760	1:200
p16/INK4a	Cell Signalling/ #4824	1:1000
p53	Santa Cruz/ sc-126	1:200
p21	Santa Cruz/ sc-6246	1:200
p14/ARF	Cell Signalling/ #2407	1:1000
ID1	Santa Cruz/ sc-488	1:200
p-Bcl-2 S70	Cell Signalling/ #2827	1:1000
p-Rb (Ser807/811)	Cell Signalling/ #9308	1:1000
CDK4	Cell Signalling/ #2906	1:1000
CDK6	Cell Signalling/ #13331	1:1000

Table 2.2 miRVANA miRNA Mimic Sequences

miRNA Mimic	Sequence
NS Cntl	Not Available
KSHV miR-K12-1	ATUACAGGAAACUGGGUGUAAGCUG
KSHV miR-K12-2	AACUGUAGUCCGGGUCGAUCUGA
KSHV miR-K12-3	UCACAUUCUGAGGACGGCAGCGACG
KSHV miR-K12-3*	UCGCGGUCACAGAAUGUGACA
KSHV miR-K12-4-3	UAGAAUACUGAGGCCUAGCUGA
KSHV miR-K12-4-5	AGCUAAACCGCAGUACUCUAGG
KSHV miR-K12-5	UAGGAUGCCUGGAACUUGCCGGU
KSHV miR-K12-6-3	UGAUGGUUUUCGGGCUGUUGAGC
KSHV miR-K12-6-5	CCAGCAGCACCUAAUCCAUCGG
KSHV miR-K12-7	UGAUCCCAUGUUGCUGGCGCUCA
KSHV miR-K12-8	CUAGGCGCGACUGAGAGAGCAC
KSHV miR-K12-9	CUGGGUAUACGCAGCUGCGUAA
KSHV miR-K12-9*	ACCCAGCUGCGUAAACCCCGCU
KSHV miR-K12-10a	UUAGUGUUGUCCCCCCGAGUGGC
KSHV miR-K12-10b	UUGGUGUUGUCCCCCCGAGUGGC
KSHV miR-K12-11	UUAAUGCUUAGCCUGUGUCCGA
KSHV miR-K12-12	AACCAGGCCACCAUUCCUCUCCG
KSHV miR-K12-12*	UGGGGGAGGGUGCCCUGGUUGA

CHAPTER 3: RESULTS

3.1 KSHV miR-K5 and miR-K11 elicit bypass of v-cyclin-induced senescence

As mentioned above, KSHV encodes a homolog of cellular D-type cyclins, v-cyclin, which deregulates cellular proliferation and triggers DNA damage checkpoint activation, resulting in irreversible cell cycle arrest and oncogene-induced senescence. Surprisingly, however, while cells latently infected with KSHV demonstrate an upregulated DDR these cells are not senescent, suggesting viral evasion of the senescence program. Leidal and colleagues screened the remaining KSHV latency locus proteins for the ability to supress v-cyclin-induced senescence and demonstrated that v-FLIP is able to subvert v-cyclin-induced autophagic responses thus blocking OIS in cells latently infected with KSHV. Interestingly, latently-infected cells expressing v-FLIP inhibitory peptides demonstrated higher levels of autophagy and senescence, compared to control cells, yet continued to demonstrate residual OIS bypass, suggesting that other factors are involved in the evasion of cellular senescence (Leidal *et al*, 2012).

Also expressed during latent KSHV infection is a cluster of viral miRNAs. Although the functions and cellular targets of many of these miRNAs remain unknown, several have been demonstrated to target pathways involved in cell cycle progression, cell survival and apoptosis, while others target tumour suppressors and oncogenes, suggesting a role for these miRNAs in cellular transformation and KSHV tumourigensis. To test whether one or more of the KSHV miRNAs may be involved in the subversion of v-cyclin-induced OIS, an OIS bypass screen was conducted.

BJ human foreskin fibroblast cells (hereafter referred to as BJ) were transduced with a retrovirus encoding v-cyclin or an empty retroviral vector and selected for 48 hours with puromycin. 72 hours post-infection the cells were transiently transfected with miRVana mimics, chemically enhanced miRNA mimics obtained from Life Technologies, of each of the individual 18 mature KSHV miRNAs and a non-specific (NS) scramble control. 48 hours post-transfection, cells were fixed and senescent cells were detected using a senescence-associated β-galactosidase

(SA-β-Gal) kit, according to manufacturers instructions. Wells were imaged by bright field microscopy (Figure 3.1B) and images were quantified using ImageJ cell counter software (Figure 3.1A). Cells transfected with v-cyclin demonstrated dramatically increased levels of senescence compared to the empty vector-infected cells. Furthermore, v-cyclin-transduced cells transiently transfected with miR-K5 and miR-K11 consistently demonstrated a significant decrease in the percentage of senescent cells compared to the NS control (Figure 3.1).

These results suggest that KSHV miR-K5 and miR-K11 do indeed allow for bypass of v-cyclin-induced senescence. This finding indicates a role for the viral miRNAs in KSHV-induced transformation and tumourigenesis.

3.2 miR-K5 and miR-K11 elicit bypass of Ras-induced senescence

To determine whether the ability of miR-K5 and miR-K11 to bypass OIS is unique to KSHV v-cyclin-induced senescence, the effect of these miRNAs on Ras-induced senescence was examined. Expression of Ras oncogene initially promotes hyperproliferation, hyper-replication and accumulation of DNA damage (Figure 1.5), which subsequently triggers activation of the cellular DDR and the p53/p21 and p16 tumour suppressor pathways (Figure 1.6), both of which are necessary in leading to the onset of senescence. To analyse the effect of miR-K5 and miR-K11 on Ras-induced senescence, an OIS bypass screen was conducted once again.

BJ cells were stably transduced with retroviruses encoding the *H-Ras* gene in an estrogen receptor inducible system. Cells were selected with blasticidin for 96 hours post-transduction then Ras expression was induced with 4-hydroxytestosterone for 72 hours. Cells were subsequently transiently transfected with miRVana mimics of miR-K5, miR-K11 and a NS control and an SA-β-Gal assay was performed according to manufacturers instructions 48 hours post-transfection. Wells were imaged by bright field microscopy and images were quantified using ImageJ cell counter software (Figure 3.2). Similar to v-cyclin-induced OIS, Ras-expressing BJ cells transfected with miR-K5 and miR-K11 revealed significantly lower levels of senescence, compared to the NS control. Intriguingly, cells transduced with Ras oncogene demonstrated lower overall levels of senescence, compared to their v-cyclin-transduced counterparts, a tribute

to the high potency of the v-cyclin oncogene. Furthermore, the efficiency of the inducible system is likely lower than the efficiency of lentiviral transduction, leading to lower expression of Ras. Cells transduced with Ras take longer to undergo senescence, compared to v-cyclin (conversation with Dr. Andrew Leidal, University of California San Francisco), meaning that prolonging the time course for this experiment may lead to increased senescence in the cells. The system may also be leaky, with low basal expression of Ras occurring even in the uninduced cells.

Together, these results suggest that miR-K5 and miR-K11 are targeting a fundamental aspect of the senescence pathway, common to both v-cyclin and Ras oncogenes, indicating a more general interference with the senescence program rather than a specific targeting of v-cyclin or v-cyclin activities. In addition to supporting a broader impact of the discovery, this finding also facilitates further investigation into the mechanism of action of the viral miRNAs in KSHV-induced bypass of OIS as either oncogene can be used in future assays.

3.3 miR-K5 and miR-K11 induce altered appearance of DDR foci

Having determined that miR-K5 and miR-K11 are targeting fundamental aspects of the senescence pathway to induce bypass of OIS, the mechanism of action of these miRNAs was next examined. Senescent cells frequently harbour extensive of DNA damage and, consequently, persistent DDR signalling (d'Adda di Fagagna, 2008). This persistent DDR is of the utmost importance in senescence, promoting chromatin remodelling and the formation of senescence-associated heterochromatin foci, in addition to inducing the senescence-associated secretory phenotype (Figure 1.5). Furthermore, DNA damage signalling activates the p53/p21 and p16/Rb tumour suppressor pathways, leading to proliferation arrest (Figure 1.6). v-cyclin induces DNA damage and its expression in primary cells results in a strong DDR (Leidal *et al*, 2012). As such, miR-K5 and miR-K11 were analysed for a potential abrogating effect on DNA damage signalling within v-cyclin transduced cells.

To examine the effect of miR-K5 and miR-K11 on the DDR, BJ cells were transduced with retroviruses encoding v-cyclin and selected for 48 hours with puromycin. 72 hours post-

infection cells were subsequently transiently transfected with miR-K5 and miR-K11 miRVana mimics as well as a NS control. 48 hours post-transfection cells were fixed and immunostained with anti-53BP1 and anti-γH2AX antibodies and counterstained with 1 μg/mL DAPI for 15 minutes. Coverslips were mounted onto slides and sealed with Prolong Gold Antifade reagent then visualized by fluorescent microscopy (Figure 3.3). DNA double stranded breaks are bound by histone H2AX which is phosphorylated following the recruitment of DDR machinery, including 53BP1. Typically, cells with DNA damage and an activated DDR display a punctate nuclear staining for both 53BP1 and γ-H2AX, as both proteins are recruited to sites of DNA damage to serve as scaffolds for repair signalling.

As expected, cells transduced with v-cyclin and transfected with the NS control are replete with 53BP1 and γH2AX foci. Intriguingly, however, cells transfected with miR-K5 and miR-K11 also demonstrate extensive punctate nuclear staining of both proteins. Furthermore, although these foci appear to be decreased in number, their size is significantly larger than those observed within the NS control (Figure 3.4). Images were quantified using a macro program in FIJI software (developed by Robertson Holden). Given their superior quality and sharpness, the 53BP1 foci were chosen for quantification. FIJI analysis demonstrated a trend towards a decrease in the number of 53BP1 foci in the KSHV miRNA-transfected cells, but a trend towards an increase in foci size for both miR-K5 and miR-K11, although both trends appear to be stronger for the miR-K5 transfected cells (Figure 3.4)

Thus, KSHV miR-K5 and miR-K11 appear to be interfering with the cellular DDR given that the DDR foci are significantly altered by concomitant v-cyclin and viral miRNA expression.

3.4 miR-K5 and miR-K11 induce increased phosphorylation of H2AX

To further study the relationship between the KSHV viral miRNAs and the DDR, expression of H2AX phosphorylation was examined in cells transiently transfected with miR-K5 and miR-K11 compared to the NS control in the presence of v-cyclin. Histone H2AX is phosphorylated by ATM kinase when bound to DSBs, allowing for the recruitment of downstream DDR signalling effectors. This means that an increase in γH2AX expression is

indicative of upregulation of DDRs. As such, expression of γH2AX was analysed by Western blot.

BJ cells were transduced with retroviruses encoding v-cyclin then selected for 48 hours with puromycin. 72 hours post-infection, cells were transiently transfected with miR-K5 and miR-K11 miRVana mimics in addition to the NS control. 48 hours post-transfection, cells were lysed and lysates were run on an SDS-PAGE gel followed by Western blot transfer. Blots were probed with anti-γH2AX antibodies and imaged with ECL2 chemiluminescent reagents according to manufacturers instructions (Figure 3.5). As expected, cells expressing the viral miRNAs demonstrated a significant increase in γH2AX expression relative to the NS control.

Together these data indicate that miR-K5 and miR-K11 are upregulating the DDR in cells undergoing v-cyclin-induced OIS. This finding runs counter to our original hypothesis which was that miRNA-mediated OIS bypass may result from suppression of OIS-initiating DDRs. However this upregulation of DNA damage signalling in the miR-K5 and miR-K11 transfected cells may represent a by-product of other cellular mechanisms targeted by these miRNAs, such as an repression of tumour suppressors or cyclin-dependent kinase inhibitors that typically prevent the onset of DNA damage in pre-cancerous cells.

3.5 miR-K5 and miR-K11 inhibit autophagic flux in an autophagy receptor cell line

Although autophagy, a major catabolic process involved in the large-scale degradation and recycling of intracellular biomass, is active at basal levels in most cell types, this homeostatic mechanism is also triggered by various cellular stresses, including an activated DDR. Autophagy has further been demonstrated to promote Ras-induced senescence (Young *et al*, 2009) by inducing rapid protein turnover in response to oncogenic stress, leading to the onset of senescence. Furthermore, KSHV encodes two proteins, viral Bcl-2 and v-FLIP which have the ability to subvert the autophagic machinery (Lee *et al*, 2009; Liang *et al*, 2008; Pattingre *et al*, 2005; Wei *et al*, 2008). Leidal and colleagues demonstrated that v-FLIP's ability to block OIS in latently-infected cells is a result of its suppression of v-cyclin-induced autophagic responses

(Leidal *et al*, 2012). As a result, cells transfected with the KSHV miRNAs were examined for autophagic activity.

The process of autophagy is initiated by the formation of a flattened isolation membrane or phagophore that expands to sequester a portion of the cytoplasm and engulfs its cargo by forming a double-membrane bound autophagosome around it. As the membrane is expanding, LC3-I, which is present on the membrane, is covalently modified with phosphatidylethanolamine forming LC3-II, which is inserted into the membrane on both the cytosolic and luminal sides, resulting in membrane curvature and eventual closure of the autophagosome. The autophagosome subsequently fuses with the lysosome, forming an autophagolysosome, the contents of which are degraded by lysosomal hydrolases and released into the cytoplasm to be recycled for future use (Figure 1.7). A common technique for visualizing the progression of autophagy is the examination of LC3 processing and degradation in cells.

To examine whether the KSHV miRNAs are interfering with autophagic flux, the effect of miR-K5 and miR-K11 on cells transfected with a dual fluorescent LC3 reporter was analysed. A stable cell line expressing this reporter was established by transducing U2OS osteosarcoma cells, a common cell line used for fluorescence microscopy owing to their large, flattened appearance, with retroviruses encoding an mCherry-GFP-LC3 reporter. Autophagosomes in these cells appear yellow, owing to the concerted fluorescence of the mCherry and GFP markers. However during autophagolysosomal acidification the GFP, which is acid sensitive, is denatured whereas the mCherry reporter is not. This selective denaturing of the green fluorescent marker results in the LC3 foci appearing red upon acidification and degradation of autophagosomal contents, thus indicating that the cell is progressing through the process of autophagy.

