DYNAMIC INTERPLAY BETWEEN KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS LATENT PROTEINS IN THE CONTROL OF ONCOGENE-INDUCED SENESCENCE

by

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ABSTRACT

Acute oncogenic stress can activate autophagy and facilitate permanent arrest of the cell cycle through a failsafe mechanism known as oncogene-induced senescence (OIS). Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiologic agent of Kaposi's Sarcoma (KS) and has been reported to encode oncoproteins within its latency program that engage host autophagy and OIS pathways; however, the mechanisms by which KSHV oncoproteins promote KS tumorigenesis remain unclear. Here, I demonstrate that ectopic expression of the latent KSHV protein viral cyclin (v-cyclin) deregulates the cell cycle, induces DNA-damage responses (DDRs) and promotes OIS through an autophagy-dependent mechanism. During latency, v-cyclin is co-expressed from a single transcript with a viral homolog of FLICE-inhibitory protein (v-FLIP) that blocks autophagy by binding and inhibiting Atg3. Co-expression of v-FLIP has no effect on DDRs, but efficiently blocks v-cyclin-induced autophagy and senescence. Remarkably, suppression of v-FLIP function during KSHV latency, through specific inhibitory peptides, rescues host cell autophagy and induces senescence of infected cells. Together, these data reveal a coordinated viral gene-expression program that subverts autophagy, impairs senescence, and facilitates the proliferation of KSHV-infected cells.

LIST OF ABBREVIATIONS AND SYMBOLS USED

°C	Degrees Celsius
%	Percent
AIDS	Acquired immune-deficiency syndrome
AIDS-KS	Acquired immune-deficiency syndrome-associated Kaposi's sarcoma
AMPK	Adenosine monophosphate activated protein kinase
ATCC	American Type Culture Collection
bFGF	Basic fibroblast growth factor
BrdU	Bromodeoxyuridine
cDNA	Cloned deoxyribonucleic acid
CKI	Cyclin-dependent kinase inhibitor
Ct	Cycle threshold
d	Day(s)
DAPI	4',6-diamidino-2-phenylindole
DE	Delayed-early genes
DED	Death-effector domain
DDR	DNA-damage response
DMEM	Dulbecco's Minimal Essential Medium
DNA	Deoxyribonucleic acid
DSBs	Double-strand DNA breaks
EBV	Epstein-Barr Virus
EM	Electron microscopy
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GFP	Green fluorescent protein
h	Hour(s)
HA	Hemagglutinin epitope
HAART	Highly active anti-retroviral therapy

HCMV	Human cytomegalovirus
HFFs	BJ fibroblasts
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HSV-1	Herpes simplex virus 1
HVS	Herpesvirus saimiri
IE	Immediate-early genes
IFN-γ	Interferon gamma
IKKγ	Inhibitor of kappa B kinase gamma
IL-6	Interleukin 6
IL-8	Interleukin 8
IRES	Internal Ribosomal Entry Site
KSHV	Kaposi's Sarcoma-associated Herpesvirus
L	Late genes
LANA	Latency-associated nuclear antigen
LC3-I	Unmodified LC3
LC3-II	Phosphatidylethanolamine-modified LC3
MAPK	Mitogen-activated protein kinase
min	Minute(s)
mRNA	Messenger ribonucleic acid
miRNA	Micro-ribonucleic acid
MK2	Mitogen-activated protein kinase-associated protein kinase 2
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target-of-rapamycin complex 1
mTORC2	Mammalian target-of-rapamycin complex 2
MV	Microvascular
NF-κB	Nuclear factor kappa B
OIS	Oncogene-induced senescence
ORF	Open-reading frame
PAMP	Pathogen-associated molecular patterns

PCR	Polymerase chain reaction
PEI	Polyethylenimine
PEL	Primary effusion lymphoma
PRR	Pattern-recognition receptors
PS	Post-selection
qRT-PCR	Quantitative real-time polymerase chain reaction
RFP	Red Fluorescent Protein
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute Medium
RTA	Replication transcription activator
SA β-gal	Senescence-associated β-galactosidase
SAHF	Senescence-associated heterochromatin foci
SASP	Senescence-associated secretory phenotype
TERT-HFFs	Telomerase-immortalized BJ fibroblasts
TIME	Telomerase-immortalized microvascular endothelial
TNFα	Tumor necrosis factor alpha
TSC2	Tuberous sclerosis 2
v-cyclin	Viral cyclin
VEGF	Vascular endothelial growth factor
v-FLIP	Viral FLICE-inhibitory protein
v-IL-6	Viral -IL-6
VSV	Vesicular stomatitis virus
VZV	Varicella zoster virus
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

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CHAPTER 1 - INTRODUCTION

1.1 Overview

Viruses are obligate intracellular parasites that exploit the energy supplies, raw materials and biosynthetic machinery of their cellular hosts, sometimes leading to cell death. Viruses and their hosts co-evolve; viruses evolve to maximize yield of progeny, whereas host cells evolve to limit viral replication and promote survival of the host organism. An unfortunate consequence of these evolutionary forces is the emergence of viruses that maximize their replication and survival by subverting host cell proliferation. These viruses, known as oncoviruses, encode genes that activate the same cellular pathways that promote deregulated proliferation of cancer cells, and thus inadvertently induce tumorigenesis in hosts.

Oncogene-induced senescence (OIS) is a cellular failsafe mechanism that protects cells against deregulated proliferation induced by oncogenes. The senescence program promotes permanent proliferation arrest in response to oncogenic stress and involves numerous effector mechanisms. Furthermore, OIS is increasingly regarded as an important barrier to tumorigenesis. Accumulating evidence indicates that oncoviruses such as Kaposi's sarcoma-associated herpesvirus (KSHV) induce cellular stresses during infection that are typically associated with trigger of OIS; however, no oncogenic virus to date has been observed to induce senescence. This suggests that either cell stresses induced by KSHV oncogenes are not sufficient to promote OIS or that KSHV, and perhaps oncoviruses in general, have evolved mechanisms to actively inhibit OIS during infection. Presently, the role of OIS in KSHV infection remains largely unexplored and may yield valuable knowledge about oncoviruses and the process of tumorigenesis.

The following introduction provides a summary of KSHV, virus-associated pathologies, replication and gene products associated with tumorigenesis. Furthermore, the mechanics of OIS are discussed in detail, highlighting KSHV oncogenes that intersect known senescence pathways. Finally, autophagy is reviewed, facilitating discussion of the potential links between KSHV modulation of autophagy, OIS and tumorigenesis.

1.2 Kaposi's Sarcoma-Associated Herpesvirus

KSHV is the most recently discovered human herpesvirus and, like all members of the *Herpesviridae* family, is a large enveloped virus with a linear double-stranded DNA (dsDNA) genome. Furthermore, being a herpesvirus, KSHV can undergo both latent and lytic infectious cycles within host cells. Herpesviruses are categorized into three subfamilies, α-herpesvirinae, β-herpesvirinae and γ-herpesvirinae, based on similarities in their size, structure, and genomic homology. Notable α-herpesviruses include herpes simplex virus 1 (HSV-1) and varicella zoster virus (VZV), the causative agent of chicken pox and shingles, whereas human cytomegalovirus (HCMV) is a member of the β-herpesvirus subfamily. Epstein-Barr Virus (EBV) and KSHV are γ-herpesviruses. Members of this subfamily preferentially undergo latent infectious cycles and have restricted host cell specificity, predominantly infecting B and T lymphocytes. Intriguingly, EBV and KSHV are the etiological agents for a number of human cancers. EBV infection is associated with the development of nasopharyngeal carcinomas, Burkitt's lymphoma and Hodgkin's lymphoma. In contrast, KSHV is the infectious causative agent of an unusual endothelial neoplasm known as Kaposi's sarcoma, as well as two lymphoproliferative disorders: Primary effusion lymphoma and Multicentric Castleman's disease (Fields et al., 1996).

1.2.1 Disease Associations

1.2.1.1 Kaposi's Sarcoma

In 1872, Hungarian dermatologist Moritz Kaposi first noted a strange "idiopathic multiple pigmented sarcoma of the skin" that afflicted some of his patients. Occasionally, patients with this apparent skin pathology would also develop lesions in their lungs and gastrointestinal tract (Kaposi, 1872). Despite the fact this disease was later found to be an endothelial neoplasm, rather than a sarcoma of the skin, it was named Kaposi's sarcoma (KS) to honor Moritz Kaposi's seminal discovery (Ganem, 2010; Mesri et al., 2010).

KS is categorized into four distinct epidemiological forms: classic KS affecting elderly men of Mediterranean or Ashkenazi Jewish decent; endemic KS, found in children and young adults throughout sub-Saharan Africa; iatrogenic KS, occurring within individuals receiving immunosuppressive drugs, such as those who have undergone organ transplant surgery; and epidemic KS, the most common epidemiological variant and predominant malignancy amongst individuals with AIDS (Acquired immune deficiency syndrome) (Antman and Chang, 2000; Boshoff and Weiss, 2002; Ganem, 2010; Mesri et al., 2010). Classic KS is a relatively mild form of the disease, with lesions commonly localized to the skin of the lower extremities. Individuals with endemic KS display broad severity ranging from benign cutaneous lesions to aggressive lesions that extend into the soft tissue, bone, and viscera. Iatrogenic, or post-transplant, KS is a rapidly progressing form of the disease; however patients frequently undergo spontaneous remission upon the discontinuation of immunosuppressive therapy. Finally, epidemic or AIDS-associated KS (AIDS-KS) is the most aggressive epidemiological variant and significantly contributes to the morbidity and mortality of AIDS patients (Beral et al., 1990). AIDS-KS typically presents cutaneously on the head, neck, torso, hard palate, and gums, and later advances to the lymph nodes, gastrointestinal tract, and lungs. Importantly, the incidence of AIDS-KS has decreased dramatically since the implementation of highly active anti-retroviral therapy (HAART). This highlights the dependence of AIDS-KS on human immunodeficiency virus (HIV) infection and its associated immunosuppression. Furthermore, KS remains a common AIDS-related comorbidity in third world countries that lack reliable access to HAART (Ganem, 2010; Mesri et al., 2010).

Despite striking differences in disease severity and outcome, at the histological level all forms of KS are quite similar. KS is an unusual malignant neoplasm of the endothelium that commonly presents cutaneously as multifocal dark purple lesions (Figures 1.1A and 1.1A B). Lesions typically progress morphologically from patches to plaques to raised nodules, and aggressive forms can lead to disease within visceral tissues (Antman and Chang, 2000; Ganem, 2010; Mesri et al., 2010). KS tumors are highly

vascularized and pervaded by inflammatory cells, including macrophages, mast cells, dendritic cells and lymphocytes (Boshoff and Weiss, 2002). The primary proliferating cells within KS tumors are the so-called 'spindle cells', abnormally elongated endothelial cells with a spindle-like morphology (Figure 1.1C) (Browning et al., 1994; Boshoff et al., 1995; Boshoff et al., 1997; Boshoff, 1998). Furthermore, spindle cells found within most individual KS lesions are clonal. Intriguingly, examination of tumors from patients with multiple KS lesions revealed some tumors to be oligoclonal, suggestive of spindle-cell metastatic dissemination (Rabkin et al., 1995; Rabkin et al., 1997; Gill et al., 1998). Identification of the precise endothelial cell lineage from which spindle cells originate has been enigmatic because these cells simultaneously express markers of various endothelial lineages. However, recent evidence suggests that the spindle cell may be derived from the lymphatic, rather than vascular, endothelial lineage (Hong et al., 2004; Weninger et al., 1999). KS spindle cells are also important modulators of the pro-inflammatory and angioproliferative secretory profile of the tumor microenvironment. In particular, spindle cells secrete high levels of vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), basic fibroblast growth factor (bFGF), tumor necrosis factor α (TNF-α), and interferon-γ (IFN-γ) (Ensoli et al., 1989; Ensoli et al., 1992; Miles et al., 1990; Salahuddin et al., 1988).

In 1994, Chang and colleagues suspecting KS risk might be linked to a sexually transmitted pathogen other than HIV, used representational difference analysis to search for DNA sequences present in the cells of KS lesions but absent in normal tissue (Chang et al., 1994). This landmark study identified herpesvirus-like DNA sequences within diseased tissue and led to the discovery of KSHV, also known as human herpesvirus-8 (HHV-8), the etiological agent of KS. To date, KSHV is the only known risk factor to be essential for KS development (Moore and Chang, 1995; Moore et al., 1996). Quickly thereafter, KSHV was also linked to two lymphoproliferative disorders (Cesarman et al., 1995), underscoring the clinical importance of this novel oncogenic herpesvirus.

1.2.1.2 Primary Effusion Lymphoma

Primary effusion lymphoma (PEL) is a rare B-cell lymphoma that often induces lymphomatous effusion in the pleural, pericardial, and peritoneal cavities (Arvanitakis et al., 1996; Knowles et al., 1989). HIV-positive patients suffering from advanced stages of immunosuppression are most susceptible to the development of PEL. Virtually all cases of PEL harbor KSHV DNA in every tumor cell (Cesarman et al., 1995; Cesarman et al., 1996; Mesri et al., 1996; Nador et al., 1996). Furthermore, many B-lymphocyte tumor cells from PEL patients are also co-infected with EBV. KSHV infection of PEL cells is predominantly latent, which has made PEL cell lines the most widely studied model for KSHV latency (Arvanitakis et al., 1996; Renne et al., 1996b). The establishment of KSHV-positive PEL cell lines that can be induced to undergo lytic replication has become an invaluable tool for the study of KSHV molecular biology and pathogenesis.