To measure autophagy flux in response to the KSHV miRNAs, the U2OS mCherry-GFP-LC3 reporter cells were transfected with the KSHV miRNAs or the NS control. 48 hours post-transfection, cells were treated with 250nM Torin 1, an mTOR inhibitor, to induce autophagy. 4 hours post-Torin 1 treatment, cells were fixed with 4% paraformaldehyde and coverslips were mounted with Prolong Gold Anti-Fade Reagent. Slides were imaged with multidimensional acquisition for both mCherry and GFP fluorescence (Figure 3.6). As expected, the NS control-

transfected cells displayed elevated levels of autophagosomes and autolysosomes upon Torin 1 treatment, as evidenced by an increase in red LC3 foci. In contrast, both the miR-K5 and miR-K11 transfected cells contained yellow, rather than red, LC3 foci, indicating that the viral miRNAs appear to be interfering with the flux of autophagy and preventing degradation of autophagosomal contents. As a further control, U2OS mCherry-GFP-LC3 cells were treated with a combination of Torin 1 and either 100nM Bafilomycin, an inhibitor of autophagosome/lysosome fusion, or 1mM Chloroquine, an inhibitor of endosomal acidification (Figure 3.6). As expected, both treatments also resulted in an increase in yellow LC3 foci, providing further evidence that the miRNAs are interfering with the final steps of autophagic degradation.

These findings suggest that both miR-K5 and miR-K11 are interfering with the flux of autophagy. It appears that the miRNAs are interfering with the degradation of autophagosomal contents, either by preventing the fusion of the autophagosome with the lysosome, or by inhibiting autophagolysosomal acidification. This hypothesis is further supported by the fact that the yellow foci observed in the miRNA-transfected U2OS cells match those observed in both the Bafilomyin and Chloroquine controls. This result is further supportive of OIS bypass in the KSHV miRNA-transfected cells as autophagy represents an important inducer of senescence.

3.6 miR-K5 and miR-K11 inhibit expression of p16 but not p21 or p53

The main hallmark of cellular senescence is an inability to progress through the cell cycle. This growth arrest is coordinated by the activation of tumour suppressor pathways, notably the p53 and p16/Rb pathways (Figure 1.6). These pathways regulate various different aspects of senescence and many genes involved in senescence feed into either pathway or act downstream to mediate the senescent phenotype. The p53 tumour suppressor pathway is critical in cellular stress responses. Activated in response to DNA damage, p53 activates p21 that in turn blocks firing from replication origins and prevents cell cycle progression and cellular proliferation. Similarly, the p16/Rb tumour suppressor pathway represses genes involved in cell cycle progression, thus reinforcing the senescent phenotype. Given the crucial importance of both

pathways in senescence, the effect of both miR-K5 and miR-K11 on these pathways was consequently analysed.

BJ cells were transduced with retroviruses encoding v-cyclin then selected for 48 hours. 72 hours post-infection cells were transfected with KSHV miRNAs and the NS control. 48 hours post-transfection, cells were lysed and lysates run on an SDS-PAGE gel followed by Western blot transfer. Blots were probed with anti-p53, anti-p21 and anti-p16 antibodies and imaged with ECL2 chemiluminescent reagent according to the manufacturer's instructions (Figure 3.7). Compared to the NS control, cells transfected with miR-K5 and miR-K11 demonstrated no change in the expression levels of p53 or p21, suggesting that this pathway is not being affected by the KSHV miRNAs. In contrast, the miR-K5 and miR-K11 transfected cells both exhibited a decrease in p16 expression level compared to the NS control, indicating that both miRNAs are repressing the activation of this pathway.

Owing to the low expression of p16 in the BJ cells, this experiment was repeated in IMR90 fibroblast cells, which express higher levels of p16 (Leidal *et al*, 2012). In addition, the effect of the miRNAs on p16 was examined in Ras and v-cyclin induced senescence. IMR90 cells were transduced with retroviruses encoding v-cyclin and Ras then selected for 48 hours. 72 hours post-infection cells were transfected with KSHV miRNAs and the NS control. 48 hours post-transfection, cells were lysed and lysates run on an SDS-PAGE gel followed by Western blot transfer. Blots were probed with anti-p16 antibody and imaged with ECL2 chemiluminescent reagent according to the manufacturer's instructions (Figure 3.8). Results demonstrate a reduction in p16 expression in both the v-cyclin and Ras transduced cells, indicating that the miRNAs are repressing the activation of this pathway in the IMR90s and in both forms of OIS. Interestingly, p16 expression is much higher in the v-cyclin-transduced cells compared to the Ras-transduced cells. v-cyclin, as a more potent oncogene, tends to induce senescence more quickly than Ras, suggesting that the cells transduced with v-cyclin are further along the pathway to senescence than the Ras-transduced cells. The higher expression of p16 in these cells suggests that the time course should be carried out for longer in future experiments to maximize the observed effect on p16.

These findings indicate that KSHV miR-K5 and miR-K11 have no effect on expression levels of p53 or p21 tumour suppressors, but repress expression of p16. This suggests a role for these miRNAs in downregulating the p16/Rb tumour suppressor pathway, while leaving the p53 tumour suppressor pathway intact. This effect may result from direct targeting of the p16 mRNA or may represent an indirect effect incurred by miRNA targeting of another regulator of p16 expression.

3.7 miR-K5 and miR-K11 have no effect on p14 expression

The repression of p16 observed above may represent direct targeting of this protein by the miRNAs. Alternatively, the miRNAs may be targeting upstream modulators of p16 that result in a decrease in its expression levels. Although mechanisms for determining miRNA target sites are beyond the scope of this thesis, p16 represents an interesting molecular situation. p16 is encoded upon the same transcript as p14 ARF, known collectively as the CDKN2A locus. Although these two proteins have distinct first exons separated by a splice acceptor site, they share a second and third exon (for more detailed information, see section 4.3.4). Direct targeting of any sequence within the CDKN2A locus common to both p14 and p16 would result in a decrease in the expression of both proteins, and would provide an indication of direct mRNA targeting by the KSHV miRNAs. As such, p14 expression was examined.

BJ cells were transduced with retroviruses encoding v-cyclin then selected for 48 hours. 72 hours post-infection cells were transfected with KSHV miRNAs and the NS control. 48 hours post-transfection, cells were lysed and lysates run on an SDS-PAGE gel followed by Western blot transfer. Blots were probed with an anti-p14 antibody (Figure 3.9). Compared to the NS control, cells transfected with miR-K5 and miR-K11 demonstrated no change in the expression levels of p14, demonstrating that they are not directly targeting sequences common to both p16 and p14 on this transcript. Direct targeting of p16 is still a possibility, in either its unique exon or the associated UTRs.

3.8 miR-K5 and miR-K11 have no effect on ID1 expression

The p16 tumour suppressor protein is regulated at multiple different levels (see section 4.3 for more detailed information). One such mechanism is transcriptional regulation by Inhibitor of DNA Binding 1 (ID1) which represses transcription of p16, but has no effect on p14 expression. ID1 is itself controlled by Smurf2 ubiquitin ligase, which results in the degradation of ID1 and allows for p16 transcription (Ramkumar *et al*., 2012). During senescence, Smurf2-mediated ubiquitination and subsequent degradation of ID1 has been linked to the regulation of p16 expression, leading to bypass of senescence (Kong *et al*., 2011). To determine, therefore, whether the KSHV miRNAs were inducing an upregulation in ID1, and thus repressing p16, protein expression of ID1 was examined.

BJ cells were transduced with retroviruses encoding v-cyclin then selected for 48 hours. 72 hours post-infection cells were transfected with KSHV miRNAs and the NS control. 48 hours post-transfection, cells were lysed and lysates run on an SDS-PAGE gel followed by Western blot transfer. Blots were probed with an anti-ID1 antibody and imaged with ECL2 chemiluminescent reagent according to the manufacturer's instructions (Figure 3.9). Compared to the NS control, cells transfected with miR-K5 and miR-K11 demonstrated no change in the expression levels of ID1, demonstrating that they are not targeting p16 by enhancing the expression of its upstream regulator ID1. The miRNAs may be targeting other upstream regulators unique to the p16 promoter. Given that no change in p14 expression was observed, this would tend to suggest that regulation is occurring at the transcriptional or post-transcriptional level.

3.9 miR-K5 and miR-K11 do not affect phosphorylation of Rb or Bcl-2

As a tumour suppressor, p16 has various different effects on the cell, and on senescence in particular. p16 binds to cyclin-dependent kinases, such as CDK4 or CDK6, thus inhibiting their ability to interact with cyclins and preventing phosphorylation of Rb. This allows Rb to remain associated with the transcription factor E2F1, localizing it to the cytoplasm and preventing the transcription of E2F1 target genes which contribute to OIS bypass (Leidal *et al*,

2012) Furthermore, in the context of KSHV, p16 binding to CDK6 inhibits the formation of the v-cyclin-CDK6 complex, which normally phosphorylates and inactivates Bcl-2 (Ojala *et al*, 2000) promoting cellular hyperproliferation. This leads to the accumulation of DNA damage and subsequent activation of the DDR, resulting in autophagy and senescence. To determine whether either of these pathways are targeting by the miRNAs, Western blots were performed to analyse levels of various key proteins downstream of p16.

BJ cells were transduced with retroviruses encoding v-cyclin then selected for 48 hours. 72 hours post-infection cells were transfected with KSHV miRNAs and the NS control. 48 hours post-transfection, cells were lysed and lysates run on an SDS-PAGE gel followed by Western blot transfer. Blots were probed with an anti-phospho-Bcl-2 and anti-phospho-Rb antibodies (Figure 3.10; Figure 3.11). Compared to the NS control, cells transfected with miR-K5 and miR-K11 demonstrated no change in the expression levels of any of these proteins.

These results suggest that p16 may not be acting through either of these pathways, but instead may be mediating senescence bypass through more direct chromatin modification or other alternate tumour suppressor pathway activities. However repeats of these experiments will be necessary to determine their validity. Additionally, other experiments, such as Western blotting or RT-PCR for key E2F1 target gene expression would provide further insight into how these pathways are being affected.

3.10 Summary

In summary, results described in this thesis demonstrate that two KSHV miRNAs, miR-K5 and miR-K11, have the ability to bypass OIS induced by both v-cyclin and Ras oncogenes. These miRNAs also upregulate the DDR, as evidenced by altered γH2AX and 53BP1 immunofluorescent foci. Both miR-K5 and miR-K11 reduce p16 levels within transfected cells, although they have no effect on p14, ID1, CDK4 or CDK6 expression or phosphorylation of Rb or Bcl-2. Finally, both miRNAs possess the ability to interfere with autophagic flux, demonstrated by a lack of LC3 degradation in cells transfected with these miRNAs. This result appears similar to Bafilomycin and Chloroquine controls, both inhibitors of autophagic flux,

suggesting that miR-K5 and miR-K11 may be contributing to OIS bypass by preventing autophagy from proceeding. However it is important to note that this interference with autophagic flux was demonstrated out of the context of v-cyclin expression and thus further experiments will be required to determine the role for this inhibition in OIS bypass.

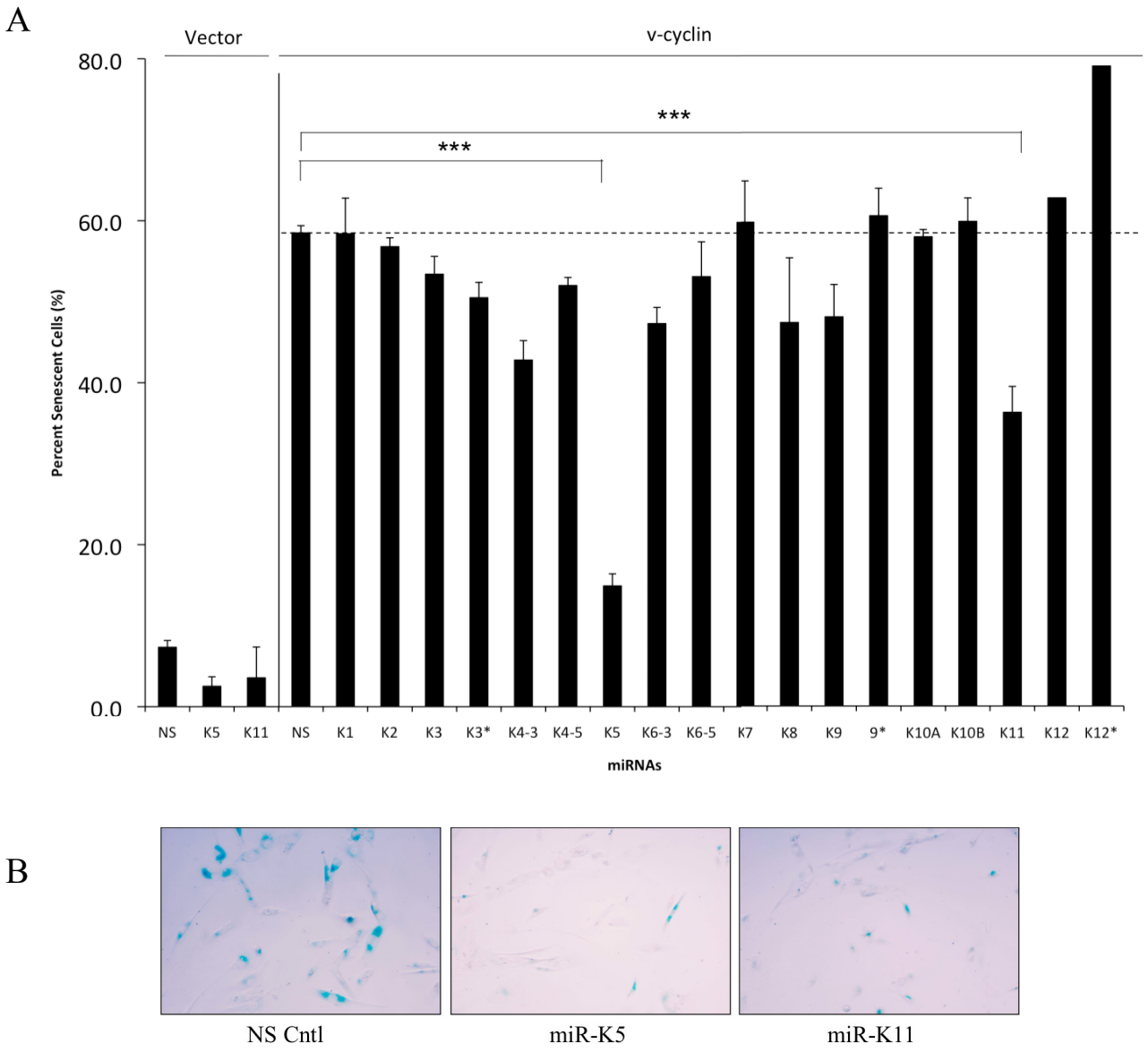


Figure 3.1 KSHV miR-K5 and miR-K11 trigger bypass of v-cyclin-induced senescence. (A) BJ cells were transduced with KSHV v-cyclin then 48 hours later transfected with various KSHV miRNA mimics (miRVana). At 96 hours post-transfection cells were stained using a senescence-associated β-galactosidase assay. Stain was removed after 24 hours and cells were left in PBS at 4^{o}C until imaged. Stained (senescent) cells were counted using ImageJ Cell Counter software. Results are reported as means ± SEM of triplicates; ns > 0.05 and *** $p<0.001$ for indicated parameters analysed. miR-K5 and miR-K11 consistently demonstrated bypass of senescence in each of three biological replicates. **(B)** Representative images of BJs transfected with non-specific miRNA, miR-K5, and miR-K11.

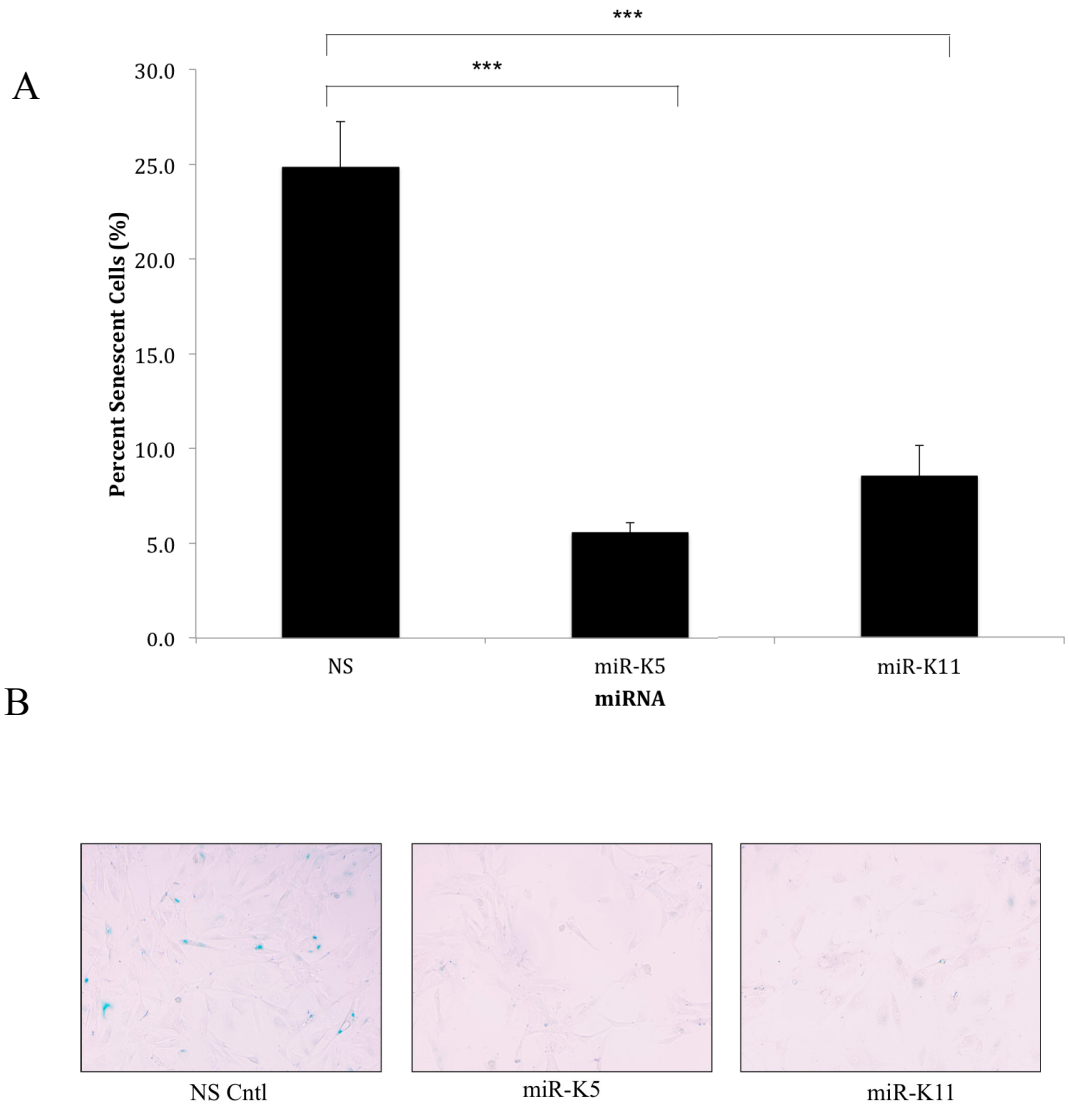


Figure 3.2 KSHV miR-K5 and miR-K11 trigger bypass of Ras-induced senescence. **(A)** BJ cells stably expressing a pLNCX-ER-Ras retroviral vector were induced with tamoxifen then 72 hours later transfected with KSHV miRNA mimics. At 96 hours post-transfection cells were stained using a senescence-associated β-galactosidase assay. Stain was removed after 24 hours and cells were left in PBS at 4^{o}C until imaged. Stained (senescent) cells were counted using ImageJ Cell Counter software. Results are reported as means ± SEM of triplicates; *** $p<0.001$ for indicated parameters analysed. miR-K5 and miR-K11 consistently demonstrated bypass of senescence in each of three biological replicates. **(B)** Representative images of BJs transfected with non-specific miRNA, miR-K5, and miR-K11.

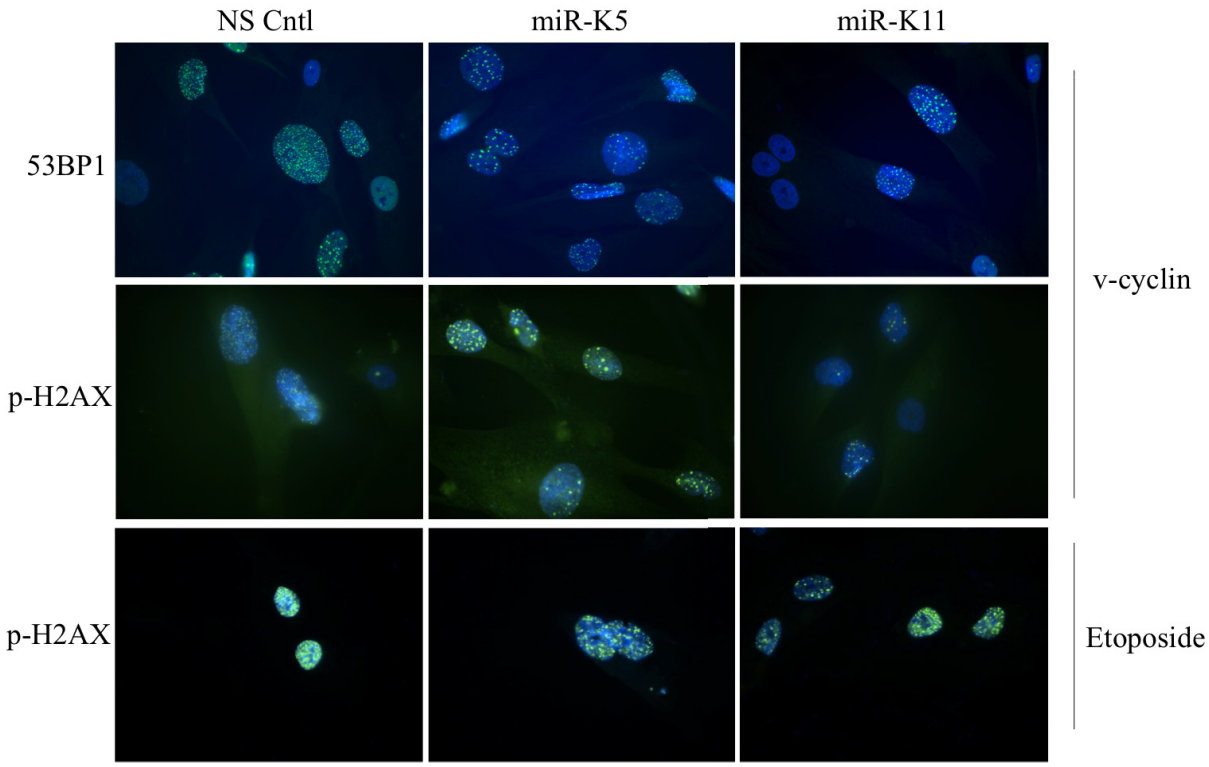


Figure 3.3 miR-K5 and miR-K11 alter the appearance of DNA damage foci. BJ fibroblast cells were transduced with KSHV v-cyclin then 48 hours later transfected with KSHV microRNA mimics (miRVana) for a non-specific control, miR-K5, miR-K11. At 96 hours post-transfection cells were fixed with 4% paraformaldehyde then permeabilized with 90% Methanol. Alternatively, BJ fibroblast cells were transfected with KSHV microRNA mimics for a non-specific control, miR-K5 and miR-K11 then 48 hours post-transfection cells were treated with 5 ng/μL Etoposide. 4 hours post-treatment cells were fixed with 4% paraformaldehyde then permeabilized with 90% Methanol. Cells were then probed with α-γH2AX primary antibody then α-Rabbit Alexa488 secondary antibody and counterstained with DAPI before mounting with Prolong Gold and visualized.

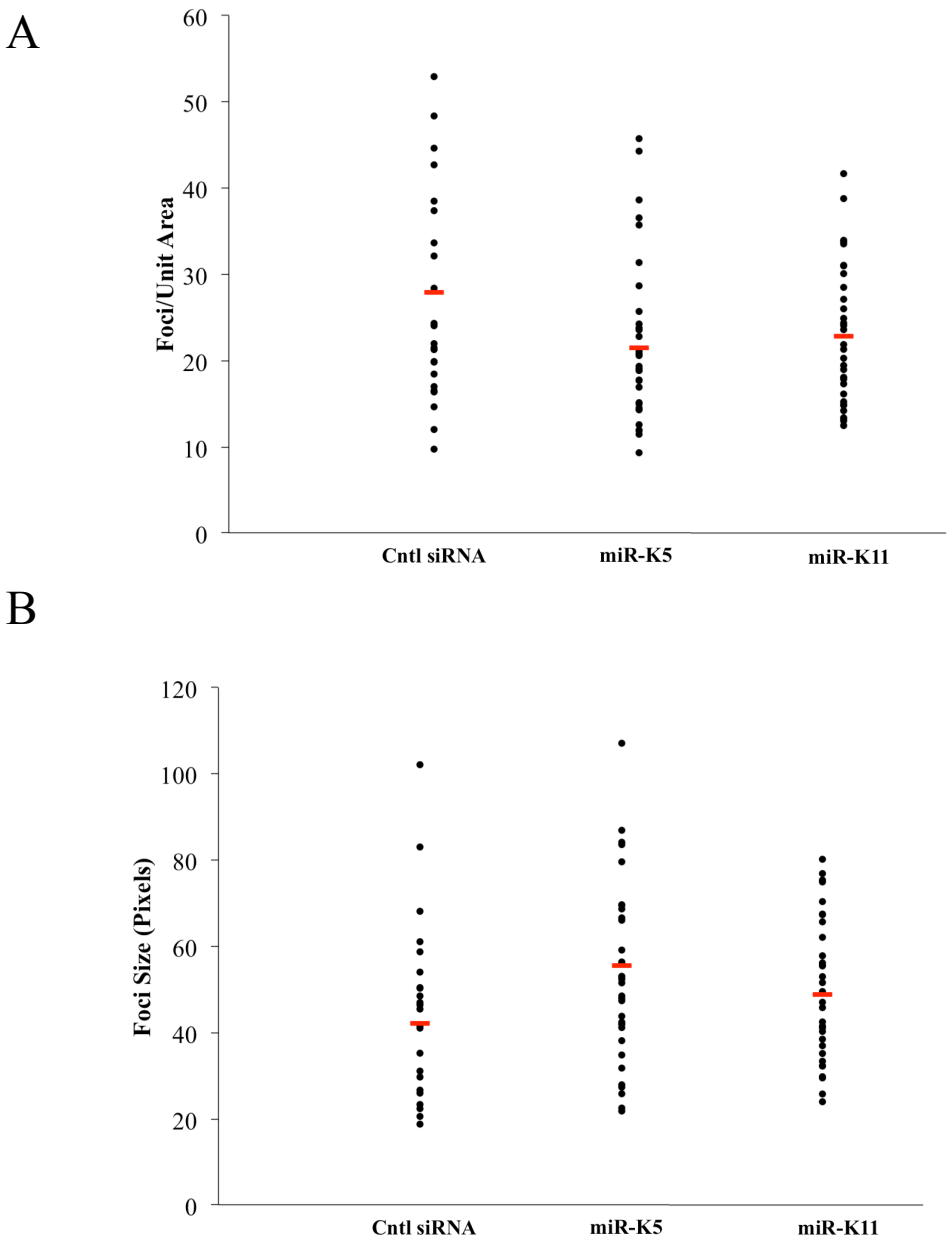


Figure 3.4 Altered DNA damage foci demonstrate a trend towards fewer, larger foci. BJ fibroblast cells were transduced with KSHV v-cyclin then 48 hours later transfected with KSHV microRNA mimics (miRVana) for a non-specific control, miR-K5 and miR-K11. At 96 hours post-transfection cells were fixed with 4% paraformaldehyde then permeabilized with 90% Methanol. Cells were then probed with α-53BP1 primary antibody then α-Rabbit Alexa488 secondary antibody and counterstained with Dapi before mounting with Prolong Gold and visualized. Images were then quantified using FIJI software. Graphs represent measurements of number of foci per unit nucleus area **(A)** and foci size **(B)**. Data points are represented in black with means represented in red. Data were not statistically significant.