1.2.1.3 Multicentric Castleman's Disease

Castleman's disease is a rare, polyclonal lymphoproliferative lesion (Du et al., 2001). Two clinical forms of Castleman's disease exist: (1) a localized form, defined by a single node or node cluster, generally found in HIV-negative patients and unlinked to KSHV; and (2) a multicentric form, found more frequently in HIV-positive patients and virtually always linked to KSHV infection (Oksenhendler et al., 1996; Soulier et al., 1995). Multicentric Castleman's disease (MCD) is an aggressive systemic illness characterized by sustained fever, sweats, weight loss, swollen lymph nodes and spleen. Intriguingly, the severity and course of MCD is closely correlated with KSHV viral load, and the disease is thought to be mediated by a viral homologue of IL-6 (v-IL-6) expressed and secreted from infected cells (Oksenhendler et al., 2000).

1.2.2 Kaposi's Sarcoma-Associated Herpesvirus Molecular Virology

1.2.2.1 Virion structure and genome organization

KSHV virions appear structurally similar to those of other herpesviruses (Figure 1.1D). Electron-microscopy analyses have shown that virions contain an electron-dense

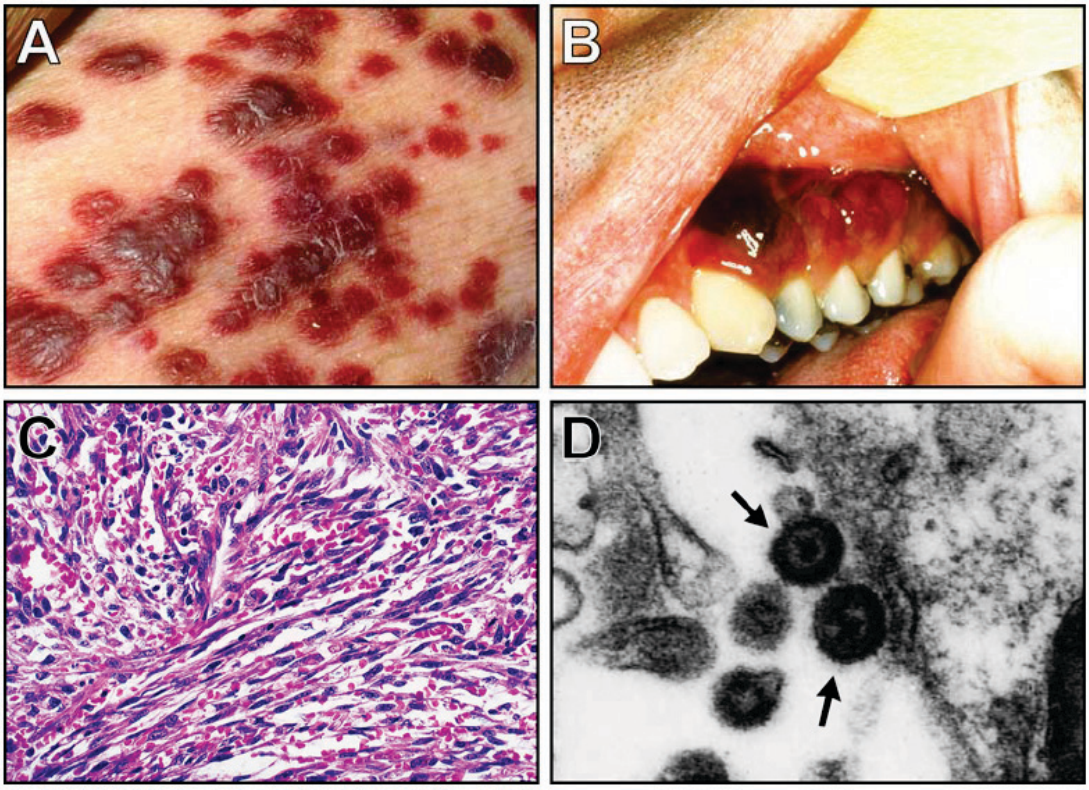

Figure 1.1
Kaposi's sarcoma
Kaposi's sarcoma (KS) often presents as nodular lesions, dark purple or red in colour, primarily localized to the (A) skin, but can also be found in the (B) oral cavity and, in advanced cases of KS, may disseminate to the lungs and gastrointestinal tract (not shown). (C) The driving force of KS is the 'spindle cell', an abnormally elongated endothelial cell that is unique to KS. (D) The etiological agent for KS development is a novel γ-herpesvirus, 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) VisualsOnline. Image for (C) was reproduced from Rosai (2004). Image for (D) was reproduced from Antman and Chang (2000).

nucleocapsid within a lipid bilayer envelope. The envelope of KSHV contains numerous glycoproteins, including glycoprotein B, which facilitate virion interaction with the cell surface (Wang et al., 2003). Between the KSHV envelope and the nucleocapsid is a proteinaceous region known as the tegument. The tegument is composed of numerous KSHV proteins, and while the functions of many remain undefined, it is likely that they participate in early events of the replicative cycle, before viral gene expression begins (Bechtel et al., 2005b). Intriguingly, KSHV virions also harbor messenger ribonucleic acids (mRNAs) corresponding to abundantly expressed viral transcripts of the lytic cycle (Bechtel et al., 2005a). Similar to tegument proteins, these mRNAs may participate in initiation of the KSHV replicative cycle. The KSHV capsid, which has been studied extensively, is structurally similar to those of HSV-1 and HCMV. The capsid forms a symmetric icosahedron approximately 110 nm in diameter and each face is triangular in shape (Nealon et al., 2001; Said et al., 1996; Trus et al., 2001; Wu et al., 2000).

The linear dsDNA genome of KSHV is approximately 170 kb in length (Renne et al., 1996a). The genome contains a central unique region of approximately 145 kb, containing all the viral open reading frames (ORFs), flanked by a series of 801-bp GC-rich direct-terminal repeats without coding potential. The complete nucleotide sequence of KSHV predicts the existence of at least 87 ORFs (Ganem, 2010; Mesri et al., 2010). Comparison of the KSHV genome sequence with those of other γ-herpesviruses demonstrates that there are four major regions of highly conserved genes. In fact, Herpesvirus saimiri (HVS), a closely related γ-herpesvirus of new-world primates, shares 68 conserved genes with KSHV that have been numbered consecutively from the 5' to 3' ends of the genome and marked by the prefix 'ORF' (Russo et al., 1996). Striking similarities in genome organization also exist between KSHV and murine γ-herpesvirus 68, as well as some other non-human γ-herpesvirus members (Alexander et al., 2000; Searles et al., 1999; Virgin et al., 1997). Viral genes that are unique to KSHV have been designated K1 to K15 (Wen and Damania, 2010). Importantly, KSHV also encodes an impressive number of ORFs that share significant homology to cellular genes (Boshoff and Weiss, 1998). It is likely that many of these virally encoded homologues have been

pirated from host cells through the course of co-evolution. Intriguingly, many KSHV gene products with cellular homologs appear to have evolved distinct functions and have been strongly implicated in viral pathogenesis (Boshoff and Weiss, 1998).

1.2.2.2 The Lytic Replication Cycle

KSHV infection initiates with the binding of virion envelope glycoproteins to cell surface proteins, often termed cellular receptors, that have been co-opted by the virus to mediate entry. Following binding, the KSHV virion either directly fuses with the plasma membrane of the host cell or is endocytosed and fuses with endosomal membranes, delivering the tegument and capsid, which encompass the viral genome, to the cytoplasm of the cell (Chandran, 2010). Subsequently, capsids are trafficked to the nuclear envelope and the viral genome is imported through nuclear pores into the nucleus. Within the nucleus, the KSHV genome is circularized and bound by host cell histones, forming viral chromatin, to facilitate transcription by RNA polymerase II.

Like all herpesviruses, KSHV can express its genes in two distinct genetic programs, lytic replication or latency, depending on the conditions of infection. The KSHV lytic replication cycle involves expression of the nearly the entire viral genome and occurs in temporally regulated transcription cascade (Deng et al., 2007). As their names would indicate, immediate-early (IE) genes are the first class to be expressed during lytic replication. Most important amongst IE genes is the one encoding replication transcription activator (RTA). The RTA protein functions as a master regulator of lytic replication by governing the activation of later gene classes within the expression cascade. The next transcripts, activated by RTA, are derived from delayed-early (DE) genes. This class includes genes that affect the abundance of nucleotide precursors, host RNA turnover, enzymes and cofactors that participate in viral DNA replication, as well as genes that facilitate immune evasion (Deng et al., 2007). The DE genes have common functions in preparing the cell for viral replication and bulk protein synthesis. Accumulation of DE transcripts through the course of infection stimulates lytic-cycle DNA replication and results in the production of many copies of the KSHV genome

within a single cell. The last genes expressed during the lytic replication cycle are the late (L) genes, which encode mostly for structural proteins (Deng et al., 2007). Synthesis of L genes stimulates the assembly and release of new virions. The assembly process involves incorporation of newly replicated genomes into viral capsids, acquisition of the tegument and budding from host cell membranes that are decorated with KSHV glycoproteins.

Although lytically infected cells are critical for viral replication and spread, their contribution to KSHV-induced tumorigenesis is poorly understood as these cells are often short-lived and destined to die. There is, however, some emerging evidence that gene products expressed during the lytic cycle, such as the DE protein viral G-protein coupled receptor (v-GPCR), strongly contribute to KS tumorigenesis by promoting inflammation, neo-angiogenesis and proliferation of adjacent, latently infected cells (Montaner et al., 2001; Montaner et al., 2003; Sodhi et al., 2004b; Sodhi et al., 2004a; Sodhi et al., 2006).

1.2.2.3 Latency

Consistent with other γ-herpesviruses, KSHV predominantly establishes latency, rather than lytic replication, within infected cells. At any given time, 85 to 99% of KSHV-infected cells harbor virus in the latent state (Dittmer et al., 1998; Miller et al., 1997; Sun et al., 1999; Zhong et al., 1996). The reasons and mechanism for preferential establishment of latency remain an enigma. Nevertheless, during latency, replication of the circularized viral genome, or episome, depends on host-cell DNA-replication machinery and is propagated during cell division (Renne et al., 1996a; Renne et al., 1996b). Moreover, latently infected cells often harbor multiple (1-20) copies of the viral episome. The KSHV latency program encodes surprisingly few genes and all are expressed from a small genomic cassette (Boshoff, 1998; Dittmer et al., 1998). In fact, this program, responsible for episome maintenance, only encodes six proteins: the latency-associated nuclear antigen (LANA), viral cyclin (v-cyclin), viral FLICE-inhibitory protein (v-FLIP), Kaposins A, B and C; and twenty-three microRNAs (miRNAs).

The strong causal relationship between KSHV latent infection and cancer suggests latent-gene products also significantly contribute to tumorigenesis. Initial characterization of KSHV latent-gene products has revealed roles in promoting cell growth and proliferation, inflammation, angiogenesis and suppression of apoptosis (Mesri et al., 2010; Speck and Ganem, 2010). Surprisingly, despite these pro-oncogenic functions, KSHV latent-gene products, expressed independently or together, are incapable of inducing cellular transformation. Studies have, however, revealed that transgenic mice engineered to express KSHV LANA, v-cyclin or v-FLIP latent proteins develop lymphoid malignancies with low frequency and after long latency (Chugh et al., 2005; Fakhari et al., 2006; Verschuren et al., 2004a). For these reasons, the group of ORFs encoding LANA, v-cyclin and v-FLIP has been coined the 'oncogenic cluster' of KSHV. Interestingly, the three ORFs of the oncogenic cluster are regulated as a common transcription unit (Dittmer et al., 1998), suggesting coordinated expression of these proteins is important for their functions. Nevertheless, great efforts have been made to elucidate the precise functions of all the genes encoded by the KSHV latency program to better understand the mechanisms that contribute to KSHV-induced tumorigenesis.

1.2.3.1 LANA

LANA (ORF73) is a large (222-232 kDa) multifunctional nuclear protein that has critical functions in regulating the persistence and segregation of the viral episome during latency (Ballestas et al., 1999). The carboxy-terminal domain of LANA binds the direct terminal repeats within the KSHV genome. This interaction is essential for the formation of the predominant viral origin of replication during latency (Ballestas and Kaye, 2001; Garber et al., 2001). The amino-terminal domain of LANA mediates direct interactions with histones H2A and H2B, and possibly linker histone H1 (Cotter et al., 2001; Piolot et al., 2001; Shinohara et al., 2002). Interactions mediated by LANA with host chromatin and with episomal DNA, together, facilitate tethering of the KSHV genome to host DNA. Importantly, these interactions persist during mitosis and facilitate segregation of the replicated KSHV genome into daughter cells as they divide. These ubiquitous functions

have rendered LANA a commonly used serological marker for latent KSHV infection (Gao et al., 1996; Kedes et al., 1996; Kedes and Ganem, 1997; Simpson et al., 1996).

LANA also has important functions in regulating transcription and proliferation of latently infected cells. Ectopically expressed LANA has been noted to bind and inhibit p53 and Retinoblastoma (Rb) tumor-suppressor proteins (Friborg et al., 1999; Radkov et al., 2000). However, the capacity of LANA to modulate these pathways in cells latently infected with KSHV remains controversial (Chen et al., 2010; Sarek et al., 2007). GSK-3β is another LANA binding partner (Fujimuro et al., 2003). This interaction stimulates the activation of β-catenin and entry into the synthesis phase of the cell cycle. Finally, LANA is able to mediate transcription regulation. This function may be important for repression of KSHV lytic-program gene expression during latency and may be involved in the deregulation of host cell gene expression (Knight et al., 2001; Schwam et al., 2000).