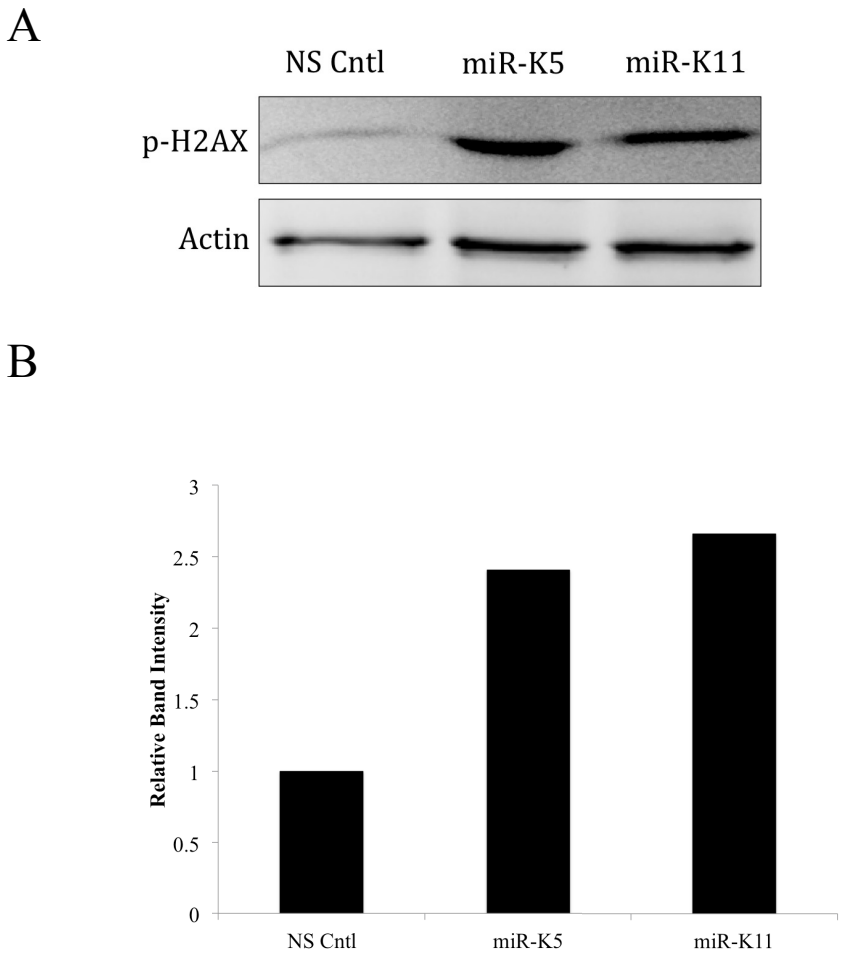


Figure 3.5 miR-K5 and miR-K11 increase expression of phosphorylated H2AX. BJ cells were transduced with retroviruses encoding v-cyclin then selected for 48 hours with puromycin. 72 hours post-infection, cells were transiently transfected with miR-K5 and miR-K11 mimics in addition to the NS control. 48 hours post-transfection, cells were lysed and lysates were run on an SDS-PAGE gel followed by Western blot transfer. Blots were probed with anti-53BP1 and anti-γH2AX antibodies and imaged with ECL2 chemiluminescent reagents according to manufacturers instructions. **(A)** Western blot image demonstrating upregulated γH2AX expression. **(B)** Images were quantified using ImageJ and band intensities normalized to actin then normalized to the NS control and represented in graphical form.

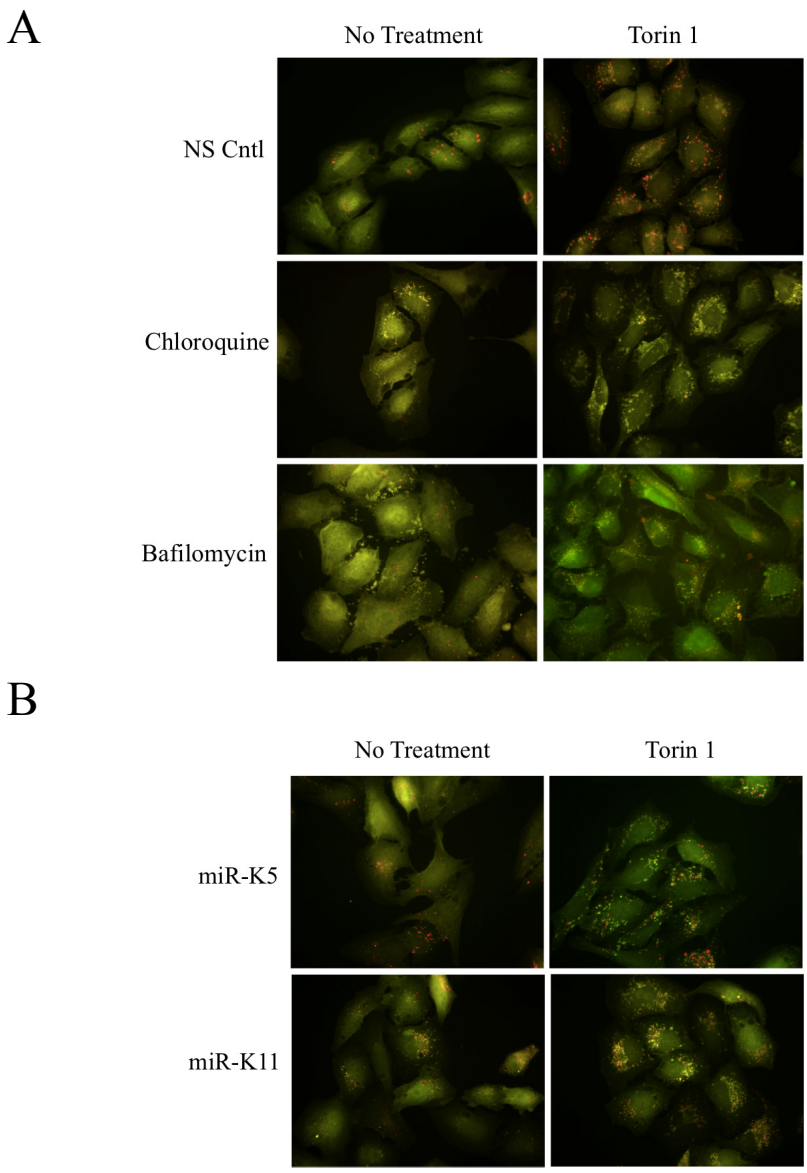


Figure 3.6 KSHV miRNAs interfere with autophagic flux. U2OS mCherry-GFP-LC3 reporter cells were transfected with the NS Cntl, miR-K5 and miR-K11 or left untransfected. 48 hours post-transfection, cells were treated with 250nM Torin 1 or left untreated in combination with 1μM Chloroquine or 100 ng/μL Bafilomycin A1 positive controls. 4 hours post-treatment, cells were fixed with 4% paraformaldehyde and coverslips were mounted with Prolong Gold Anti-Fade Reagent. Slides were imaged with multidimensional acquisition for both mCherry and GFP fluorescence. Red foci indicate functional autolysosomes whereas green and yellow foci indicate autophagosomes. **(A)** Negative and positive controls for autophagic flux compared to **(B)** KSHV miRNA transfections.

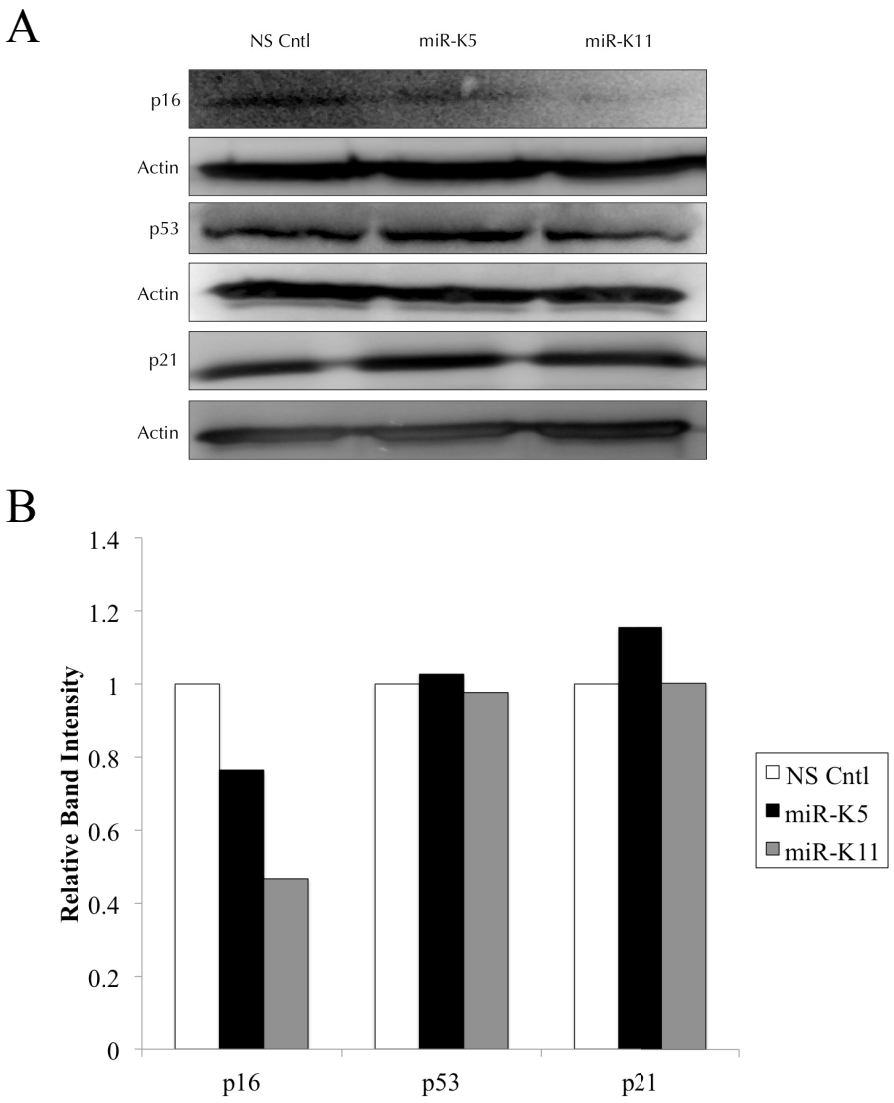


Figure 3.7 miR-K5 and miR-K11 reduce expression of tumour suppressor p16 but have no effect on p21 or p53. BJ fibroblast cells were transduced with v-cyclin then, 72 hours later, transfected with KSHV miRNAs and the NS control. 48 hours post-transfection cells were lysed and lysates run on an SDS-PAGE gel followed by Western blot transfer. Blots were probed with anti-p53, anti-p21 and anti-p16 antibodies and imaged with ECL2 chemiluminescent reagents according to manufacturers instructions. Western blot images demonstrate a reduction in p16 expression but no change in p53 or p21 **(A)**. Images were quantified using ImageJ and band normalized to the actin control then normalized to the NS control and represented in graphical form **(B)**.