1.2.3.2 v-Cyclin

The KSHV latency program encodes a homolog of cellular D-type cyclins, v-cyclin, that deregulates cellular proliferation (Chang et al., 1996). v-Cyclin 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), but has also been reported to weakly associate with CDK2, CDK4 and CDK9; however, the significance of these observations remains unknown (Chang and Li, 2008; Platt et al., 2000). v-Cyclin-CDK6 heterodimers have an expanded substrate range relative to cellular D-type cyclin-CDK6 complexes, functioning more similarly to cyclin E-CDK2 complexes, and phosphorylate numerous cellular effectors, including the Rb protein, Cdc6 and Orc1 of the origin recognition complex, and Bcl-2 (Child and Mann, 2001; Ojala et al., 2000; Laman et al., 2001; Li et al., 1997). Importantly, v-cyclin-CDK6 complexes are refractory to the action of Cip/Kip and INK4 families of cyclin-dependent kinase inhibitors (CKIs) (Swanton et al., 1997). These properties enable v-cyclin to potently drive DNA synthesis in a variety of cancer cell lines when expressed ectopically;

however, immortalized cells expressing v-cyclin frequently succumb to apoptotic cell death (Ojala et al., 1999; Ojala et al., 2000). By contrast, v-cyclin expression in primary cells triggers robust DNA-damage-checkpoint activation and these cells appear to undergo a p53-dependent form of irreversible cell-cycle arrest that has hallmarks of oncogene-induced senescence (OIS) (Koopal et al., 2007; Verschuren et al., 2002). Presently the contributions of v-cyclin to KSHV latency remain unknown.

1.2.3.3 v-FLIP

The latent v-FLIP protein (ORF 72) encoded by KSHV was first identified for its homology to cellular FLICE-inhibitory proteins (cFLIPs) (Djerbi et al., 1999; Irmler et al., 1997). However, this viral protein appears to have lost its functions regulating caspase-8 over the course of KSHV evolution (Chugh et al., 2005). v-FLIP still retains two functional death-effector domains (DEDs) and, similar to its cellular homolog, these have been shown to mediate interactions with the autophagy regulatory protein Atg3 (Lee et al., 2009). Studies have shown that v-FLIP interaction with Atg3 potently inhibits lipid modification of LC3 and elongation of the autophagosomal membrane, thus blocking cellular autophagic degradation. Despite understanding of the mechanisms of v-FLIP inhibition of autophagy, its importance to KSHV latency and pathogenesis remains unclear.

v-FLIP is also a potent activator of the NF-κB pathway. To trigger NF-κB signal transduction, v-FLIP binds and activates the inhibitor of κB kinase-γ (IKKγ), leading to the derepression of this pathway (Chaudhary et al., 1999; Liu et al., 2002; Sun et al., 2003). Activation of NF-κB by v-FLIP serves two important functions in latency: first, it antagonizes entry into lytic replication, thereby preserving KSHV latency (Brown et al., 2003; Grossmann and Ganem, 2008); second, it renders infected cells resistant to apoptosis through upregulation of Bcl-2 and Bcl-XL (Guasparri et al., 2004; Sakakibara et al., 2009; Sun et al., 2006). Intriguingly, the ‘spindled’ shape of latently infected endothelial cells is also linked to NF-κB activation by v-FLIP; however, the functional

relevance of this morphological change is unknown (Grossmann et al., 2006; Matta et al., 2007).

1.2.3.4 Kaposins

As a result of a complex translation initiation program and alternative splicing, expression of the kaposin locus generates a number of unique gene products; the principal protein species are kaposins A, B, and C (Muralidhar et al., 2000; Sadler et al., 1999). Kaposin A (K12) is a small transmembrane protein that can induce some characteristics of transformation in the Rat-3 cell line, and injecting these cells into athymic nude mice induces the formation of small angiogenic neoplasms (Muralidhar et al., 1998). The predominant product from the kaposin locus is kaposin B, a cytosolic protein that is composed of two sets of direct repeats and shares no homology with kaposin A (Sadler et al., 1999). Kaposin B may contribute to the secretory profile of the KS microenvironment by stabilizing mRNAs encoding cytokines and growth factors. Interestingly, kaposin B binds to and activates mitogen-activated protein kinase (MAPK)-associated protein kinase 2 (MK2), effectively blocking the degradation of normally labile AU-rich-element mRNAs (McCormick and Ganem, 2005; McCormick and Ganem, 2006). Enhancement of the stability of these transcripts ensures their translation, promoting increased cytokine release, such as IL-6 and VEGF, to drive KS development and tumorigenesis. The function of kaposin C remains unknown.

1.2.3.5 microRNAs

microRNAs (miRNAs) are short (~19 to 23 nucleotides) non-coding RNAs that post-transcriptionally regulate gene expression by either targeting mRNAs for degradation or suppressing their translation (Cullen, 2006). Interestingly, many herpesviruses have adapted miRNAs to establish latency and evade immune recognition (Cullen, 2006); indeed, KSHV encodes 12 pre-miRNAs (miR-K1 to miR-K12), which result in 23 mature miRNAs that are expressed from the kaposin transcript in latently infected cells (Cai et al., 2005; Cai and Cullen, 2006; Pfeffer et al., 2005; Samols et al., 2005; Umbach and

Cullen, 2010). Although the functions and cellular targets of most of these viral miRNAs are unknown, some have been identified. For example, miR-K1 targets the CKI p21, and, because p21 activity is important for cell cycle-arrest, miR-K1 is implicated in promoting spindle-cell proliferation (Gottwein and Cullen, 2010). A number of other miRNAs have been found to target thrombospondin 1, an important tumor suppressor and anti-angiogenic factor (Samols et al., 2007). These viral miRNAs may influence spindle-cell proliferation and differentiation and reinforce KSHV latency, contributing to KS tumorigenesis (Ziegelbauer, 2011).

1.2.2.6 KSHV Latent Gene Products Regulate Proliferation Pathways

A common trait amongst KSHV latent-gene products is their ability to engage pathways that regulate cell proliferation. For example, ectopically expressed LANA protein has been reported to inhibit the tumor suppressors p53 and Rb (Friborg et al., 1999; Radkov et al., 2000); and to stabilize β-catenin and facilitate cell-cycle progression (Fujimuro et al., 2003). KSHV v-FLIP regulates the NF-κB pathway and is an inhibitor of autophagy (Chaudhary et al., 1999; Lee et al., 2009). The NF-κB pathway and autophagy have roles in restricting cell proliferation. The KSHV latency program has even been shown to encode a miRNA that specifically represses expression of the CKI p21 (Gottwein and Cullen, 2010).

The most profound link between KSHV latent-gene products and proliferation pathways involves the v-cyclin protein (reviewed in Verschuren et al., 2004b). As a homologue of cellular D-type cyclins, v-cyclin complexes with CDK6 and stimulates cell-cycle progression by inducing phosphorylation of Rb-family proteins and components of the origin-recognition complex, including Cdc6 and Orc1. Furthermore, v-cyclin has evolved to activate CDK6 in a manner that renders this heterodimeric complex refractory to inhibition by Cip/Kip and INK4 family members. These functions of v-cyclin should promote cell proliferation very efficiently; however, ectopic expression of v-cyclin, paradoxically, induces robust proliferation arrest (Koopal et al., 2007; Verschuren et al., 2002).

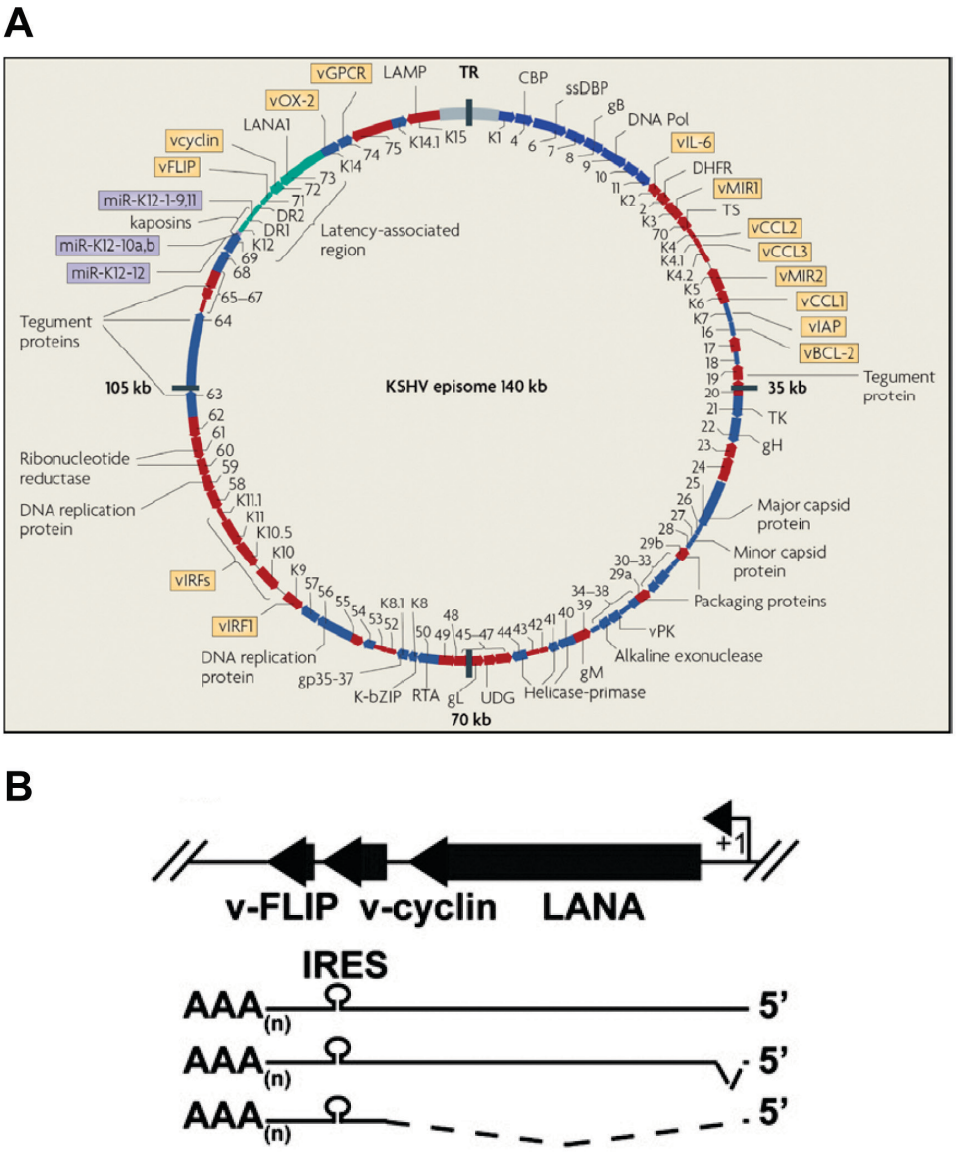

Figure 1.2
The KSHV Episome and 'Oncogenic Cluster'
(A) During latency, the KSHV genome is circularized by way of the terminal repeat (TR) region and maintained as an episome. Like many viral genomes, the KSHV genes are predominantly clustered based on their expression profiles; notably, all of the latent genes are expressed from a latency-associated genomic cassette. Identified ORFs and 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 expressed from this locus. Alternative splicing produces three transcript variants. v-FLIP translation initiates from an IRES within the transcripts. Figure was reproduced from Leidal et al. (2012).

The evolution of a cohort of KSHV latency genes that subvert proliferation pathways highlights their importance to viral fitness. Furthermore, it suggests that host cells may have evolved to control virus spread by undergoing proliferation arrest through mechanisms like oncogene-induced senescence.

1.3 Oncogene-Induced Senescence

Cellular senescence refers to the permanent growth arrest that dividing cells effectuate in response to oncogenic stress (reviewed in Adams, 2009; Campisi and d'Adda di Fagagna, 2007; Kuilman et al., 2010). Senescent cells exhibit dramatic phenotypic changes that include permanent withdrawal from the cell cycle, large and flattened appearance because of alterations in cytoskeletal structures, resistance to apoptosis and the capacity to remain alive *in vitro* for extended periods of time, even years in some instances.

The essentially irreversible proliferation arrest of primary human cells in culture was first noted by Leonard Hayflick in 1965, who coined the term "cellular senescence" to describe the phenomenon (Hayflick, 1965). For many years, Hayflick's *in vitro* observations of cellular replicative exhaustion were largely attributed to an inability of research scientists to properly mimic a cell's natural environment *in vivo*, and thus were largely dismissed. From these early repudiated findings, the process of cellular senescence is now understood to occur in a broad range of cell types and settings, and be much more than a simple cessation of cell growth. Roles for senescence have been defined in tumor suppression, aging and a number of other human pathologies, making it fundamentally important for human health.

1.3.1 Senescence-Inducing Stimuli

Transition to the senescent state can be triggered by a multitude of acute stimuli. For example, the proliferation arrest of many primary cells in culture, as noted by Hayflick, is directly attributable to telomere erosion that occurs at chromosome ends as cells replicate their DNA. Dangerously short telomeres are detected by the cell as DNA damage, and this results in activation of DNA-damage responses (DDRs) (d'Adda di Fagagna et al.,

2003). Importantly, DDRs triggered by telomere erosion persist long after damage detection and these signals are necessary for telomere erosion-induced senescence, more commonly known as replicative senescence. Severe or irreparable damage to genomic DNA far removed from telomeres can also induce senescence. Pharmacological agents, such as chemotherapeutics, that damage DNA are incredibly potent inducers of senescence (Chang et al., 1999; Kuilman et al., 2010; Schmitt et al., 2002). Surprisingly, even strong mitogenic signals, such as deregulated oncogenes, can create extensive DNA damage and persistent DDR signaling by inducing aberrant firing of replication origins and replication-fork collapse (Di Micco et al., 2006). Senescence in response to strong mitogenic signals is often referred to as oncogene-induced senescence (OIS). Irrespective of the context, a common characteristic of many senescence stimuli is their ability to evoke profound genomic damage.