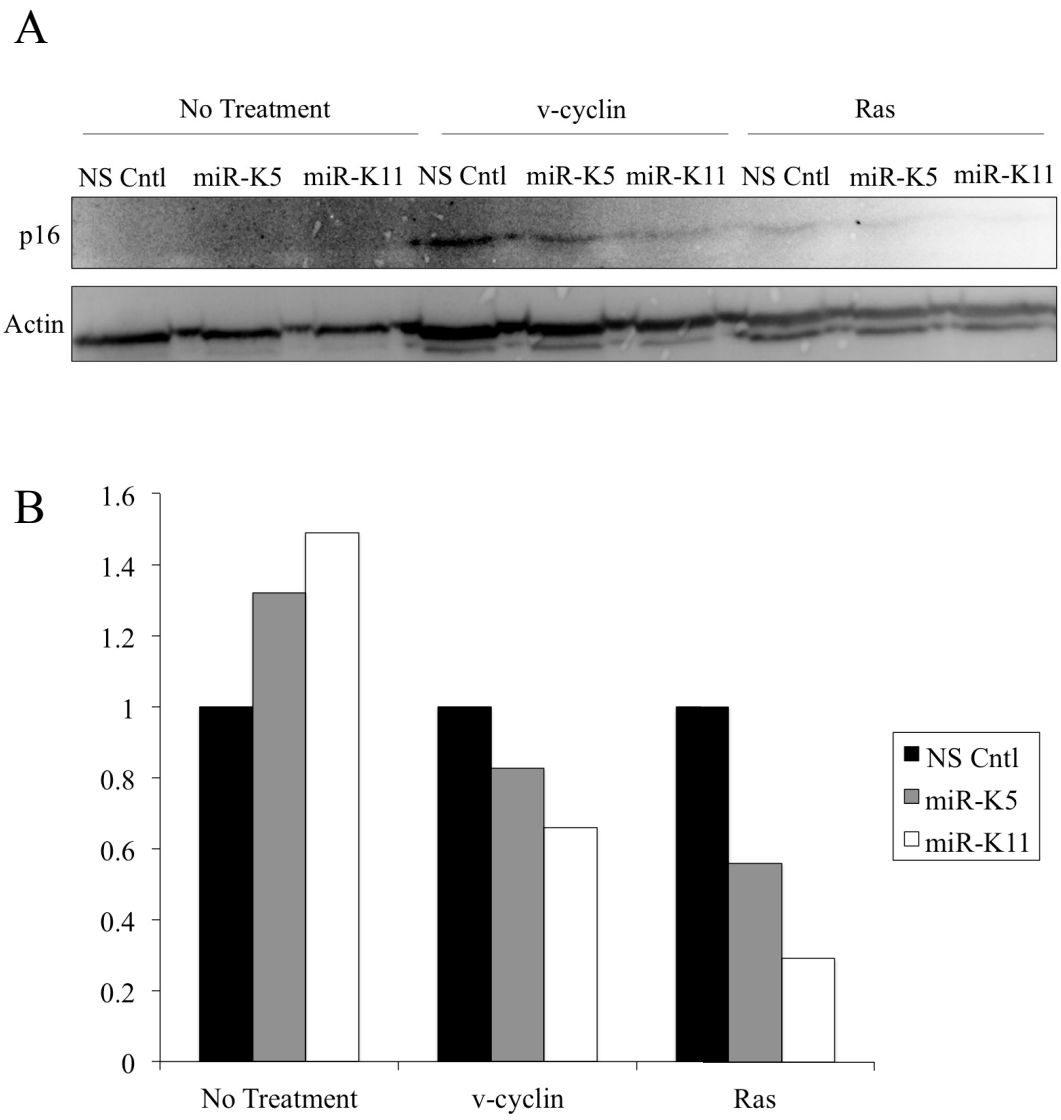


Figure 3.8 miR-K5 and miR-K11 reduce expression of tumour suppressor p16 in IMR90 Fibroblast cells. IMR90 fibroblast cells were transduced with v-cyclin then, 72 hours later, transfected with KSHV miRNAs and the NS control. 48 hours post-transfection cells were lysed and lysates run on an SDS-PAGE gel followed by Western blot transfer. Blots were probed with anti-p16 antibodiy and imaged with ECL2 chemiluminescent reagents according to manufacturers instructions. Western blot images demonstrate a reduction in p16 expression **(A)**. Images were quantified using ImageJ and band normalized to the actin control then normalized to the NS control and represented in graphical form **(B)**.

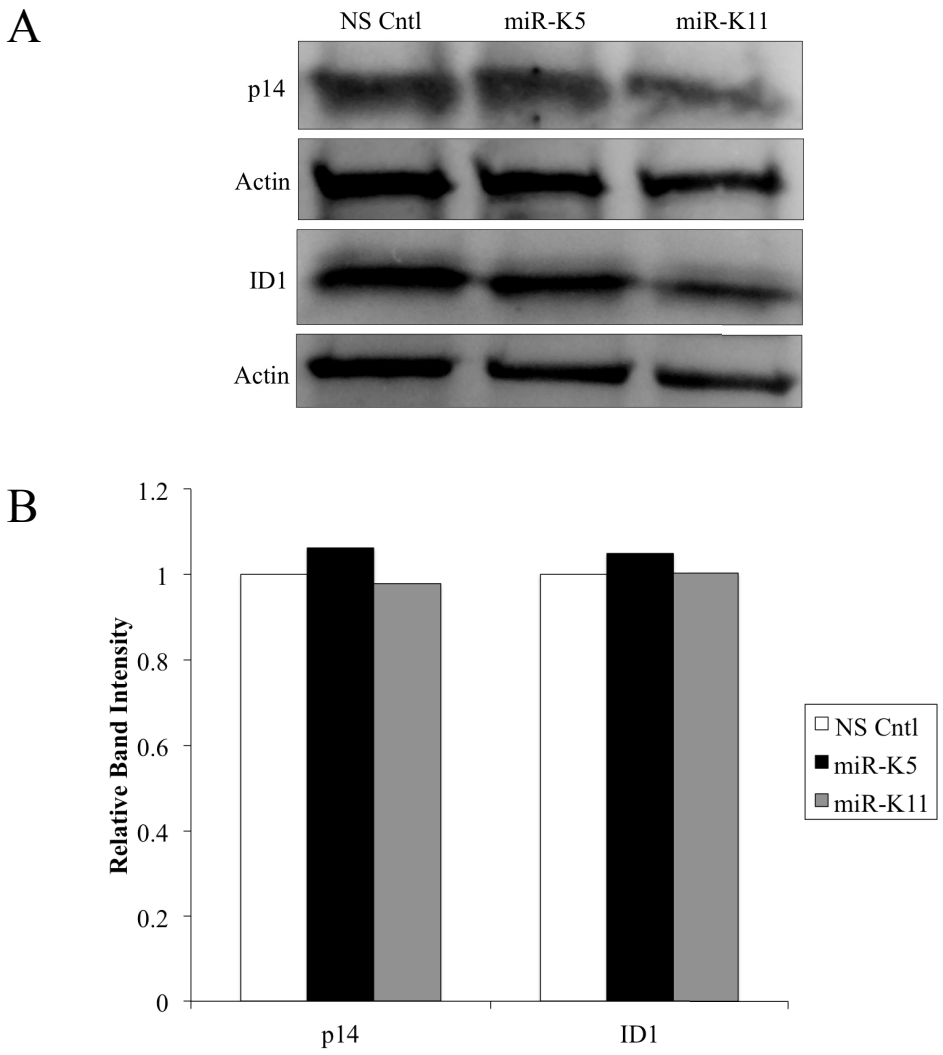


Figure 3.9 miR-K5 and miR-K11 do not affect p14 of ID1 expression. BJ fibroblast cells were transduced with v-cyclin then, 72 hours later, transfected with KSHV miRNAs and the NS control. 48 hours post-transfection cells were lysed and lysates run on an SDS-PAGE gel followed by Western blot transfer. Blots were probed with anti-p14 and anti-ID1 antibodies and imaged with ECL2 chemiluminescent reagents according to manufacturers instructions. Western blot images demonstrate no change in p14 or ID1 expression levels **(A)**. Images were quantified using ImageJ and band normalized to the actin control then represented in graphical form **(B)**.

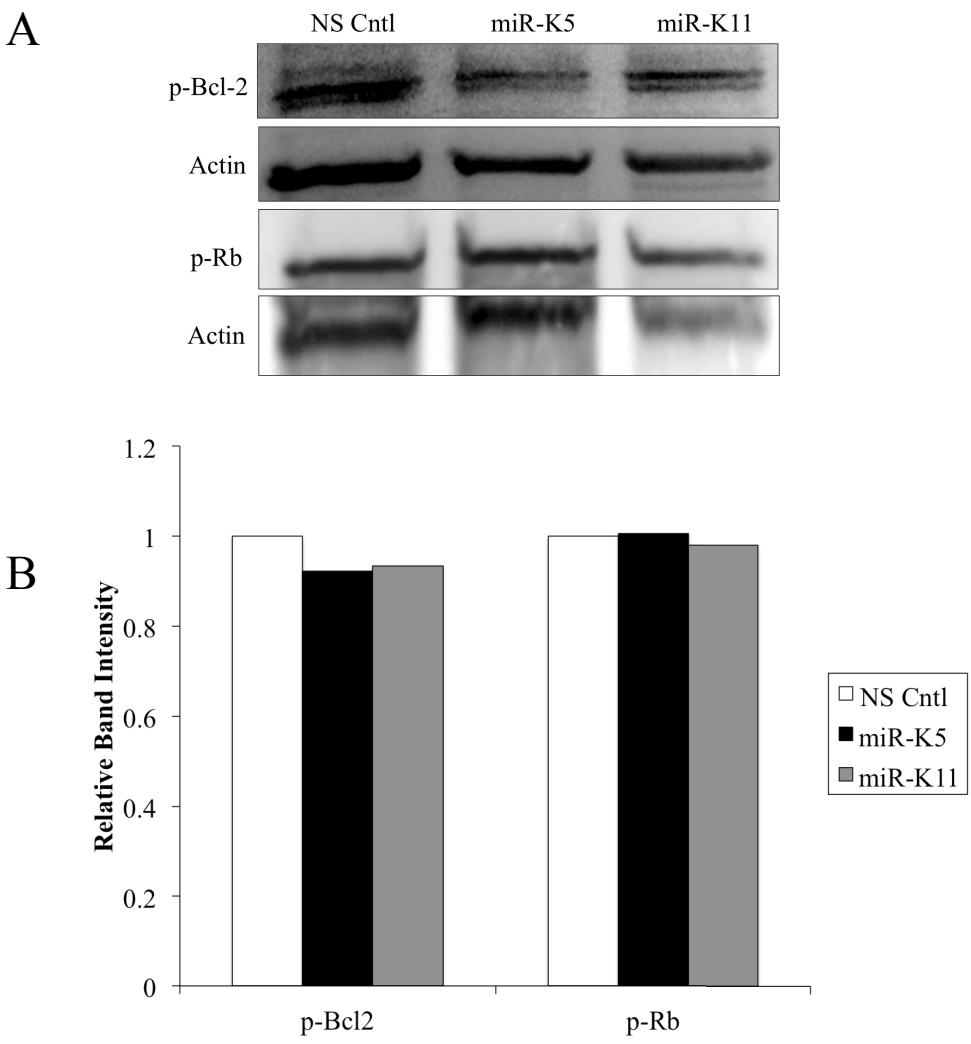


Figure 3.10 miR-K5 and miR-K11 have no effect on phosphorylation of Rb or Bcl-2, both downstream effectors of p16. BJ fibroblast cells were transduced with v-cyclin then, 72 hours later, transfected with KSHV miRNAs and the NS control. 48 hours post-transfection cells were lysed and lysates run on an SDS-PAGE gel followed by Western blot transfer. Blots were probed with anti-phospho-Bcl2 and anti-phospho-Rb antibodies and imaged with ECL2 chemiluminescent reagents according to manufacturers instructions. Western blot images demonstrate no change in Rb or Bcl2 phosphorylation **(A)**. Images were quantified using ImageJ and band normalized to the actin control then represented in graphical form **(B)**.

CHAPTER 4: DISCUSSION

4.1 miR-K5 and miR-K11 bypass OIS by supressing p16

Chronic oncogene expression is known to trigger OIS, an anti-tumour defence program designed to arrest cells in a metabolically active state (Adams, 2009). The KSHV latency locus encodes a potent oncogene, v-cyclin, which, in a similar fashion to cellular oncogene Ras, induces OIS. Initially, expression of these oncogenes results in hyperproliferation and hyperreplication, leading to an accrual of DNA damage and subsequently triggering cellular DDRs. The activation of DDRs leads to the p53-dependent induction of autophagy that, by degrading cytoplasmic contents, eventually releases factors such as IL-6 and IL-8, in addition to other cytokines important for the SASP, leading to the initiation of senescence. However as OIS is not conducive to cellular transformation, oncogenic viruses must find ways of subverting this anti-cancer defence mechanism during latency. Studies targeting this pathway have demonstrated that, by inhibiting one or more components, OIS bypass can be induced. shRNA knockdown of Rb1, a tumour suppressor protein that binds to E2F transcription factors and transcriptionally activates important DDR and cell cycle progression proteins, results in inhibition of the DDR and thus OIS bypass (Leidal *et al*, 2012; Shi *et al*, 2013). The KSHV latency locus also encodes v-FLIP, which suppresses v-cyclin-induced autophagy and thus plays a role in abrogating OIS. Furthermore, there exists in the literature a long precedent for viral miRNAs in establishing an intracellular environment conducive to transformation, although no role for KSHV miRNAs in OIS has yet been described. Work included in this thesis indicates a role for two viral miRNAs in bypassing OIS. KSHV miR-K5 and miR-K11 both demonstrated the ability to bypass v-cyclin- and Ras-induced senescence (Figure 3.1; Figure 3.2), thus suggesting that the actions of these miRNAs are not unique to bypass induced by the virus, but instead represent targeting of fundamental aspects of the senescence pathway. Following identification of these two miRNAs capable of OIS bypass, further investigation into the mechanism of action of each individual miRNA was conducted. Cells transiently transfected with either miR-K5 or miR-K11 display an upregulated DDR evidenced by increased γ-H2AX expression (Figure 3.5) and an altered appearance for γ-H2AX and 53BP1 foci (Figure 3.3). Furthermore, these cells express lower levels of p16 (Figure 3.7), suggesting that both miRNAs target cellular tumour suppressor

pathways. This suppression of p16 is consistent with an upregulated DDR. As expression of tumour suppressors is important in maintaining OIS, this decrease in p16 levels represents a plausible mechanism by which the miRNAs are inducing senescence bypass (Wajapeyee *et al*, 2008).

4.2 Possible mechanisms of miRNA-mediated p16 suppression inducing OIS bypass

The tumour suppressor protein p16 mediates senescence through multiple different mechanisms. In the context of KSHV infection, the actions of p16 are twofold (Figure 4.1). Firstly, p16 binds to, and sequesters, cyclin-dependent kinases thus inhibiting their activity. In KSHV-infected cells p16 binding to CDK6 should prevent it from interacting with v-cyclin to form the v-cyclin-CDK6 complex and thus blocking the phosphorylation of Rb. This allows Rb to remain associated with the transcription factor E2F1, localizing it to the cytoplasm and preventing the transcription of E2F1 target genes which contribute to G1/S transition (Leidal *et al,* 2012; Rayess *et al*, 2012). Indeed, Leidal and colleagues demonstrated that inhibition of Rb resulted in OIS bypass (Leidal *et al*, 2012). By reducing p16 expression, miR-K5 and miR-K11 may induce Rb phosphorylation and E2F target gene transcription, thus inducing senescence bypass (Figure 4.1). Although we detected no increase in Rb or Bcl-2 phosphorylation in the miRNA-transfected cells (Figure 3.10) a methodical analysis of v-cyclin target protein phosphorylation in control or miR-K5/miR-K11-transfected cells over an extended time course is necessary. Interestingly, the interaction of IL-6 and its receptor has been linked to the activation of the p16 tumour suppressor pathway reinforcing growth arrest (Kuilman *et al*, 2008). Given that IL-6 is released into the cytoplasm from autophagolysosomes following acidification, miRNA-mediated inhibition of autophagic flux may result in a loss of IL-6 release leading to a decrease in p16 expression (Figure 4.2).

4.3 Possible mechanisms for miR-K5- and miR-K11-mediated p16 repression

4.3.1 Chromatin Silencing

The regulation of p16 protein levels is complex and carried out at multiple different levels. The majority of p16 regulation occurs as a result of either histone modification or DNA methylation at the chromatin level, although oncogene activation regulates p16 expression levels mainly via histone modification, and not DNA methylation (Yamakoshi *et al*, 2009). The latter is primarily controlled by the polycomb repressor complex (PRC) (Schwartz *et al*, 2007; Sparmann and Van Louhizen, 2006). The PRC, made up of PRC1 and PRC2, plays a crucial role in silencing the INK4/ARF locus, which encodes the p16 protein. Interestingly, oncogenic homeobox proteins were recently shown to bypass senescence by recruiting PRCs to the p16 promoter to repress its expression (Martin *et al*, 2013). Several transcription factors within PRC1 inhibit transcription of p16 including Bmi-1, Mel18, CXB7 and CXB8 (Maertens *et al*, 2009; Quelle *et al*, 1995). Proteins associated with PRC2, including polycomb-like protein 2 (Pcl2), decrease the catalytic activity of the PRC, resulting in decreased promoter methylation. Furthermore, PRC2, along with ZBP-89 and YY1, interacts with class 1 histone deacetylases (HDACs) at the p16 promoter site to further regulate its activity. (Bhalla, 2005; Kuzmichev *et al*, 2002; Van der Vlag and Otte, 1999). This epigenetic silencing is countered by the actions of H2A.Z, which prevents modifications by remodelling chromatin at the p16 locus (Filippova, 2008; Raisner and Madhani, 2007; Wallace and Felsenfeld, 2007). Another possibility is the fact that a DNA replication origin within the p16 locus, known as the INK4a/ARF Regulatory Domain ($RD^{INK4A/ARF}$), recruits cell division cycle 6 (Cdc6) which represses the locus by recruiting histone deacetylases, resulting in heterochromatin formation (Gonzalez *et al*, 2006). Although none of these chromatin remodellers are known targets of miR-K5 and miR-K11, miRNA targeting of upstream silencers of p16 remodelling represents a viable explanation for the reduced p16 levels observed in the miR-K5 and miR-K11 transfected cells. Further experiments will be necessary to examine the effect of the miRNAs on several of these proteins.

4.3.2 Targeting of Upstream Regulators

In addition to epigenetic repression of p16 at the chromatin level, p16 expression is enhanced by actions of PPAR-gamma, the AP-1 transcription factors and members of the E26-transformation specific (Ets) family of transcription factors (Gan *et al*, 2008; Gil and Peters, 2006). The Ets transcription factors are regulated by Inhibitor of DNA Binding 1 (ID1), another transcription factor which represses transcription of p16 (Figure 4.3B). ID1 suppresses p16 by sequestering the transcription factor E47 and preventing it from transactivating p16. ID1 is itself controlled by Smurf2 ubiquitin ligase, which results in the degradation of ID1 and allows for p16 transcription (Ramkumar *et al*, 2012). During senescence, Smurf2-mediated ubiquitination, and subsequent degradation, of ID1 has been linked to the regulation of p16 expression, leading to bypass of senescence (Kong *et al*, 2011). Intriguingly, however, no changes in ID1 levels were observed in the miR-K5 and miR-K11 transfected cells (Figure 3.9), suggesting that miRNA-mediated repression of p16 is not occurring as a result of this upstream regulation. Further analysis over an extended time course will be necessary to determine whether the kinetics of this pathway are affected in the miRNA transfected cells

4.3.3 Interference with p16 mRNA Processing

Evidence also exists in the literature for p16 regulation at the mRNA level. ARE/poly(U)-binding/degradation factor 1 (AUF1) is known to degrade p16 mRNA (Gratacos and Brewer, 2011). p16 mRNA splicing is regulated in part by BRCA1 (Stefansson *et al*, 2011), a scaffolding protein that facilitates assembly of multiprotein complexes involved in the DDR, cell cycle checkpoint activation, transcriptional regulation and maintaining genomic stability. Formation of these complexes depends upon BRCA1 phosphorylation by ATM, ATR or Chk2 protein kinases. Once phosphorylated, BRCA1 interacts with BCLAF1, a protein commonly known for inducing resistance to DNA damage and required for efficient DDR (Liu *et al*, 2007). BCLAF1 mediates the formation of BRCA1-mRNA splicing complexes following DNA damage. This results in the recruitment of mRNA splicing machinery leading to enhanced pre-mRNA splicing of BRCA1/BCLAF1 target genes, thus promoting stability of the transcript and protein expression. Interestingly, since many of these target genes are involved in the DDR, inhibition of

BRCA1/BCLAF1 complex formation often results in sensitivity to DNA damage and defective DDR (Savage *et al*, 2014). BCLAF1 is a known target of miR-K5 suggesting that, by inhibiting BRCA1/BCLAF1 complex formation, miR-K5 may be interfering with p16 mRNA splicing, resulting in the observed decrease in p16 expression (Ziegelbauer, *et al*, 2009). However there is not yet any direct evidence that BCLAF1 is required for proper p16 RNA splicing and it will have to be experimentally confirmed.

4.3.4 Direct Targeting of p16 by RNA Silencing

Interestingly, p16 is encoded upon the same transcript as p14 (INK4/ARF transcript, referred to collectively as the CDKN2A locus). The p14 and p16 proteins are transcribed from individual promoters and use alternative first exons, 1β and 1α respectively, with exon 1β located 20 Kb upstream of exon 1α (Figure 4.3). These exons are joined by a splice acceptor site to the coding sequence and therefore both p16 and p14 share exons 2 and 3. A one-nucleotide shift in the segment encoding p14 means that these two genes are translated in different open reading frames and therefore produce two entirely different proteins (Rizos *et al,* 2001; Tannapfel *et al*, 2001). Given their separate promoters and lack of similarity in their sequences, these two proteins have the ability to be regulated independently. For example, the aforementioned transcription factor ID1 specifically represses the p16 promoter, but has no effect on p14 (Kong *et al*, 2011). p14 shares a similar role to p16 in that it is able to act as a negative regulator of cell cycle proliferation. In addition, p14 interacts with mouse double minute 2 homolog (mdm2) and blocks mdm2-induced degradation of p53, resulting in an increase in p53 expression and its subsequent tumour suppressor activities (Ivanchuk *et al*, 2001). miRANDA miRNA target analysis, a tool devised to identify possible miRNA targets based on sequence analysis, reveals a binding site for miR-K5 and miR-K11 in the shared 3'UTR, suggesting that this repression would not be unique to p16 (Figure 4.3C). This is consistent with the fact that no decrease in p14 expression was observed in miR-K5 and miR-K11 transfected cells. This binding site does, however, suggest direct targeting of p16 by miR-K5 and miR-K11, although further validation studies will be needed. Given the lack of change in p14 and ID1 expression levels (Figure 3.9), data certainly point to more direct targeting of p16 by both KSHV miRNAs. The necessary validation could be achieved through luciferase reporter assays with both the wild-type and

versions of the p16 locus with point mutations in the possible miRNA target sites. In addition, applying miRNA target identification methods such as PAR-CLIP, pSILAC and RIP-CHIP would provide more insight into the nature of miR-K5 and miR-K11 mediated targeting of p16. From a more functional perspective, knocking out p16 expression with miRNA or siRNA and performing an OIS bypass experiment would offer further insight as to whether this is indeed the pathway through which the miRNAs are acting. The data described above certainly point to direct targeting of p16 by miR-K5 and miR-K11, resulting in the induction of E2F1 target gene transcription. Many of these genes are involved in processes such as autophagy that initiate the senescence program. Therefore, by repressing the p16 tumour suppressor, both miRNAs facilitate OIS bypass. Furthermore, direct targeting of p16 by the miRNAs would also be consistent with the upregulated DDR observed in the miR-K5 and miR-K11 transfected cells.

4.4 The role of autophagy flux inhibition on senescence bypass

In addition to evidence suggesting that KSHV miR-K5 and miR-K11 target both the DDR and p16 tumour suppressor, results reported in this thesis indicate that both miRNAs are also targeting autophagy (Figure 3.6). U2OS cells stably expressing an mCherry-GFP-LC3 dual reporter construct demonstrate reduced LC3 degradation when transfected with either miR-K5 or miR-K11, suggesting that these miRNAs are interfering with autophagic flux. Although some ambiguity remains in the link between autophagy and senescence, increasing evidence points to autophagy as a direct inducer of senescence. In the context of KSHV, Leidal and colleagues discovered that cellular levels of autophagy increase in v-cyclin-transduced cells and that deficiencies in autophagy signalling impaired the onset of v-cyclin-induced OIS. Furthermore, the KSHV v-FLIP protein was shown to repress v-cyclin-induced autophagy as a mechanism of inhibiting OIS (Leidal *et al*, 2012). Given the strong evidence in the literature for autophagy as an inducer of senescence, by preventing autophagic flux, miR-K5 and miR-K11 would be able to prevent the induction of senescence and therefore facilitate bypass. This is primarily thought to be due to the fact that the amino acids and other molecules released from autophagosomes following acidification and degradation include multiple pro-senescence factors, including IL-6, IL-8, chromatin remodellers and members of the DDR (Leidal *et al*, 2012). Therefore, by

inhibiting autophagic flux, miR-K5 and miR-K11 may contribute to the senescence bypass phenotype, suggesting that OIS bypass by the miRNAs is occurring at multiple levels.

4.5 Possible mechanisms for miR-K5 and miR-K11 mediated inhibition of autophagic flux

There exist several possibilities as to how the KSHV miRNAs are preventing autophagic flux. Firstly, miR-K5 and miR-K11 may be interfering with autophagosome trafficking. By inhibiting the transport of autophagosomes to lysosomes, these miRNAs would prevent autophagosome-lysosome fusion and hence the degradation of autophagosomal contents. Several cellular components are necessary for this process. In particular, the endosomal sorting complex required for transport (ESCRT) machinery is believed to play a role in autophagosome trafficking. Deletion of several key members of this machinery resulted in a build-up of non-degradative autophagosomes (Rusten *et al*, 2007). Similarly, Rab GTPases and soluble N-ethylmadeimide-sensitive factor attachment protein receptors (SNAREs) are also key to autophagosome transport. Finally, cytoskeletal motor proteins ensure directional transport of autophagosomes (Cai *et al*, 2007). Targeting of any of these proteins, or their upstream regulators, would result in the observed phenotype. In addition to preventing autophagosome trafficking, miR-K5 and miR-K11 may be inhibiting autophagosomal-lysosomal fusion. This process is mediated by Rab7 GTPase on the autophagosomal membrane, and Lamp-1 and Lamp-2 on the lysosome (Gutierrez *et al*, 2004). Evidence also suggests that the Beclin 1/class III PI3K complex may play a role in this process (Kroemer *et al*, 2010). Beclin 1 is bound, and thus inhibited, by Bcl-2. Bcl-2 itself is inhibited by BCLAF1, a target of miR-K5. Thus miR-K5 transfection could result in derepression of Bcl-2 by BCLAF1. Finally, miR-K5 and miR-K11 could inhibiting autophagic flux by preventing acidification of the autophagolysosome by lysosomal hydrolases. This may be accomplished by targeting proteins involved in maintaining a low pH within the lysosome, allowing for activation of hydrolytic enzymes requiring an acidic pH. Alternatively, the miRNAs may be inhibiting hydrogen ATPases within the autophagolysosome. Interestingly, U2OS mCherry-GFP-LC3 cells treated with Bafilomycin A1, an inhibitor of autophagosome-lysosme fusion, or Chloroquine, an inhibitor of autophagolysosomal acidification, display an identical phenotype to those transfected with miR-K5 and miR-K11, suggesting that the miRNAs may be targeting autophagic flux at this late stage.

4.6 miR-K5 and miR-K11 target the DDR

Owing to the crucial role of the DDR in initiating and maintaining senescence, its components represent attractive targets for KSHV miRNA-mediated gene repression. By inhibiting the cellular DDR, these miRNAs would prevent the formation of SAHF, the induction of the SASP and tumour suppressor pathways and senescence-associated proliferation arrest. Surprisingly, as discussed above, observations in cells transfected with miR-K5 and miR-K11 tend to suggest an upregulation in the DDR, given an altered foci appearance and increase in γ-H2AX expression (Figure 3.3; Figure 3.5). Intriguingly, immunofluorescence staining for 53BP1 and γ-H2AX resulted in no obvious change in foci number, but a visible change in foci size, a result which appeared stronger in the miR-K5 transfected cells. The nuclear puncta in miR-K5 and miR-K11-transfected cells appear larger and brighter than those in the NS control-transfected cells. Quantification demonstrated a trend towards fewer, larger foci for the KSHV miRNA transfections. Interestingly, these foci most closely resemble DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS). DNA-SCARS represent persistent severe or irreparable DNA damage preceding the establishment of senescence-associated growth arrest and SASP. Interestingly, the formation of DNA-SCARS is independent of functional p53 or RB tumour suppressor pathways and cells deficient for these pathways continued to proliferate despite harbouring senescence levels of DNA-SCARS, suggesting that DNA-SCARS can be uncoupled from senescence-associated growth arrest. Furthermore, cells deficient in crucial DDR kinases formed DNA-SCARS faster than wild-type cells, suggesting that these DNA-SCARS may also arise from faulty DNA repair machinery. Formation of DNA-SCARS was accelerated in cells lacking certain DNA repair proteins, meaning that it is indeed defective repair that initiates the formation of these structures (Rodier *et al*, 2010). Rodier and colleagues therefore proposed that following senescence-inducing DNA damage, a general repair phase occurs for approximately 48 hours, after which remaining foci contain unresolved but stable chromatin modifications resulting from damage which persist indefinitely (Rodier *et al*, 2010). Owing to the striking similarity in their appearance, and the fact that foci induced by the KSHV miRNAs are uncoupled from senescence, the 53BP1 foci in the miR-K5 and miR-K11 transfected cells may represent DNA-SCARS. Furthermore, concurrent SAHF staining with immunofluorescence for 53BP1 demonstrated that these foci are appearing in proliferating, and