1.3.2 The Senescence Phenotype

Senescent cells display a unique set of molecular features that generally enables discrimination of senescence from other forms of proliferation arrest, including quiescence, differentiation and transient checkpoint arrest (Adams, 2009; Campisi and d'Adda di Fagagna, 2007; Kuilman et al., 2010). However, it is important to consider that, to date, no hallmark or phenotypic feature of senescence is exclusive to the senescent state. Therefore, senescence is fundamentally defined by the aggregate of several phenotypes. The most prominent phenotypic features of senescent cells include: (1) persistent DDR signaling; (2) permanent proliferation arrest; (3) the accumulation of distinct chromatin structures, referred to as senescence-associated heterochromatin foci (SAHF); (4) secretion of cytokines, growth factors, proteases and other factors, through a pathway known as the senescence-associated secretory phenotype (SASP); and (5) expression of a β-galactosidase variant known as senescence-associated β-galactosidase (SA β-gal) (Figure 1.3A). Importantly, each of the phenotypic features of senescence is rendered by profound changes in cellular signaling pathways during transition to the senescent state.

1.3.1.1 Persistent DNA-Damage Signaling

Initiation of senescence most often begins with detection of severe or irreparable genomic damage. Deregulated oncogenes frequently induce double-strand DNA (DSBs) breaks or single-strand DNA breaks (SSBs) through aberrant DNA synthesis (Di Micco et al., 2006; d'Adda di Fagagna, 2008). Sites of DNA damage recruit and promote the activation of large multiprotein complexes that function as both sensors and amplifiers of the DDR signal (Figure 1.3B). Signaling through these complexes becomes persistent and senescence-inducing when the DNA damage cannot be repaired. Interestingly, the nature of DNA damage, be it DSBs or SSBs, affects the complexes that signal damage (d'Adda di Fagagna, 2008). Histone variant H2AX is amongst the first proteins to bind DSBs (Figure 1.3B *left*). Together with the MRN complex, H2AX recruits and activates the apical DDR kinase, Ataxia telangiectasia mutated (ATM). In the process, H2AX becomes phosphorylated by ATM, forming γH2AX, and this event facilitates the recruitment of DDR signaling mediators such as 53BP1. Mediators serve to amplify the DNA-damage signal and facilitate transmission to effector proteins, including Checkpoint kinase 2 (CHK2) and the tumor suppressor p53. Contrastingly, at SSBs within the genome, replication protein A (RPA) coats single-strand DNA (Figure 1.3B *right*) and recruits the ATM-related kinase, ATR (d'Adda di Fagagna, 2008). In collaboration with the 9-1-1 complex, ATR recruits and phosphorylates H2AX and 53BP1 to transmit and amplify the DDR signal to effector proteins, including Checkpoint kinase 1 (CHK1) and p53. Persistent activation of DDRs has been shown, in many contexts, to be essential for senescence; signaling by ATM and ATR helps promote (i) the induction of SAHF, (ii) the characteristic cytokine secretion profile of the SASP and (iii) proliferation arrest through the activation of p53 and Retinoblastoma (Rb)/INK4a tumor suppressor pathways.

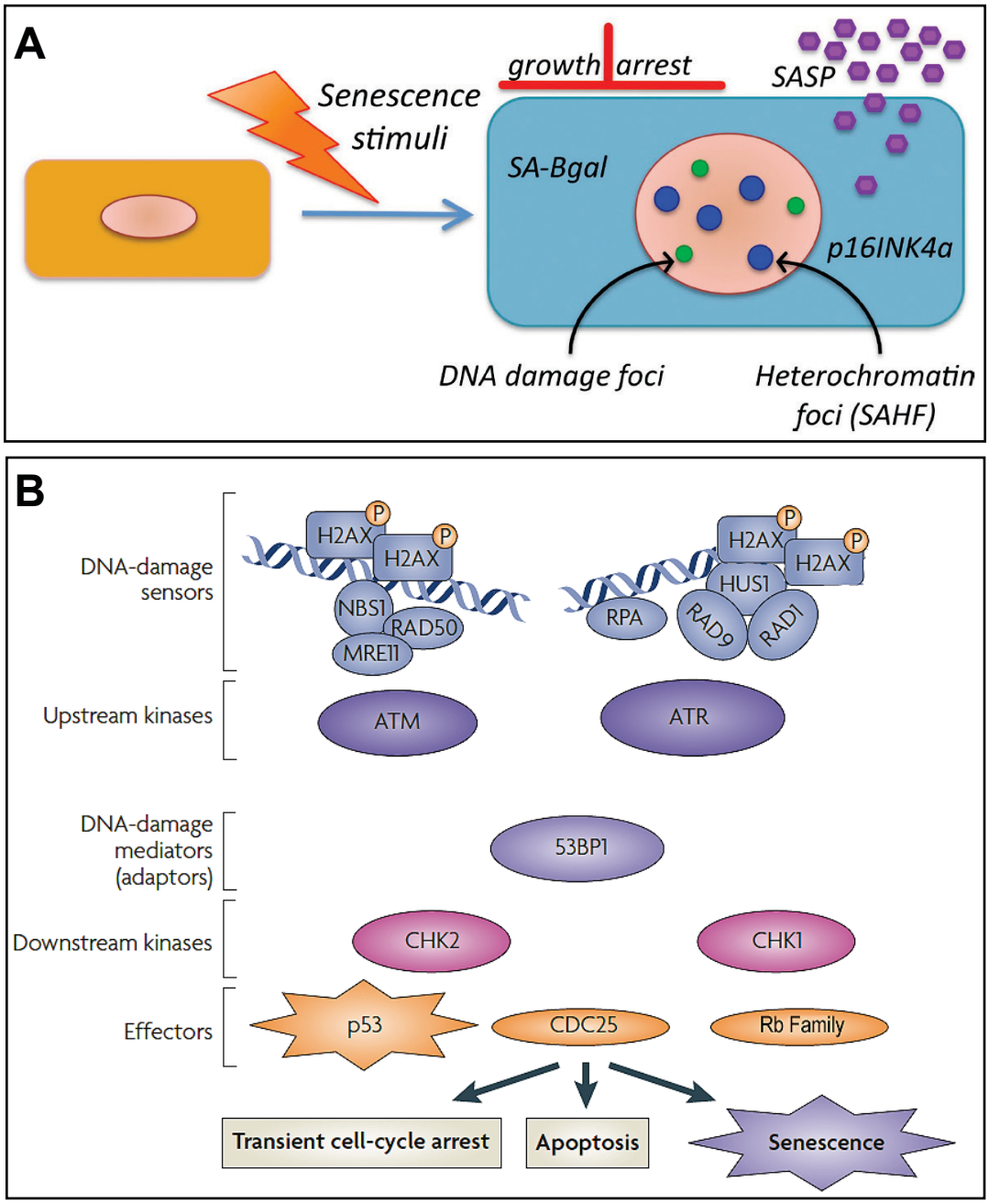

Figure 1.3

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 upregulate INK4a. Adapted from Rodier and Campisi (2011). (B) DNA damage in the form of dsDNA breaks (left) is bound by H2AX and the MRN complex to promote activation of ATM, which signals to downstream effectors; 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. Adapted from Campisi and d'Adda di Fagagna (2007).

1.3.1.2 Proliferation Arrest: Central Roles for p53 and Rb/INK4a Pathways

To achieve senescence, DNA-damage signals contribute to that activation of p53 and Retinoblastoma (Rb)/INK4a tumor suppressor pathways, critical regulators of numerous arms of the senescence program. Genes involved in senescence have been reviewed recently, and most of the relevant genes encode proteins that modulate, are involved in or feed into the p53 and/or Rb/INK4a pathways or are downstream effectors. As might be expected from the profound changes that occur in transition to senescence, many of these genes regulate cell-cycle arrest, the cytoskeleton, chromatin structure and the secretory pathway.

The tumor suppressor p53 is a transcription factor central to the integrated cellular stress response. DNA damage induces phosphorylation of p53, on serine 15, by ATM and ATR. This event stabilizes p53 and facilitates its functions in transcription regulation. Although p53 is a master regulator of gene expression, estimated to affect as many as 1500 distinct genomic loci (Veprintsev and Fersht, 2008), its most important function in senescence is transactivation of the gene encoding p21. The p21 protein is a CKI, and through attenuation of CDK activity, most notably CDK2, it impairs cell-cycle progression and proliferation. p21-mediated inhibition of CDK2 blocks loading and firing of replication origins, and also activates the Rb family of tumor suppressors (Campisi and d'Adda di Fagagna, 2007). Rb-family proteins are also master regulators of gene expression. However, in contrast to p53, the Rb family predominantly function as repressors of gene expression. Many downstream targets of the Rb-family, including genes regulated by E2F-family transcription factors, encode proteins necessary for cell-cycle progression (Campisi and d'Adda di Fagagna, 2007). Therefore, activation of the Rb-family represses the expression of genes necessary for proliferation. Together, p21 and Rb-family proteins block the cell cycle in response to oncogene-induced DNA damage and help enforce senescence-associated proliferation arrest.

The INK4a protein constitutes another important inhibitor of proliferation during senescence. INK4a, similar to p21, arrests the cell cycle by attenuating CDK activity and activating Rb-family proteins (Campisi and d'Adda di Fagagna, 2007). However, INK4a

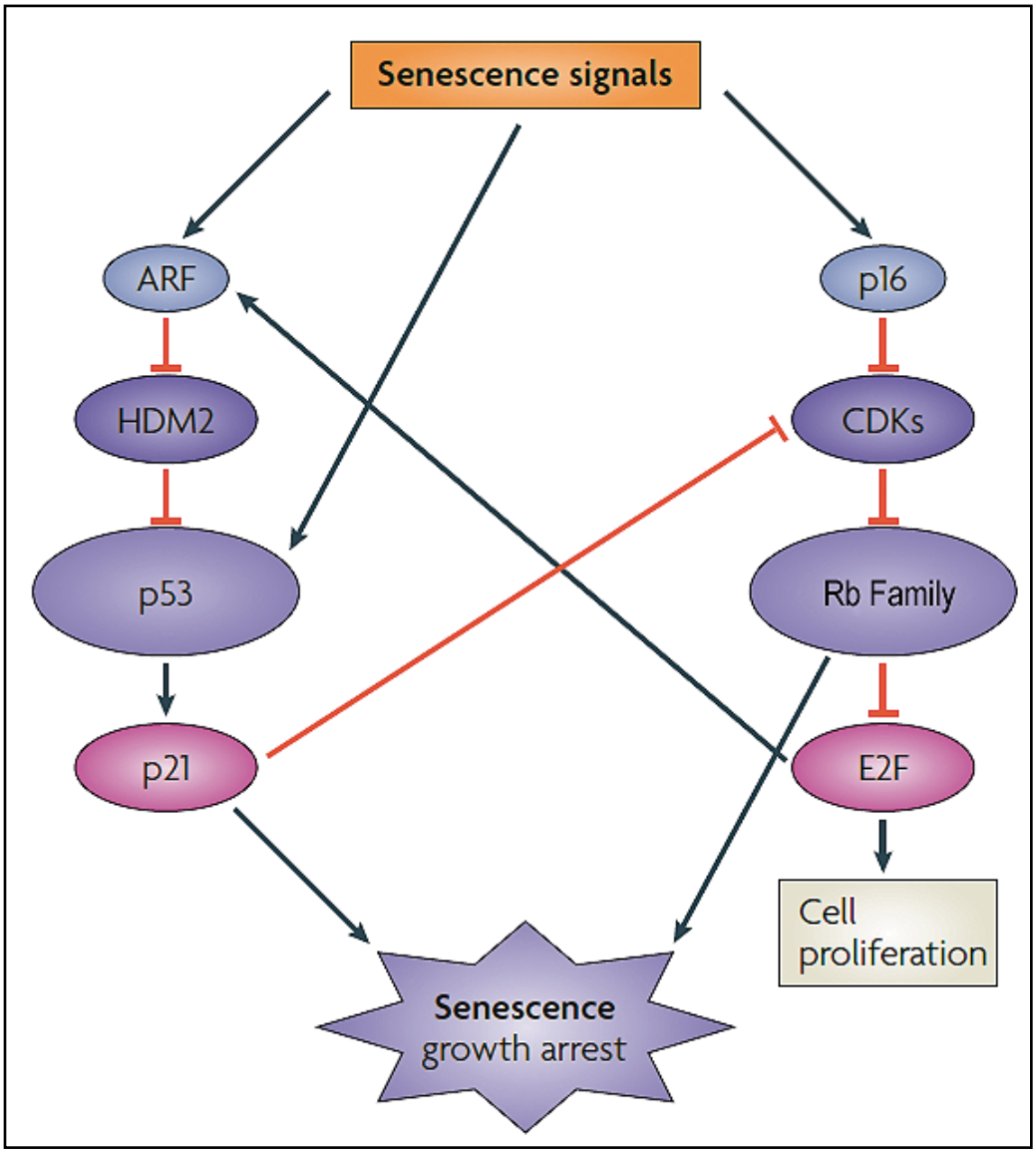

Figure 1.4
Control of Senescence by the p53 and Rb/p16INK4 Pathways
Senescence stimuli trigger DDRs and engage the p53 and Rb/p16INK4a tumor-suppressor pathways to induce arrest. p53 is stabilized and activated by phospho-signals and proteins, such as ARF, that disrupt its interaction with HDM2. Subsequently, 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 p16INK4a expression. Subsequently, p16INK4a 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).

is not particularly responsive to DNA damage and is expressed independently of p53. Alternatively, INK4a expression is regulated by diverse stresses, most notably oncogenic stress (Ferbeyre et al., 2000; Lin et al., 1998; Serrano et al., 1997), through pathways that control chromatin structure (Gil and Peters, 2006). In proliferating cells, the polycomb group of transcription repressor complexes tightly inhibit expression from the *INK4* locus (Bracken et al., 2007; Gil et al., 2004; Gil and Peters, 2006; Jacobs et al., 1999); however, deregulated proliferation appears to result in the disassembly of *INK4*-proximal polycomb complexes, in part because of the loss of EZH2 and the specific histone methylation pattern with which it is associated (Bracken et al., 2007). Importantly, the intricate mechanisms leading to INK4a expression, Rb activation and proliferation arrest illustrate signaling pathways that can trigger senescence without obvious DNA damage.

1.3.1.2 Senescence-Associated Heterochromatin Foci

The functions of polycomb complexes in INK4a expression point to a more profound role for chromatin in modulating senescence. Transition to the senescent state is marked by significant changes in cellular chromatin structure; within senescent cells, vast expanses of chromatin are condensed into discrete domains throughout the nucleus (Narita et al., 2003). Studies have revealed that punctate domains within the chromatin of senescent cells, termed SAHF, do not form arbitrarily on DNA, and have dramatic influences on gene expression. Importantly, genes within chromatin incorporated into SAHF are strongly silenced and frequently encode proteins that function in proliferation (Narita et al., 2003). Recent emerging evidence also suggests that SAHF may have important roles in controlling the intensity of DNA-damage signaling during senescence (Di Micco et al., 2011). Therefore, SAHF are another mechanism by which senescent cells enforce growth arrest.

The proteins that compose SAHF, not surprisingly, have extensive overlap with common heterochromatin markers. For example, SAHF are enriched for histones that are hypoacetylated, methylated on lysine 9 of histone H3 (H3K9Me), and bound by Heterochromatin Protein 1 (Adams, 2009). However, other components of SAHF,

including high-mobility-group AT-hook proteins 1 and 2 (HMGA1; HMGA2), macroH2A and histone 3 variant H3.3, may render this heterochromatin structure unique (Narita et al., 2006; Zhang et al., 2005). The Rb family of tumor suppressors colocalizes with SAHF, and loss of Rb impairs SAHF formation (Narita et al., 2003; Ye et al., 2007b). Other regulators of SAHF formation include components of the HUCA histone-chaperone complex, including HIRA, UBN1 and ASF1a, as well as the Wnt signaling pathway and PML bodies (Banumathy et al., 2009; Ye et al., 2007a; Ye et al., 2007a). Intriguingly, linker histone H1 is absent from SAHF, which thus may also be considered a characteristic feature of these heterochromatin domains (Funayama et al., 2006). Future characterization of SAHF will hopefully shed more light on the role of these unique nuclear structures within the senescence program.

1.3.1.3 Senescence-Associated Secretory Phenotype

Cells undergoing senescence exhibit profound changes in their transcriptome that not only reflect proliferation arrest, but also robust activation of inflammatory responses. Consequently, senescent cells secrete high levels of numerous factors, including cytokines, chemokines, growth factors and proteases. The profoundly altered secretome of senescent cells is often referred to as the SASP, and has significant similarity to the wound-healing response. Components of the SASP include many factors that affect cell proliferation, remodel the extracellular matrix and modulate immune cells (reviewed in Kuilman and Peeper, 2009).

Interleukins are the most abundant and prominent factors within the SASP. This group of cytokines has important immune regulation functions and modulate inflammation. Intriguingly, the secretion of interleukins, and other factors within the SASP, stimulates immune-cell clearance of senescent cells *in vivo* (Kang et al., 2011; Rakhra et al., 2010; Xue et al., 2007). Clearance has been reported to involve both innate and adaptive components of the immune system, and suggests that the SASP coordinates removal of senescent cells and tissue repair. Senescence robustly up-regulates and 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 found at low amounts within the SASP (Coppe et al., 2008). Surprisingly, in addition to regulating immune clearance, IL-6 and IL-8 secretion by senescing cells is necessary for proliferation arrest. Cells deficient for IL-6 or the IL-8 receptor CXCR2 fail to efficiently senesce in response to oncogenic stress (Acosta et al., 2008; Kuilman et al., 2008). Mechanistically, IL-6 and IL-8 promote senescence through autocrine feedback signaling; surface bound IL-6 and IL-8 enhance activation of the INK4-family CKIs and p53, respectively, which enforce proliferation arrest (Acosta et al., 2008; Kuilman et al., 2008). Although the SASP helps reinforce senescence, it is not sufficient to induce senescence. Paracrine signaling by the SASP can, in some contexts, induce proliferation and invasiveness of adjacent cells (Coppe et al., 2008; Krtolica et al., 2001). These mechanisms likely contribute to tissue repair, but may also inadvertently create a pro-oncogenic microenvironment.

The nuclear factor-κB (NF-κB) signaling pathway is a well-described positive regulator of IL-6 and IL-8 expression, and of many other factors common to the SASP (Hoffmann and Baltimore, 2006). Nevertheless, the potential contributions of NF-κB to senescence remain controversial. This is partly due to reports that, in some contexts, NF-κB can promote senescence bypass (Batsi et al., 2009; Guerra et al., 2011). However, striking accumulation of NF-κB within the chromatin of senescent cells and impaired proliferation arrest upon repression of NF-κB signaling suggest a critical role for this transcription factor in the senescence program (Chien et al., 2011; Rovillain et al., 2011). NF-κB complexes are dimers composed of five members: p65, p50, p52, RelB and c-Rel. In the absence of activating signals, NF-κB complexes are repressed through sequestration in the cytoplasm by inhibitor-of-κB (IκB) proteins. Activation of NF-κB occurs when stress stimuli activate the inhibitor-of-κB-kinase γ (IKKγ), which phosphorylates IκB, promoting its degradation and NF-κB-complex nuclear important (Hoffmann and Baltimore, 2006). Subsequently, NF-κB complexes transactivate genes that encode many pro-inflammatory factors. Despite extensive understanding of this

pathway, it remains largely unclear how NF-κB is activated during senescence. Recent reports suggest this may be achieved through direct DDR activation of IKKγ, possibly through the coordinated activities of PARP-1 and ATM (Stilmann et al., 2009; Wu et al., 2006). Intriguingly, the mechanisms leading to IKKγ activation appear to influence NF-κB function. Therefore, fine tuning of the NF-κB pathway may be critical to controlling its functions in SASP gene expression.

1.3.1.4 SA β-gal and Expansion of the Lysosomal Compartment

SA β-gal is the most commonly employed senescence biomarker (Dimri et al., 1995). Surprisingly, all cells, including non-senescent cells, express β-galactosidase enzymes. The SA β-gal protein is the only lysosomal β-D-galactosidase, encoded by the *GLB1* gene, that becomes strongly up-regulated during senescence (Lee et al., 2006). Lysosomal β-D-galactosidase, not surprisingly, evolved to be maximally active within the low pH environment of the lysosome (pH 4.8). However, the enzyme still retains low level activity at more neutral pH conditions (Debacq-Chainiaux et al., 2009). Thus, SA β-gal assays, which measure the abundance of lysosomal β-D-galactosidase through cleavage of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal), are conducted at pH 6.0 to eliminate basal activity. In fact, increased SA β-gal activity within senescent cells is largely indicative of massive expansion of the lysosomal compartment during senescence (Lee et al., 2006), .

Until very recently, the mechanisms responsible for expansion of the lysosomal content and its functions in senescence were unknown. It now appears that senescent cells activate autophagy, a pathway responsible for the degradation and recycling of cytoplasmic components through the lysosome (Young et al., 2009). Surprisingly, autophagy is necessary for efficient execution of senescence and appears to regulate the SASP. It is postulated that the massive secretion and remodeling that occurs within cells during transition to the senescent phenotype requires coupling of catabolic and anabolic mechanisms, thus justifying the activation of this pathway (Narita et al., 2011).

1.3.3 Senescence and Tumor Suppression

Cancer is a disease defined by cells that have acquired the ability to grow and divide indefinitely, even in the presence of extensive genomic damage (Hanahan and Weinberg, 2000). Therefore, the process of senescence, being directly opposed to limitless replication of cells with DNA damage, should be tumor-suppressive. Although this notion seems obvious, evidence supportive of senescence as a tumor suppressor mechanism has only recently been discovered.

Studies of human tissues and tumorigenesis in mice have revealed that senescence is a critical anticancer mechanism that prevents the growth of cells at risk to neoplastic transformation. Perhaps the most striking examples of senescence in humans are melanocytic nevi or moles, which are composed almost exclusively of senescent cells (Michaloglou et al., 2005). Human melanocytes frequently become mutated in the *Ras* or *BRAF* oncogenes and this results in their rapid proliferative expansion, activation of oncogenic stress responses and ultimately, OIS. Analysis of tissue sections from human moles revealed markers of senescence, including robust activation of SA β-gal (Michaloglou et al., 2005). Importantly, studies have also shown that human melanomas frequently harbor mutations within effectors of senescence, including p53 and Rb, and inactivation of these pathways in mouse models leads to cancerous progression of deregulated melanocytes (Ha et al., 2008). Cells displaying hallmarks of senescence, including activated DDRs and SAHF, have also been discovered in early benign stages of bladder and colorectal cancers, whereas malignant adenocarcinomas derived from consistent tissues were found to be largely devoid of senescent markers (Bartkova et al., 2005). Bladder and colorectal tumors commonly demonstrate constitutive activation of the Ras pathway and harbor mutations within the p53 tumor suppressor. Collectively, these seminal discoveries serve to not only highlight OIS as an anticancer mechanism, but also demonstrate that inactivation of critical effectors of the senescence program predisposes to the development of cancer.

Cellular senescence is thought to suppress tumorigenesis through at least three mechanisms. First, and most obvious, senescence-associated proliferation arrest should,

in a cell-autonomous manner, block the growth and proliferation of incipient cancer cells. Second, arrest of the cell cycle and senescent-cell DNA heterochromatization appears to limit further DNA damage and genomic instability (Di Micco et al., 2011). Third, the SASP elaborated by senescent cells appears to trigger elimination of cells susceptible to neoplastic transformation by the immune system (Xue et al., 2007). The elucidation of a cooperative mechanism between cellular senescence and the immune system for clearance of deregulated and damaged cells is an exciting new frontier that may help explain why senescent cells have not been observed more frequently. Current evidence suggests that both the innate and adaptive immune system can promote clearance of senescent cells and even tumor regression if the cells that compose cancerous lesions can be induced to undergo senescence (Kang et al., 2011; Rakhra et al., 2010; Xue et al., 2007).

The 'Achilles heel' of the senescence program, and a reason cancers still arise, involves the genetic inactivation or suppression of senescence effectors. It is not surprising that a majority of tumors harbor genetic and/or epigenetic alterations that inactivate the p53 and Rb/INK4a pathways (Campisi and d'Adda di Fagagna, 2007). In fact, many viruses have evolved to specifically target these precise pathways to facilitate viral replication and inadvertently induce oncogenesis. Identification of autophagy as an effector mechanism for OIS suggests that viral modulation or epigenetic silencing of genes essential for autophagic degradation may also contribute to tumorigenesis. Finally, emerging evidence suggests that the cellular microenvironment can have an impact on the sensitivity of cells to senescence stimuli, perhaps blunting the action of critical senescence effectors (Krtolica et al., 2001). Ironically, components of the SASP have been shown to promote the malignant progression of incipient cancer cells through paracrine signaling (Coppe et al., 2008; Krtolica et al., 2001). Furthermore, secretion of these cytokines frequently does not stop once senescence has failed (Coppe et al., 2008). These observations suggest that tumor suppression by way of senescence is not without risks, and may actually promote tumorigenesis when critical senescence effectors cease functioning properly.

1.4 Autophagy

Autophagy (Greek for 'self-eating') is a process of cellular self-cannibalization, whereby cells capture their own cytoplasm and organelles within membranous sacs and then consume the contents within lysosomes. This evolutionarily conserved process is an important means of controlling the quality and quantity of intracellular biomass.

In recent years, autophagy has been found to have critical roles in diverse biological processes, including metabolism (Rabinowitz and White, 2010; Vousden and Ryan, 2009), integrated stress responses (Kroemer et al., 2010), development and differentiation (Mizushima and Levine, 2010) and immunity (Deretic and Levine, 2009; Levine et al., 2011). Furthermore, deregulated autophagy is implicated in the pathogenesis of many human diseases, such as neurodegeneration (Nixon, 2006), microbial infection (Deretic and Levine, 2009; Deretic, 2010), metabolic disorders (Rabinowitz and White, 2010), aging (Madeo et al., 2010) and cancer (Jin and White, 2007; Levine, 2007; Liang and Jung, 2010; Maiuri et al., 2009b; Mathew et al., 2007a). As a result, autophagic pathways are the subject of intensive characterization with the hope that new therapeutic targets will emerge for the treatment of broad disease classes.