not senescent, cells consistent with the hypothesis that they represent DNA-SCARS. This suggests a defective DDR in these cells, meaning that the miRNAs are targeting the DDR or one of its many upstream modulators. Despite this conviction, several other theories exist which may also explain the observations. These larger foci may represent accumulation of multiple 53BP1 or γ-H2AX molecules around the same DNA break, also suggesting impaired DDR. Alternatively, these larger foci might indicate an upregulated DDR within the KSHV miRNA-transfected cells. This latter hypothesis is further supported by the fact that cells transfected with miR-K5 and miR-K11 demonstrate dramatically increased levels of γ-H2AX compared to the NS control. However, an increase in γ-H2AX may also represent persistent DNA damage and DDR activation resulting from faulty DDRs. This would be consistent with the DNA-SCARS hypothesis. To determine whether the foci are DNA-SCARS, further validation studies are necessary. One major difference between regular 53BP1 foci and DNA-SCARS is that the latter co-localize with PML bodies. Thus, co-localization studies between 53BP1 foci and PML bodies should be performed (Rodier *et al*, 2010). DNA-SCARS also lack the DNA repair proteins RPA and RAD51, and thus immunofluorescence for these proteins should be performed to determine whether the foci observed in the miRNA-transfected cells are DNA-SCARS (Rodier *et al,* 2010). Furthermore, DNA-SCARS lack single-stranded DNA and DNA synthesis and thus bromodeoxyuridine assays and probing for the single-stranded DNA binding protein RPA70 would further distinguish between regular 53BP1 foci and DNA-SCARS (Rodier *et al,* 2010). Several limitations of this experiment will also need to be clarified in future experiments. In particular, thresholding for image quantification remained problematic. In order to assure that the smaller foci were distinguishable from each other when in close proximity, the threshold was set relatively high, meaning that the size of the larger foci was reduced and any smaller, fainter foci all but disappeared. However it was determined that this was preferable to setting a low threshold and risking the small foci being indistinguishable, and therefore being considered as one foci, thereby creating error in both the total number and size of the foci.

The observation of faulty DNA damage repair would also be consistent with an inhibition of autophagic flux. Recently, autophagy has been linked to the activation of the DDR. Inhibition of histone deacetylases (HDACs) induces autophagy, which is known to degrade several key DDR proteins including Sae2, a target of cyclin-dependent kinase 1 involved in the processing of

DSBs. The autophagic degradation of these repair proteins leads to reduced processing of DSBs by the repair machinery and an impaired DDR (Robert *et al*, 2011). If necessary DDR proteins such as Sae2 are sequestered in accumulating non-degradative autophagosomes, resulting from flux inhibition, then they are prevented from completing repair. This would result in an increase in DNA damage within the cell and a subsequent upregulation of initiation of the DDR.

4.7 A Precedent for miRNAs in senescence bypass

Multiple human miRNAs have been linked to senescence bypass. The miR-17-92 gene cluster is upregulated in many different forms of cancer and has been demonstrated to play a role in oncogenic transformation and the inhibition of apoptosis. Interestingly, this miRNA cluster also has the ability to bypass Ras-induced senescence by targeting the p21 tumour suppressor protein to facilitate tumourigenesis (Hong *et al*, 2011). Additionally, miR-17-5p, miR-20a-b, miR-93, miR-106a-b, miR-130b, miR-302a-d, miR-372, miR-373, miR-512-3p, miR-515-3p, miR-519c-e, miR-520a-g, miR-526b*, and miR-146a-b have the ability to bypass Ras-induced senescence. These miRNAs also primarily function by targeting the p21 tumour suppressor protein and they are upregulated in many different forms of cancer (Borgdorff *et al*, 2010; Cho *et al*, 2009; Feliciano *et al*, 2011; Lee *et al*, 2009; Voorhoeve *et al*, 2006).

4.8 miR-K5 and miR-K11 target proteins involved in tumourigenesis and senescence

The work included in this thesis is the first study to link viral miRNAs to bypass of OIS. However, precedent exists in the literature for a role for miR-K5 and miR-K11 in oncogenesis. miR-K5 targets two proteins with relevance to senescence. Firstly, miR-K5 inhibits Myeloid Differentiation Primary Response Gene 88 (MyD88), a positive regulator of the NF-κB pathway. In addition to its role in the host immune response, the NF-κB pathway is crucial to the SASP, an important feature of senescence. Additionally, miR-K5 targets BCLAF1, which interacts with Bcl-2, thus preventing its interaction with beclin 1 and inhibiting autophagy, an important inducer of senescence. BCLAF1 also functions as an anti-apoptotic protein through its regulation of the DDR. It recruits γ-H2AX to the sites of double stranded DNA breaks, allowing for efficient DNA damage repair (Lee *et al*, 2012). Intriguingly, miR-K5 is expressed at the lowest

levels of any of the KSHV miRNAs during latent infection. This is owing to an A to G polymorphism in the miR-K5 passenger strand found in approximately twenty percent of all KSHV genomes (Marshall *et al*, 2007; Marshall *et al*, 2010). This single nucleotide polymorphism results in dramatically reduced processing of primary-miR-K5 by Drosha in the nucleus and leads to lower levels of miR-K5 expression (Cai *et al*, 2005, Gottwein *et al*, 2006; Gottwein *et al*, 2011).

Perhaps even more interesting, is a strong link between miR-K11 and cancer. miR-K11 shares perfect seed sequence homology with human hsa-miR-155 (Gottwein *et al*, 2007; Skalsky *et al*, 2007). Intriguingly, miR-155 is an oncomiR and is upregulated in many B-cell lymphomas (Eis *et al*, 2005; Kluiver *et al*, 2005). Indeed EBV, which in contrast to KSHV does not express an ortholog of miR-155, upregulates expression of miR-155 upon infection, an important step in B-cell immortalization (Linnstaedt *et al*, 2010). Additionally, Marek's Disease Virus (MDV), an α-herpesvirus that causes T-cell lymphomas in chickens, expresses MDV-miR-M4, another ortholog of miR-155. These studies suggest that viral orthologs of miR-155 may be important in the development of these virally induced cancers. miR-K11 and miR-155 regulate a similar subset of genes, several of which exhibit relevance to senescence. Both miRNAs regulate TBT and CNC homology 1 (BACH1), a transcriptional repressor that regulates genes involved in the cell cycle and oxidative stress (Warnatz *et al*, 2011). In addition, both target IKKε, involved in NF-κB activation and the SASP, tumour suppressor protein THBS1, Fos, which controls cell cycle regulation and proliferation and cell proliferation and the oncogene MAF (Gottwein *et al*, 2007; Hansen *et al*, 2010; Liang *et al,* 2011; Liu *et al*, 2012; Samols *et al*, 2007). Recently, both miR-K11 and miR-155 have been shown to directly target SMAD5 (Rai *et al*, 2010). This repression allows cells expressing either miRNA to overcome TGF-β-induced growth arrest ensuring continued cell division and tumour development (Rai *et al*, 2010; Liu *et al*, 2012). Interestingly, BACH1 is among the most strongly regulated targets of all the KSHV miRNAs. The most well characterized target of BACH1-mediated transcriptional repression is hemeoxygenase 1 (HMOX1), an enzyme necessary for heme catabolism whose expression is strongly upregulated in KS (McAllister *et al*, 2004). Given that DNA damage is a crucial mediator of the senescence pathway, increased reactive oxygen species resulting from de-repression of HMOX1 by miR-K11 may be relevant to miR-K11-mediated bypass of OIS.

Furthermore, miR-155 is known to target the Suppressor of Cytokine Signalling 1 (SOCS1), which acts as a scaffolding protein for the phosphorylation, and consequent activation, of the tumour suppressor p53. Calabrese *et al*. demonstrated that SOCS1 activation of p53 is sufficient to induce senescence in fibroblast cells (Calabrese *et al*, 2009).

Recently, miR-K5 and miR-K11 have been coupled together in another study examining the role of miRNAs in KSHV infection and tumourigenesis. Bekerman and colleagues demonstrated that miR-K5 and miR-K11 translationally repress activation-induced cytidine (AID) by interacting with the 3'UTR of this gene. AID is typically expressed in germinal centre B cells, although recent studies suggest it may also be expressed in lower levels in other cell types, including fibroblast cells. It is involved in class-switch recombination and somatic hypermutation, both of which serve to increase antibody diversity and strengthen antibody affinity. KSHV infection results in an upregulation of AID, and AID-induced DNA damage, resulting in defective lytic replication and infectivity in primary B cells. Although AID expression has been linked to transformation in the past, given its ability to induce DSBs and a subsequent DDR, it plays a protective role in KSHV pathogenesis, marking infected cells for elimination, and therefore inhibiting viral tumourigenesis. As such, AID may actually limit the oncogenic potential of KSHV. Thus by targeting AID expression, miR-K5 and miR-K11 contribute to KS tumourigenesis. Further evidence for this role exists in the fact that EBV upregulates miR-155 during EBV-mediated oncogenesis, resulting in a reduction in AID expression (Bekerman *et al*, 2013). Interestingly, transient transfection of both miR-K5 and miR-K11 in concert resulted in an additive effect on AID suppression. This would be an interesting phenomenon to pursue with respect to OIS bypass. This could be accomplished by transiently transfecting both miR-K5 and miR-K11 into v-cyclin-transduced cells and comparing the OIS bypass to cells transfected with only one of the miRNAs.

4.9 Predicting targets for miR-K5 and miR-K11

Predicting targets for miRNAs is an extremely difficult process. Several miRNA target prediction strategies do exist but all are wrought with complications and uncertainties. The fastest method for predicting mRNA targets is to use sequence comparison, a process known as

seed-matching, with a focus on mRNAs containing sequences complementary to the 5' end of the miRNA under investigation. Despite numerous successes, however, this method has a significant false-positive and false-negative rate and also misses mRNA targets, which do not have perfect sequence complementarity (Lal *et al*, 2009 ; Ziegelbauer, 2014). Another common method is expression analysis at the mRNA level, using microarrays and RNA sequencing, or with proteomics (Ziegelbauer, 2014). However these methods identify both direct and indirect targets, although proteomic screening has demonstrated promise in identifying miRNA targets, which are missed at the mRNA level (Gallaher *et al*, 2013; Grey *et al*, 2007). The redundancy of most miRNAs also represents a significant problem for these methods. More recent methods of identifying miRNA targets centre around studying miRNA:mRNA complexes, followed by deep sequencing (Ziegelbauer, 2014). One such approach, known as RIP-Seq, involves cross-linking miRNAs, mRNAs and the RISC followed by immunoprecipitation for RISC. All RNA sequences associated with RISC are enriched and subsequently sequenced to find targets. Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) is also a common method for identifying targets, particularly of viral miRNAs, by crosslinking RISC complexes with miRNAs then converting associated mRNAs into a cDNA library for deep sequencing analysis. PAR-CLIP is frequently used in models where specific viral miRNAs are deleted then enriched mRNAs compared to wild type infections to determine targets of those same miRNAs (Pavelin *et al*, 2013). Following identification of miRNAs of interest, targets must be validated, which poses another significant challenge. The most common method of analysis consists of luciferase reporter assays wherein the region of interest, usually the 3' UTR, is cloned downstream of a luciferase reporter then exposed to the miRNAs. If a target sequence is located within the cloned region, luciferase expression will be reduced. This is often followed by site-directed mutagenesis to further validate the target by demonstrating that mutations to certain bases within the region of interest result in a restoration of luciferase activity (Ziegelbauer, 2014).

4.10 Limitations and Future Directions

Owing to the immense difficulty in identifying miRNA targets, this investigation focuses instead on functional studies of miR-K5 and miR-K11. As both these miRNAs have multiple known, and hundreds of unknown, targets, it is difficult to address whether the phenotypic

readout obtained in the OIS bypass screen is due to one specific miRNA target or multiple targets within the same pathway, or multiple pathways resulting in similar physiological readouts. Indeed, the data presented in this thesis suggests that miR-K5 and miR-K11 are, in fact, targeting three distinct cellular pathways to obtain bypass of OIS: the DDR, p16 tumour suppressor and autophagy. However, these three targets may be reconciled into one conceivable model as described above. Further validation, as discussed, will be necessary to determine the nature of miR-K5 and miR-K11 mediated repression of each of these pathways and their respective effects on OIS. Furthermore, the OIS screen itself will require additional confirmation via alternative methods. The most important experiment that must be performed is to determine the effects of these miRNAs in the context of KSHV infection. One experiment to address this question could be a study involving miRNA sponges, known as antagomiRs. These antagomiRs are designed to bind and sequester a particular miRNA. A control experiment wherein cells are infected with KSHV then transfected with antagomiRs against miR-K5 or miR-K11 will be necessary to determine whether the original wild-type phenotype, OIS, can be rescued. This will allow for increased confidence into the physiological readout obtained with the KSHV miRNAs. Further validation studies will also involve obtaining or cloning miR-K5 and miR-K11 knockout viruses to determine whether these viruses have the ability to bypass OIS. Alternatively, KSHV miR mutant bacmids, developed by the lab of Dr. Rolf Renne, with the removal of either miR-K5 or miR-K11 could be tested. Additionally, as these miRNAs are believed to be acting in conjunction with v-FLIP to bypass OIS, studies involving co-transfection or co-transduction of miR-K5, miR-K11 and v-FLIP will be required to confirm the original hypothesis that these miRNAs play a role in aiding v-FLIP bypass v-cyclin-induced OIS. Following this crucial validation of the initial OIS bypass screen, further experiments to determine the effects of this miRNAs over the course of senescence and beyond will also be necessary. By studying the DDR, p16 expression and downstream effects and autophagy along a time course, further insight will be gained into where miR-K5 and miR-K11 are inducing their respective effects. Following these time course experiments, studies examining the effect of the miRNAs on the DDR foci, autophagic flux and both downstream and upstream effectors of p16 will be necessary to determine the exact effects mediated by both miR-K5 and miR-K11.