1.4.1 The Fundamental Mechanisms of Mammalian Autophagy

Degradation of cytoplasmic material in the autophagy pathway occurs through at least three distinct mechanisms, including macroautophagy, chaperone-mediated autophagy and microautophagy. Macroautophagy, usually referred to simply as autophagy, is the subject most pertinent to this work, and thus will be the primary focus. Briefly, chaperone-mediated autophagy selectively targets cytosolic components to the lysosome for degradation, while microautophagy involves the lysosomal degradation of cytosolic components that become trapped in nascent multivesicular bodies. By contrast, classical autophagy involves the engulfment of a portion of the cytoplasm by an isolation membrane or 'phagophore', resulting in the formation of a double-membrane vesicle known as the autophagosome. Fusion of the autophagosome with a lysosome creates an

autolysosome and results in the degradation of autolysosomal contents by lysosome-derived hydrolytic enzymes (Figure 1.5).

Autophagosome biogenesis and fusion with lysosomes requires the function of at least four distinct molecular components, including (1) the unc-51-like kinase (ULK) complex; (2) the Beclin 1/ class III phosphatidylinositol 3-kinase (PI3K) complex; (3) two transmembrane proteins, Atg9 and VMP1; and (4) two ubiquitin-like protein (Atg12 and LC3) conjugation systems (reviewed in Kroemer et al., 2010). Importantly, many components of the core autophagy machinery are directly targeted by stress-signaling pathways.

The ULK complex plays a pivotal role in the initiation of autophagy, and is a primary integration point for stress-signaling pathways that trigger autophagy. ULK-complex activity is inhibited by the mammalian target-of-rapamycin (mTOR) complex 1 (mTORC1) and activated by the adenosine-monophosphate-activated protein kinase (AMPK) (Egan et al., 2011; Hosokawa et al., 2009; Kim et al., 2011). Upon activation, the ULK complex phosphorylates components of the Beclin 1/ class III PI3K complex promoting their relocalization to the endoplasmic reticulum (ER) (Axe et al., 2008).

At the ER, the Beclin 1/ class III PI3K complex serves important functions in nucleation of the phagophore membrane, a precursor to autophagosome formation. Although the precise origins of the isolation membrane remain controversial, and may even be derived from disparate cytoplasmic sources, nucleation of this membrane often occurs proximal to the ER (Axe et al., 2008; Yla-Anttila et al., 2009b). Bcl-2-family proteins, including Bcl-2, Mcl-1 and Bcl-XL, are potent inhibitors of the Beclin 1/ class III PI3K complex (Levine et al., 2008). Stress signals that activate the c-Jun N-terminal kinase (JNK) relieve repression of the Beclin 1/ class III PI3K complex by Bcl-2 proteins (Pattingre et al., 2005; Wei et al., 2008), facilitating activation of downstream components of the autophagy pathway, including transmembrane proteins Atg9 and VMP1.

Both Atg9 and VMP1 have important functions in elongation of the isolation membrane. Atg9 appears to facilitate elongation through the recruitment of lipids from

distinct subcellular compartments (Tooze, 2010). Furthermore, interaction of Atg9 and VMP1 with Beclin 1 facilitates recruitment of proteins, such as Bif-1/endophilin, that likely help to curve the elongating membrane (Kroemer et al., 2010). Recently, VMP1 has also been shown to be essential for translocation of LC3 to immature autophagosomes (Yang and Klionsky, 2010).

Membrane elongation, and ultimately closure, are also stimulated by the activities of two ubiquitin-like conjugation systems (Yang and Klionsky, 2010). The first ubiquitin-like conjugation system covalently links Atg12 to Atg5, which is subsequently bound by Atg16, forming an E3 ligase for a protein known as LC3 (LC3-I). In association with E1-like enzyme Atg7 and E2-like enzyme Atg3, the E3 ligase formed in the first ubiquitin pathway, Atg12-Atg5:Atg16, catalyzes the covalent lipid modification of LC3 with phosphatidylethanolamine (LC3-II) (Yang and Klionsky, 2010). This complicated process of lipid modification serves to insert LC3-II into the luminal and cytosolic sides of the autophagosomal membrane, facilitating recruitment of cytoplasmic cargo for digestion and closure of the autophagosome. LC3-II also binds to the actin cytoskeleton and regulates autophagosome trafficking within the cytoplasm.

Fusion of the autophagosome with the lysosome is poorly understood. There is some evidence, however, that the Beclin / class III PI3K complex through interactions with class C Vps/HOPS complexes may regulate this process (Kroemer et al., 2010). The fusion event exposes the content of the autophagosome to lysosomal hydrolyases, leading to content degradation. Degraded material is then returned to cytoplasm by permeases in the membrane of the autolysosome.

1.4.2 Stress Signaling and the Activation of Autophagy

A plethora of distinct stresses activate autophagy, including nutrient and energy deficiencies, unfolded proteins, pathogen infection, hypoxia, mitochondrial damage and DNA damage. Under difficult conditions, autophagy functions to mitigate stress and reestablish homeostasis. Autophagic degradation serves two important cytoprotective functions: first, autophagy facilitates clearance of damaged organelles, protein aggregates

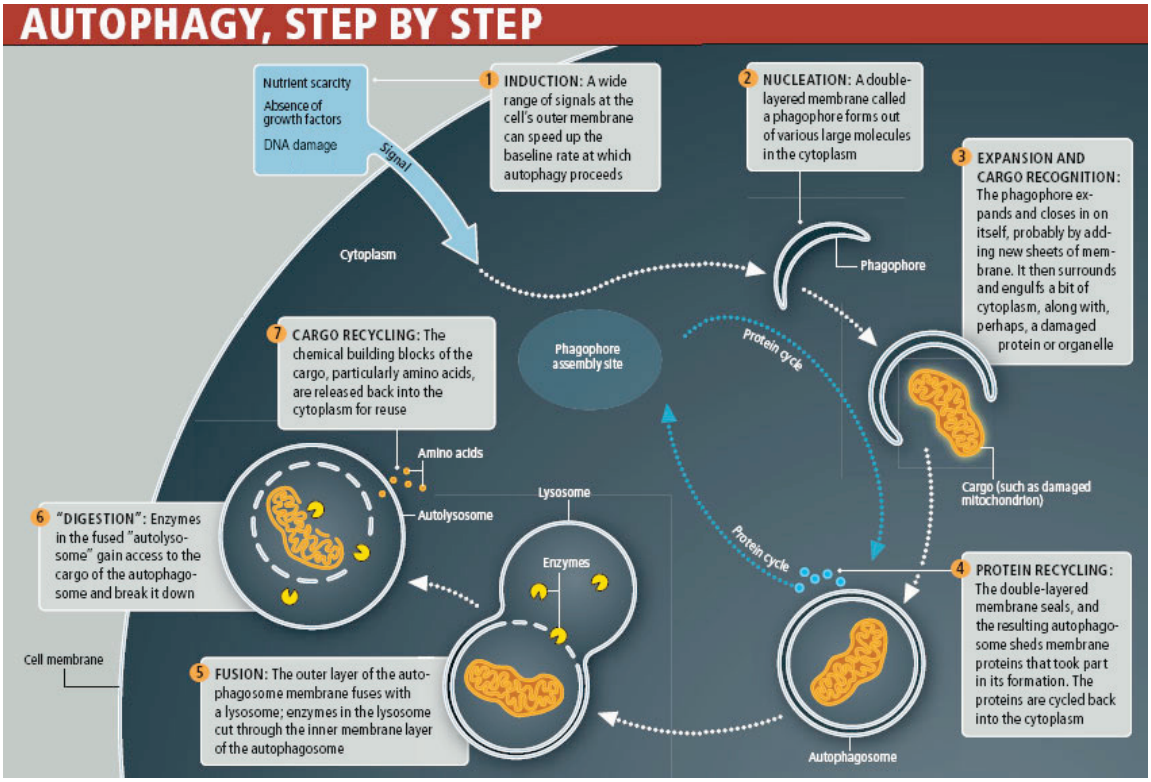

Figure 1.5
Steps Involved in the Biogenesis and Maturation of Autophagosomes
Cellular stress stimuli signal to the ULK complex and promote the activation of the Beclin 1/ class III PI3K complex, inducing phagophore nucleation. Subsequently, Atg9 and VMP1 with the aid of the LC3 lipid conjugation system promote phagophore membrane elongation, and ultimately closure. The autophagosome, and its sequestered contents, fuse with lysosomes. This event creates the autolysosome and triggers the degradation of the contents by hydrolytic enzymes. Finally, the degraded content of the autolysosome is recycled to the cytoplasm for use by the cell. Adapted from Klionsky and Deretic (2008).

and pathogens which are clearly detrimental to cellular function; second, degraded intracellular components are recycled to facilitate energy production and synthesis of nascent molecules for stress responses.

Stress signaling triggers autophagy through predominantly two mechanisms: activation of the ULK complex and Beclin 1/ class III PI3K complex. ULK activation is directly controlled by mTORC1 and AMPK, master regulators of cellular growth and energy homeostasis, respectively. mTORC1 is downstream of PI3K/AKT pathway and is directly activated by growth-factor signaling. AMPK is a sensor of cellular energy levels that is activated under conditions of stress through phosphorylation of threonine 172. In the absence of stress, mTORC1 is active and phosphorylates the ULK complex, inhibiting its interaction with AMPK (Egan et al., 2011; Hosokawa et al., 2009; Kim et al., 2011). Upon stress stimuli, AMPK is robustly activated and phosphorylates the mTORC1 repressor Tuberous sclerosis 2 (TSC2), which ultimately attenuates mTORC1 signaling to all downstream targets (Gao et al., 2002; Inoki et al., 2002), including the ULK complex. This important event renders ULK competent for interaction with AMPK and culminates in ULK activation (Figure 1.6) (Egan et al., 2011; Kim et al., 2011).

Regulation of the Beclin 1/ class III PI3K complex, independently of ULK, predominantly occurs through interaction of Bcl-2-family proteins with the core Beclin 1 component (Levine et al., 2008). Bcl-2 repression of Beclin 1 serves as the paradigm case for this regulatory mechanism. In the absence of stress, Bcl-2 binds Beclin 1(Pattingre et al., 2005), preventing full formation the complex required for allosteric activation of the class III PI3K. In response to stress, Bcl-2 is phosphorylated on serines 69, 70 and 87, predominately by the c-Jun N-terminal kinase (JNK) (Wei et al., 2008), promoting dissociation of Bcl-2 and enabling Beclin 1 to interact with all partner proteins required for class III PI3K activation (Figure 1.6).

Not surprisingly, many stress-signaling pathways converge upon the mTOR/ AMPK or JNK/Bcl-2 pathways to activate autophagy. For example, the tumor suppressor p53 has been shown to activate autophagy in response to DNA damage (Maiuri et al., 2010). Mechanistically, this has been shown to involve regulation of the mTOR/AMPK

signaling axis. DNA-damage-induced activation of p53 promotes transactivation of p53-dependent target genes. Amongst the genes up-regulated are those that encode PTEN, TSC2, AMPKβ1 (Feng et al., 2005), and lesser known proteins, including Sestrin 1, Sestrin 2 and DRAM (Budanov and Karin, 2008; Crighton et al., 2006). The tumor suppressors PTEN, TSC2 and AMPKβ1 all have well-defined roles negatively regulating mTORC1 signaling; furthermore, Sestrin 1 and Sestrin 2 have been demonstrated to facilitate AMPK activation and phosophorylation of TSC2 (Figure 1.6) (Budanov and Karin, 2008). By contrast, DRAM appears to be a protein targeted to lysosomes and has unknown functions in autophagy (Crighton et al., 2006). Together, the proteins up-regulated by p53 in response to DNA damage activate AMPK, attenuate mTOR and promote the activation of autophagy.

1.4.3 Autophagy and Cancer

The functions of tumor suppressors, including AMPK, PTEN and p53, in the activation of autophagy point to a broader role for this intracellular recycling process as an anticancer mechanism. Accumulating evidence indicates that autophagy pathways are intimately linked to the control of cancer. Key autophagy regulatory proteins, such as Beclin-1, are allelically deleted or expressed at significantly reduced levels in numerous types of cancer, including a majority of breast carcinomas (Debnath, 2011; Liang et al., 1999; Mathew and White, 2011). Furthermore, some tumor cells harness autophagy to adapt to hypoxia and extracellular matrix detachment, and to maintain metabolic fitness during oncogenic transformation (Debnath, 2011; Mathew and White, 2011). Paradoxical evidence supporting autophagy as a mechanism that both suppresses and promotes tumors has been reconciled with a context-dependent model in which autophagy suppresses tumor initiation, but is also important for stress adaptation in advanced tumors (Debnath, 2011). The autophagic mechanisms thought to contribute to tumor suppression are briefly described.

Autophagy is predominantly characterized as a pro-survival mechanism for the

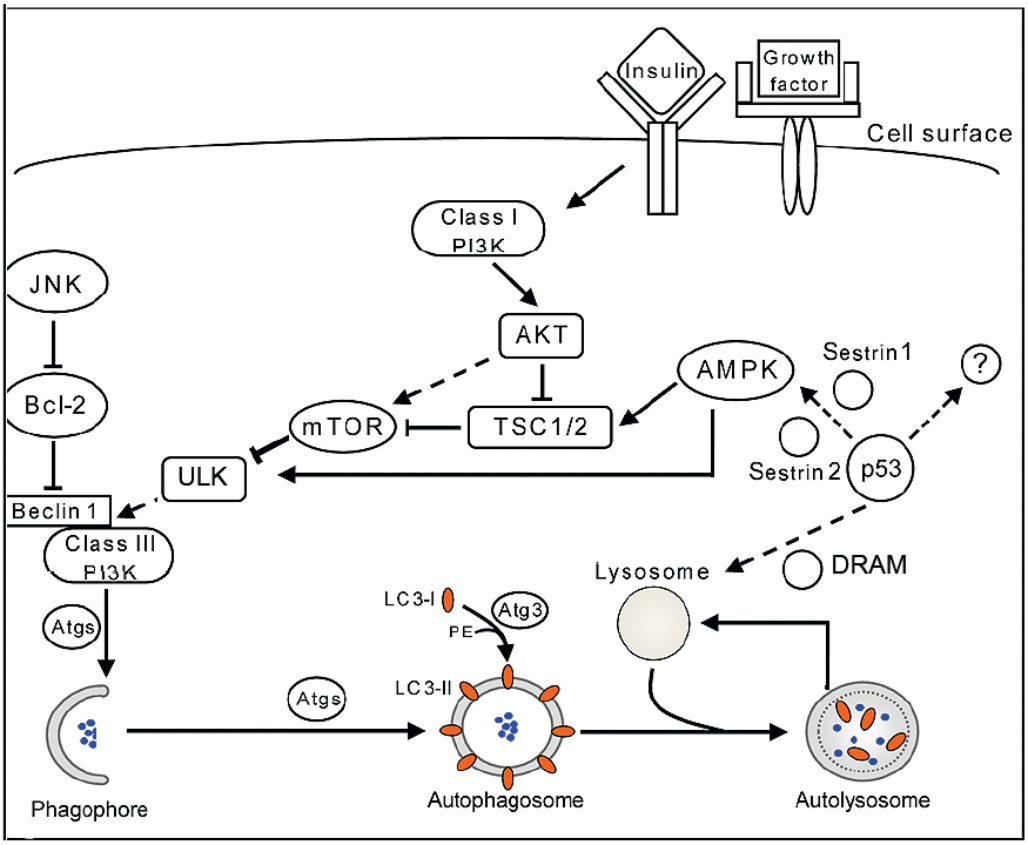

Figure 1.6
Stress Signaling and the Activation of Autophagy
A simplified schematic of the regulation of autophagy by the PI3K/Akt/mTOR and AMPK signaling axes through the ULK complex; JNK-mediated repression of Bcl-2, a negative regulator of the Beclin 1/ class III PI3K complex; and the novel modulatory roles of p53 promoting the expression of Sestrin 1, Sestrin 2 and DRAM; 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.

cell; indeed, basal levels of autophagy are homeostatic, and activated autophagy facilitates adaptation to stress (Levine and Kroemer, 2008; Mizushima et al., 2008; Moreau et al., 2010). Not surprisingly, cellular defects in autophagy increase the incidence of necrotic cell death. On its own, this would be relatively innocuous; however, in the context of a nascent tumor, increased necrotic cell death acts as an accelerant by inducing inflammation. Mice defective for autophagy and apoptosis show greatly increased tumorigenesis as compared to mice only defective for apoptosis, and this appears mainly due to increased inflammation with the tumor microenvironment (Degenhardt et al., 2006). Therefore, autophagy suppresses tumorigenesis by preventing the potentially detrimental inflammation that is associated with non-apoptotic cell death.

Recent evidence also suggests that loss of autophagy promotes genomic instability and aneuploidy (Mathew et al., 2007b; Mathew et al., 2009). p62 is a multifunctional protein that recruits protein aggregates to the autophagosome for clearance and modulates the NF-κB signaling pathway. In autophagy defective-cells, p62 accumulates, leading to deregulated NF-κB gene expression, increases in reactive oxygen species and DNA damage (Mathew et al., 2009). Elevated p62 levels have also been linked to the persistent upregulation of the transcription factor NRF2. This event appears critical for anchorage-independent growth of hepatocellular carcinoma cells where p62 is overexpressed (Inami et al., 2011).

Recently, autophagy has been shown to be an effector mechanism of OIS. Elegant work by Young *et al.* demonstrated that oncogenic *Ras* triggers robust increases in autophagic flux within cells as they transition from hyper-proliferation to the senescent state. In this system, activation of autophagy was correlated with negative feedback on the mTOR pathway. Remarkably, autophagy was also demonstrated to be necessary for senescence, since cells deficient for the essential autophagy proteins failed to efficiently undergo senescence (Young et al., 2009). Presently, it is unclear how autophagy promotes OIS, although emerging links between autophagy and cellular remodeling suggest this may play a part (Young et al., 2009; White and Lowe, 2009). Given that senescence is thought to be a barrier to malignant transformation, the loss of autophagy may allow the

escape of cells that have developed an oncogenic mutation that would normally trigger OIS and stop proliferating.

1.4.4 Autophagy and Immunity

Autophagy has recently emerged as an important defense against intracellular pathogens (reviewed in Deretic and Levine, 2009; Levine et al., 2011). The cellular autophagic machinery has been shown to eliminate pathogens by their selective sequestration/ degradation in autophagic vesicles (a process known as xenophagy: 'foreign-eat'), and the delivery of partially degraded microbial nucleic acids and peptide antigens to autophagic structures for activation of innate and adaptive immune responses (Paludan et al., 2005; Wild et al., 2011). Intriguingly, autophagy has also recently been linked to the suppression of immune and inflammatory responses (Levine et al., 2011), suggesting an exceedingly complex role for this cellular recycling mechanism in the control of immunity.

Xenophagy enables cell-autonomous elimination of invading pathogens by autophagic sequestration and degradation within lysosomes (Levine, 2005; Mizushima et al., 2008; Lin et al., 2010). This mechanism has been demonstrated to sequester and degrade invading bacteria and parasites following entry, as well as newly synthesized virions assembling within the cytoplasm (Levine and Deretic, 2007). Although the exact mechanisms that the autophagic machinery uses to specifically target free, cytosolic pathogens remain poorly defined, there are several possibilities. Recent evidence suggests that microbes may be marked by different modifications, such as ubiquitination or other molecular tags, and selected for xenophagy (Kirkin et al., 2009; Dupont et al., 2009; Fujita and Yoshimori, 2011; Wild et al., 2011). Alternatively, pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), may recognize pathogen-associated molecular patterns (PAMPs) on the surface of bacteria or viruses, consequently stimulating autophagic activity (Levine and Deretic, 2007). Although xenophagy is well characterized in limiting bacterial infections (Nakagawa et al., 2004; Gutierrez et al., 2004), supportive evidence for the clearance of viruses has only recently emerged

(Orvedahl et al., 2010); nevertheless, xenophagy is considered to be an important mechanism for capturing and degrading newly assembled virions in host cells (Kirkegaard et al., 2004; Levine, 2005; Wileman, 2007).

Autophagy also contributes to immune surveillance of infected host cells. Activation of the innate immune response, in part, depends on PRR sensing of microbial PAMPs; notably, a subset of TLRs that sense viral nucleic acids has been found to function in the lumen of endosomes (Kawai and Akira, 2006). However, viral nucleic acids are frequently released into the cytoplasm, and therefore the mechanism of endosomal TLR activation by these PAMPs was unclear. A landmark study recently demonstrated that autophagy mediates the recognition of viral ssRNAs by TLR7 in endosomal compartments during vesicular stomatitis virus (VSV) and Sendai virus infections (Lee et al., 2007). Importantly, TLR7 activation by viral ssRNAs within the endosomal compartment facilitates the triggering of a robust type I IFN antiviral response (Lee et al., 2007). Novel studies are also starting to reveal that the innate immune system can also promote the activation of autophagy; detection of PAMPS by their cognate PRRs serves to upregulate autophagy in a positive feedback loop, thus generating a switch-like mechanism for controlling pathogen clearance (Sanjuan et al., 2007; Delgado et al., 2008).

The functions of autophagy in adaptive immunity are only beginning to be elucidated. One prominent role for autophagy in adaptive immune responses involves the degradation of endogenously synthesized viral antigens and delivery of foreign peptides to class II MHC loading compartments (late endosomes), facilitating antigen presentation in host cells. Host cells displaying foreign antigens subsequently activate CD4+ T lymphocytes and elicit an adaptive immune response (reviewed in (Schmid and Munz, 2007). This process has been demonstrated with a number of important viral pathogens, including influenza (Schmid et al., 2007) and EBV (Paludan et al., 2005). The participation of autophagy in the direct elimination of intracellular pathogens and the induction of both innate and adaptive immune responses underscores the therapeutic potential of targeting autophagy to combat infectious diseases.

1.4.5 Viral Adaptations to Autophagy

Considering the functions of autophagy in pathogen clearance, it should not be surprising that many bacteria and viruses have evolved sophisticated strategies to subvert cellular autophagic defenses. Although too numerous to warrant detailed description in this discourse, many of these diverse and elegant microbial operations have been reviewed elsewhere (Kirkegaard et al., 2004; Orvedahl and Levine, 2009; Levine and Deretic, 2007; Dreux and Chisari, 2009; Lin et al., 2010).

One common mechanism that emerges from a survey of microbial strategies for evasion of autophagy involves subversion of the essential autophagy protein Beclin 1. For example, Beclin 1 is directly inhibited by the herpes simplex virus 1 (HSV-1) infected cell protein (ICP)34.5 (Orvedahl et al., 2007) and the viral Bcl-2 homologs encoded by KSHV and murine γ-herpesvirus 68 (γ-HV68) (Pattingre et al., 2005; Sinha et al., 2008; Ku et al., 2008). Furthermore, direct interaction between Beclin 1 and HSV-1 ICP34.5 or murine γ-HV68 vBcl-2 is critical for the respective viral infection and pathogenesis in mouse models, thus highlighting the importance of viral antagonism of host autophagy *in vivo* (Orvedahl et al., 2007; Leib et al., 2009). Recently, KSHV was found to encode a second inhibitor of autophagy (Lee et al., 2009); the latent v-FLIP protein binds and blocks Atg3 functions in LC3 lipid modification, thus preventing elongation of the autophagosomal membrane. It remains unclear how v-FLIP inhibition of autophagy contributes to KSHV latency.

Interestingly, not all viruses seek to block autophagic activity and evade the associated antiviral defences; some viruses actively exploit autophagy and have co-opted autophagic machinery to facilitate replication (reviewed in Kirkegaard et al., 2004; Wileman, 2007; Lin et al., 2010). Many positive-sense RNA viruses have been found to utilize host endosomal and secretory compartments for membrane scaffolding to support replication and assembly (Miller and Krijnse-Locker, 2008). Indeed, evidence suggests that some of these membranous compartments are autophagosomes, and several RNA viruses, including poliovirus (Schlegel et al., 1996; Suhy et al., 2000), hepatitis C

virus (Sir et al., 2008; Dreux et al., 2009), and Dengue virus (Heaton and Randall, 2010), induce host-cell autophagy following infection. Although it has been postulated that the double membranes of autophagosomes may serve as structural support necessary for the genome replication of some viruses, whether these double-membrane vesicles remain as functional components of the autophagic pathway is unknown (Kirkegaard et al., 2004; Lin et al., 2010).

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The emergence of microbes that inhibit autophagy creates a multifaceted problem for hosts. In particular, the fundamental importance of autophagy to metabolism, immunity, tumor suppression and other processes render viruses and bacteria that usurp autophagic pathways capable of broad pleiotropic effects. I became intrigued by the functions of autophagy in OIS, and the notion that oncoviruses might exploit this relationship to evade oncogenic stress responses and promote host cell proliferation. The remainder of this thesis describes my exploration of this hypothesis in the context of latent KSHV infection.

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1.5 Rationale and Objectives

At the time this study was initiated, the mechanisms that KSHV latent-gene products use to drive spindle cell proliferation were poorly defined. Understanding how viral and cellular oncogenes modulate host-pathogen interactions constitutes the primary focus of the Lee and McCormick laboratories. Seminal discoveries made by the Lee laboratory over a decade ago demonstrated that cancer cells harboring oncogenic *Ras* were selectively killed by the wild-type Respiratory Enteric Orphan virus (reovirus) (Coffey et al., 1998; Strong et al., 1998). Profound phenotypic changes in the proliferation of cells expressing the Ras oncoprotein render them acutely susceptible to reovirus infection, replication and lysis, and highlight the dual role of cell proliferation in viral life cycles and cancer development. Some viruses, such as KSHV, have evolved to drive

proliferation of host cells by encoding their own oncogenes. Unfortunately, this class of viruses, rather than killing cancer cells, often results in the malignant transformation of healthy cells. Groundbreaking work in the McCormick laboratory has led to the characterization KSHV oncoproteins, including Kaposin B, that promote expression of growth factors and cytokines that reinforce host cell proliferation and are intimately linked with KS tumorigenesis (McCormick and Ganem, 2005; McCormick and Ganem, 2006). However, eukaryotic cells are not simple bystanders to the actions of oncogenes, and have evolved a repertoire of intrinsic defenses, such as apoptosis and oncogene-induced senescence. Intriguingly, cells latently infected with EBV or KSHV γ-herpesviruses display hallmarks of oncogene-induced stress (Koopal et al., 2007; Nikitin et al., 2010a). It is presently unclear whether oncogenes encoded by viruses, like KSHV, trigger senescence responses in host cells and how these defenses are ultimately breached to promote tumorigenesis.

To address this problem, I characterized KSHV latent gene-products for their capacity to modulate OIS. I reasoned that expression of KSHV oncogenes would either induce senescence or elicit unique responses that permit senescence bypass. An ectopic expression screen of KSHV latent-gene products revealed that the v-cyclin protein potently induces OIS. v-Cyclin was previously linked to deregulated spindle cell proliferation and can promote the development of lymphoid malignancies with long latencies.

Full characterization of cellular responses to the v-cyclin oncoprotein revealed that autophagy is necessary for v-cyclin oncogene-induced senescence. Autophagic degradation was previously demonstrated to be an effector mechanism of senescence induced by oncogenic *Ras*. The link between autophagy and OIS was particularly intriguing because KSHV, like many other viruses, was shown to encode modulators of autophagy, namely the v-Bcl-2 and v-FLIP proteins.