Although the strong phenotypic result from the screen, and significant difference observed for the miRNAs compared to the NS control in all of the experiments described above, suggest that the miRNA mimics are being delivered into the cells, one major limitation of this OIS bypass screen is a lack of information on the efficiency of miRNA delivery. Discussions with Dr. Eva Gottwein, of Northwestern University, and Dr. Ziegelbauer, of the National Institutes of Health, revealed that both researchers frequently observe miRNAs being delivered to 100% of the cells using the same transfection mechanisms as discussed in this thesis. However studies wherein a fluorescent report tag is conjugated to the miRNA of interest to allow for gauging of transfection efficiency may be performed to validate this. These experiments are not without challenges, as the danger is that these additional tags may affect miRNA function and targeting. Furthermore, the miRANDA miRNA mimics may not function in an identical fashion to the viral miRNAs themselves. Although designed to mimic the viral miRNAs, these miRANDA mimics are chemically enhanced to ensure efficient delivery of the active strand to the RISC and to minimize cellular interferon responses. Little information is available comparing their relative functions to the miRNAs they mimic. Furthermore, the targets of miRNAs often vary depending on the cellular environment and experimental set-up. Often these miRNAs have multiple functional targets on one mRNA, although usually one site is preferred over the others (Abend *et al,* 2010; Dahike *et al*, 2012). Most miRNAs target the 3' UTR that is known to change during development or with different cellular growth conditions (Mayr and Bartel, 2009; Sandberg *et al,* 2008). Sometimes mRNAs of interest are also not expressed in laboratory cell culture settings or in particular cell types. All of the above represented significant challenges when designing the OIS bypass screen. Multiple versions of the experimental set-up used in the screen were tested and the most successful protocol was chosen based on lowest levels of cell death and strongest physiological readouts.

4.11 Conclusion

In conclusion, this thesis highlights the role of two KSHV miRNAs, miR-K5 and miR-K11, in bypassing OIS (Figure 4.4). Both miRNAs repress the expression of the tumour suppressor protein, p16, which may result in an increase in E2F target gene expression, leading to OIS bypass. Additionally, miR-K5 and miR-K11 inhibit autophagic flux supportive of

senescence bypass. Further validation studies will be required to determine the ramifications of miRNA-mediated OIS bypass in the context of viral infection and to elucidate the precise mechanism by which the miRNAs are inducing senescence bypass.

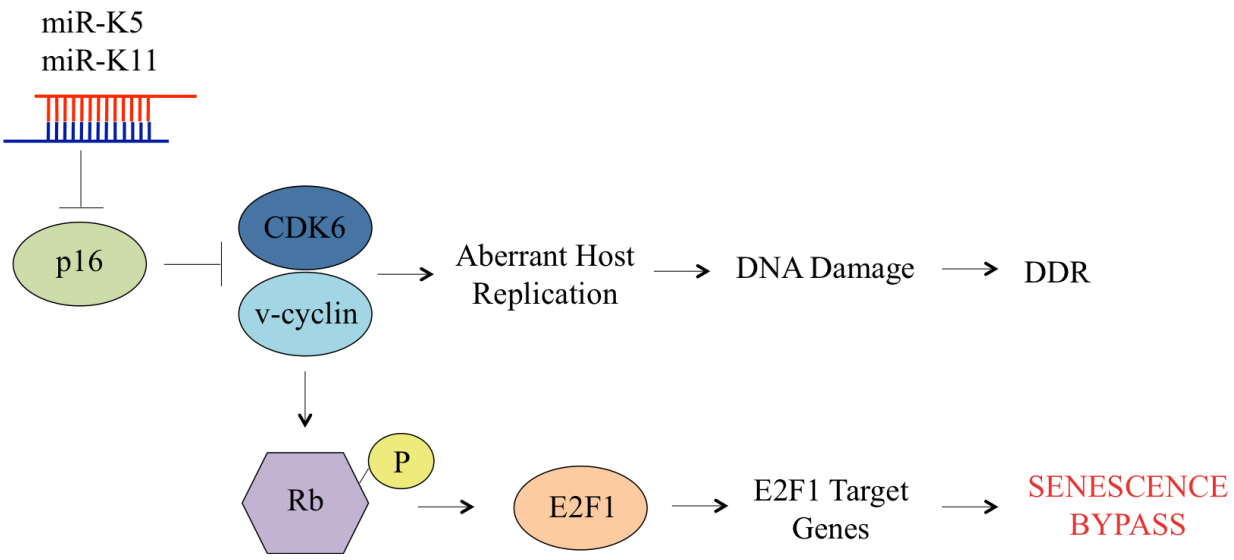


Figure 4.1 miRNA-mediated repression of p16 induces OIS bypass and upregulates the DDR. miR-K5 and miR-K11 suppress p16 expression thus allowing for the formation of the v-cyclin-CDK6 complex. This complex has two-fold effects. Firstly, it induces hyperproliferation and hyperreplication resulting in increased DNA damage and an upregulated DDR. Additionally, it allows for the phosphorylation of Rb, thus preventing its interaction with E2F1 and allowing for the transcription of E2F target genes, many of which are involved in G1/S transition and therefore proliferation.

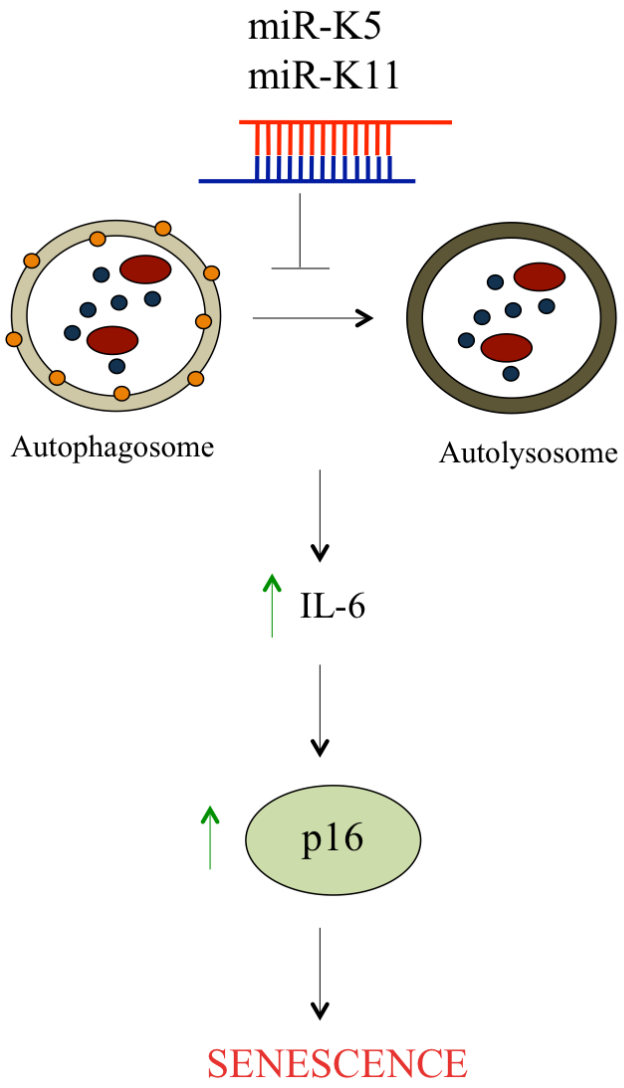


Figure 4.2 miRNA-mediated repression of autophagic flux leads to reduction in p16 and OIS bypass. miR-K5 and miR-K11 suppress autophagic flux thus preventing the release of IL-6 from autolysosomes. IL-6 has been linked to the activity of the p16 tumour suppressor pathway. This reduction in IL-6 therefore may explain the decrease in p16 expression and may subsequently lead to bypass of senescence.

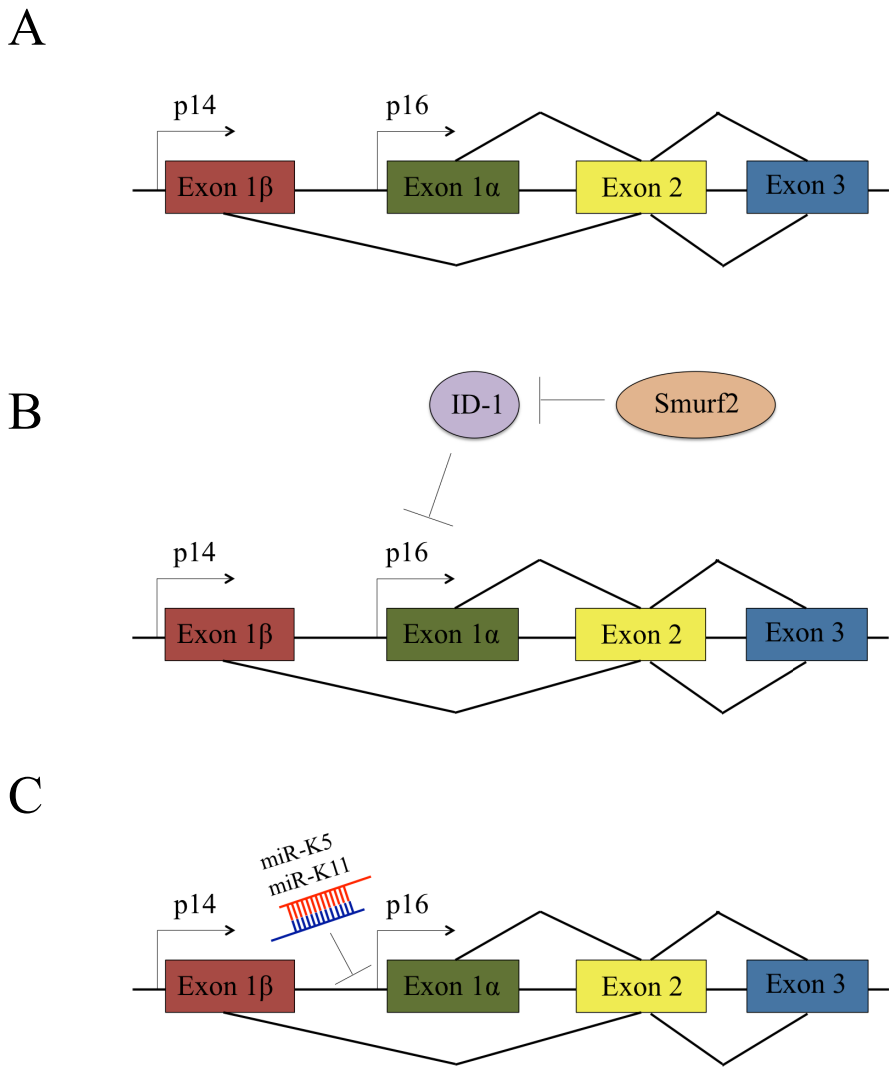


Figure 4.3 The CDKN2A Locus. **(A)** CDKN2A locus encoding p16 (INK4A) and p14 (ARF). The p14 and p16 proteins are transcribed from individual promoters and use alternative first exons, 1β and 1α respectively. These exons are joined by a splice acceptor site to the coding sequence and therefore both p16 and p14 share exons 2 and 3. **(B)** Id1 is a transcriptional repressor which suppresses expression of p16. Id1 is itself controlled by Smurf2 ubiquitin ligase, which results in the degradation of Id1 and allows for p16 transcription (Ramkumar *et al.*, 2012). During senescence, Smurf2-mediated ubiquitination, and subsequent degradation, of Id1 has been linked to the regulation of p16 expression, leading to bypass of senescence (Kong *et al.*, 2011). **(C)** miRANDA analysis suggests a target site for miR-K5 and miR-K11 in the 3'UTR of the CDKN2A locus.

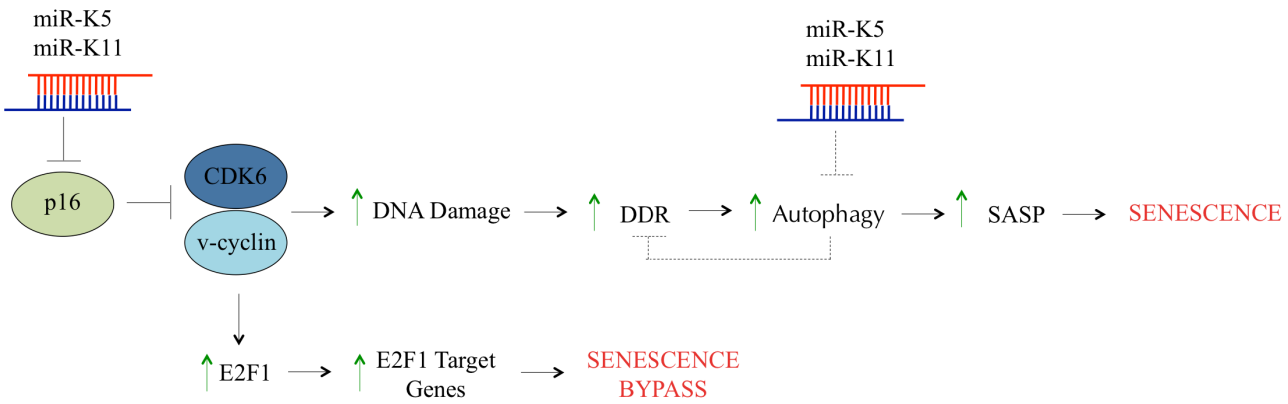


Figure 4.4 A model for viral miRNA-mediated bypass of OIS by targeting p16 and autophagic flux. miR-K5 and miR-K11 suppress p16 expression thus allowing for actions of v-cyclin. This complex has two-fold effects. Firstly, it induces hyperproliferation and hyperreplication resulting in increased DNA damage and an upregulated DDR. Additionally it prevents the transcription of E2F target genes, many of which are involved in the induction of senescence, thus leading to senescence bypass and the phenotypic outcome observed upon miRNA transfection in the OIS bypass screen. miR-K5 and miR-K11 also inhibit autophagic flux, thus sequestering key DDR factors in non-degradative autophagosomes and inhibiting the DDR. This secondary action of the miRNAs is further supportive of OIS bypass.

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