My work, and that of others, has shown that KSHV latent infection induces negligible senescence despite robust activation of precursory DDRs; I reasoned that additional KSHV latent-gene products were suppressing v-cyclin OIS. To this end, I

screened KSHV latent-gene products for the ability to suppress v-cyclin-induced senescence. Remarkably, v-FLIP, an inhibitor of autophagy encoded on the same transcript as v-cyclin during latency, was found to potently inhibit v-cyclin-induced senescence.

Following the identification of v-FLIP as a potent inhibitor of v-cyclin OIS, I explored the possibility that v-FLIP suppression of autophagy interfers with senescence induction. Analysis of autophagy and senescence within cells co-expressing v-cyclin and v-FLIP revealed this to be the case. Furthermore, I found that other functions of v-FLIP, in particular modulation of NF-κB activity, also facilitate suppression of v-cyclin OIS.

Despite having a compelling ectopic-expression model for suppression of v-cyclin OIS by v-FLIP, it remained unclear if this actually occurs in cells latently infected with KSHV. To address this problem, I used short peptides derived from v-FLIP itself, which were shown to interfere specifically with v-FLIP anti-autophagy functions, to rescue host autophagy within KSHV-infected cells. Strikingly, not only did v-FLIP inhibitory peptides rescue autophagy within infected cells, but they also promoted more senescence. Thus, v-FLIP suppression of autophagy during KSHV latency helps impair OIS.

The results presented in this thesis provide compelling evidence that KSHV has evolved to suppress OIS and facilitate spindle-cell proliferation through modulation of autophagy. These insights help to justify the coordinated expression of the v-cyclin and v-FLIP oncoproteins during latency and suggests other oncoviruses may employ similar mechanisms to mitigate cell stress responses activated through the course of infection. Furthermore, therapeutic intervention targeted at inducing autophagy or senescence within infected cells may prove beneficial toward combating virus-associated cancers.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Cell Culture and Chemicals

All senescence and autophagy experiments in this study involved the use of human diploid cells with tumor-suppressor pathways that appeared to be fully intact. Furthermore, efforts were made to employ both microvascular endothelial cells and fibroblasts, which have both been shown to demonstrate efficient establishment of latency upon *de novo* infection with KSHV. Human telomerase-immortalized microvascular endothelial (TIME) cells, BCBL-1 and Phoenix retroviral-packaging cells were kind gifts from Don Ganem, University of California, San Francisco (UCSF). BJ fibroblasts (HFFs) were obtained from American Type Culture Collection (ATCC) and telomerase-immortalized BJ cells (TERT-HFFs) were a kind gift from William Hahn, Harvard University. TIME cells were cultured in EGM-2 medium (Lonza) supplemented with microvascular (MV) endothelial cell bullets (Lonza) and fetal bovine serum (FBS) to 5 percent (%). Tissue culture plates for TIME cells were coated with 0.1% gelatin prior to cell seeding. This has been found to promote healthy attachment of TIME cells to culture plates and other substrates. HFFs and TERT-HFFs were cultured in a 4:1 mixture of knockout Dulbecco's Minimal Essential Medium (DMEM) (Invitrogen) and M199 medium (Invitrogen) containing 15% FBS and 4 mM L-glutamine according to suggestions by Dr. Hahn. BCBL-1 cells were carried in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS. Finally, 293T and Phoenix cells were cultured in DMEM (Invitrogen) containing 10% FBS. Importantly, unless otherwise noted, media were refreshed every second day to ensure activation of autophagy or senescence was not due to culturing-associated stresses. For the bulk of experiments, penicillin and streptomycin were excluded from media to ensure that low-level culture contamination was not present. Furthermore, cells used in these studies were tested and found to be mycoplasma free.

2.2 Vectors and Cloning

The pBMN retroviral vector system was used to stably express oncogenes in this study (Gary Nolan, Stanford University). To ensure transduction and expression of each oncogene in singly or multiply infected cells, I utilized a suite of retroviral vectors engineered to express neomycin- (neo), puromycin- (puro), blasticidin- (blast) resistance genes or Green Fluorescent Protein (GFP) from an internal ribosomal entry site (IRES) within vector-derived transcripts. Gary Nolan of Stanford University (from Addgene) provided pBMN-IRES-neomycin and pBMN-IRES-GFP vectors. To generate pBMN-IRES-puromycin and pBMN-IRES-blast vectors, I amplified puromycin- and blasticidin-resistance genes from pMSCV-puromycin (Clontech) and pCMV-blasticidin (Invitrogen), respectively, using primers to generate products with 5' PciI and 3' SalI restriction sites. These products were then cloned between the NcoI and SalI sites of pBMN-IRES-GFP, replacing the GFP open reading frame (ORF) downstream of the IRES element. KSHV v-cyclin, v-FLIP, LANA and cellular H-Ras^{V12} oncogenes were amplified from a KSHV genomic fragment encompassing open-reading frames 71-73 (Don Ganem, UCSF) and the pBABE-puro-H-Ras^{V12} vector (Robert Weinberg, Whitehead Institute), respectively. Primers containing an amino-terminal hemagglutinin (HA) epitope tag were used in amplification reactions for v-cyclin and H-Ras^{V12}, whereas v-FLIP was amplified with primers generating a carboxy-terminal myc epitope tag, and the resulting products were cloned into the pBMN retroviral vector between either the EcoRI and XhoI or BamHI and EcoRI restriction sites.

To generate the neomycin-selectable shRNA retroviral expression vector the AgeI-ClaI fragment of pSMP (Open Biosystems), encompassing the puromycin-resistance gene, was replaced with the AgeI-ClaI fragment of pMSCV-neomycin (Clontech). Subsequently, a miR-30 polymerase chain reaction (PCR) 'SHAGging' protocol was used to amplify specific target sequences from single-strand DNA primers and enable cloning into XhoI-EcoRI digested vector (Paddison et al., 2004). Target sequences for shRNAs have been previously published (Young *et al.* 2009) and can be found in Table 2.1.

For inducible oncogene expression, HA-v-cyclin and HA-H-Ras^{V12} were amplified

using primers that created 5' NotI and 3' EcoRI restriction sites and cloned into the corresponding region of the pRetroX-Tight-Puromycin vector (Clontech). In this system, expression of v-cyclin and Ras was driven by a second vector, pRetroX Tet-Off Advanced (Clontech), encoding the tetracycline-controlled transactivator.

v-FLIP 58AAA was cloned by Quickchange PCR of pBMN-v-FLIP-myc-IRES-puromycin using Pfu turbo (Stratagene), and v-FLIP Δα2α4 was generated using 5' phosphorylated primers bordering the regions to be deleted and Phusion polymerase (Finnzymes) was used for amplification. Products were purified and ligated, and isolated clones were sequenced. For expression of the v-FLIP dominant negative peptides we employed the pMSCV-puromycin retroviral vector (Clontech). Positive- and negative-strand primers encompassing the entirety of α2 or α4 helices of v-FLIP with a glycine-serine linker were annealed and cloned as a carboxy-terminal fusion to the ORF for Red Fluorescent Protein (RFP) using 5' EcoRI and 3' XhoI restriction sites. Sequencing was used to verify in-frame cloning of the constructs.

2.3 Virus Infections and Chemical Treatments

For packaging of retroviruses, Phoenix cells (4.5×10^6) were seeded in a 100 mm culture dishes, incubated for 24 hours, and then transfected with 6 μg of a retroviral plasmid, in the absence of serum, using polyethylenimine (PEI) (Sigma). Transfection medium was removed 6 hours later and replaced with complete medium. Viral supernatants were collected at 48 hours post-transfection, filtered (0.45 μm filter, Millipore) and then supplemented with 4 μg/mL sequabrene (Sigma). In most instances, fresh retroviral preparations were used for transductions. Occasionally, however, frozen stocks were used to satisfy the bulk volumes required for certain experiments.

Target cells were seeded at a density of 8×10^5 per six well dish 24 hours prior to retroviral infection. For all infections, culture media was replaced with viral supernatants and 'spinfected' onto the cells at 2000 revolutions per minute (rpm) for 2 hours. Subsequently, the viral supernatant was removed and cells were given 24 hours to recover in fresh culture medium and then selected in 800 μg/mL G418 (Sigma), 1 μg/mL

Table 2.1 shRNA target sequences

Target	Sequence
Scramble (Scr.)	AGCACAAGCTGGAGTACAACTA
p53	CGGAGGATTTCATCTCTTGTAT
Rb1	CGCAGTTCGATATCTACTGAAA
ATG5	CTTTGATAATGAACAGTGAGA
ATG7	GGAGTCACAGCTCTTCCTTAC

(Young *et al.* 2009)

puromycin (Sigma) and/or 5 μg/mL blasticidin (Sigma). After selection, cells were re-seed into tissue culture dishes appropriate for individual assays. For coinfection with puromycin-, neomycin- and/or blasticidin-selectable retroviral vectors, cells were infected sequentially with separate viral supernatants, and then simultaneously selected as above.

Clonal v-cyclin- and Ras-inducible cells were generated by serially diluting selected cells in 96-well dishes and isolating cells from wells rendering an obvious single clone. This labour-intensive task took approximately 1.5 months and yielded five v-cyclin clones and 3 Ras^{V12} clones. High-level, doxycycline-regulated expression was verified for all clones and the best were selected for population expansion.

KSHV preparation and infections were performed essentially as described (Lagunoff et al., 2002). Briefly, viral supernatants were obtained by inducing BCBL-1 cells, subcultured to 2×10^5 to 3×10^5 cells per mL, with 0.3 mM valproic acid for five days. After induction, lytically reactivated cells were pelleted, the supernatant was filtered through a 0.45-μm pore size filter, and the virions were pelleted at 15,000 rpm for 2 hours using an SW28 rotor in a Beckman-Coulter ultracentrifuge. Virion pellets were resuspended in phosphate-buffered saline and 'snap' frozen. For infections, culture medium was replaced with mediium containing diluted viral supernatants supplemented with 4 μg/mL sequabrene (Sigma) and 'spinfected' onto the cells at 2000 revolutions per minute (rpm) for 2 hours. Infected cells were then monitored for establishment of latency after 24 hours.

For drug treatment of KSHV-infected cells, rapamycin (Sigma) was dissolved in dimethylsulfoxide (DMSO) to a stock concentration of 100 μM and then diluted to the indicated concentrations in medium. Rapamycin (Sigma) treatments were refreshed daily in an attempt to mitigate any issues with drug stability. The concentrations of rapamycin chosen for my studies have been shown to be sufficient to repress mTORC1 activity and phosphorylation of downstream targets (Kennedy et al., 2011).

It can sometimes be difficult to distinguish between autophagy activation and repression, since both give rise to an accumulation of autophagosomes. For this reason,

bafilomycin (Sigma), an inhibitor of lysosomal acidification, is commonly used to block autophagy for determination of whether autophagic structures or markers increase, indicative of autophagic activation, or remain at a constant level, indicative of an upstream blockade in autophagic degradation. In my studies, bafilomycin was prepared in DMSO and diluted from frozen stocks into culture medium to a final concentration of 100 nM. Cells were treated for 3 hours prior to lysis or fixation for routine autophagy analysis.

2.4 Proliferation, Colony Formation, SA β-Gal and SAHF Assays

Bromodeoxyuridine (BrdU) incorporation and immunolabelling assays were performed according to the manufacturer's instructions (BD). Briefly, cells were pulsed with 100 μM BrdU for 4 hours or 8 hours, for HFFs and TIME cells, respectively, due to differences in their proliferation rates. Subsequently, samples were sequentially fixed and permeablized with 3.7% formaldehyde and 90% methanol. Cells were blocked in 3% FBS and then DNA was cleaved (to expose BrdU) with DNase I for 1 hour. The fluorescently labeled anti-BrdU antibody (1:10) was then incubated with cells overnight. Cells were washed, nuclear DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI) and then samples were visualized using a Zeiss Axiovert 200 microscope with a Hamamatsu Orca camera. Ten fields of approximately 25-50 cells per field were enumerated for each of three independent experiments.

To carry out colony-formation assays, transduced TERT-HFFs 8 days post-selection were seeded in duplicate at 2500 or 5000 cells/ 35-mm well. Two weeks later, cells were fixed and stained with 0.5 % crystal violet (Sigma). Washed and dried plates from two independent experiments were scanned for visualization.

Senescent cells were detected using an SA β-gal kit according to manufacturer's instructions (NEB). Briefly, cells were washed once and fixed in a 2% formaldehyde, 0.2% glutaraldehyde solution in phosphate-buffered saline for 10-15 minutes. After washing, the staining solution, consisting of 1 mg/mL X-gal, 50 mM potassium ferrocyanide, 50 mM potassium ferricyanide diluted in 40 mM citric acid/sodium phosphate pH 6.0, was applied to cells. Plates were sealed with tape and incubated at

37°C in bacterial incubator for 12-16 hours. After washing in phosphate-buffered saline, plates were sealed and stored at 4°C in the dark until microscopic analysis. Ten fields of approximately 50 cells each were enumerated in three separate experiments; images were taken for each field under all conditions. Furthermore, SA β-gal activity was assayed separately from other assays using cells derived from a single population due to reagent autofluorescence.

For detection of SAHF, cells were fixed and permeablized in 90% methanol and stained with 0.13 μg/mL DAPI for 2 minutes. Ten fields of 25 cells/field were enumerated in three independent experiments.

2.5 Immunofluorescence and Electron Microscopy

For immunofluorescence experiments, cells seeded on gelatin-coated coverslips were fixed in 4% paraformaldehyde, permeabilized in 90% methanol and blocked in 3% FBS. Primary antibodies were incubated with samples overnight at 4˚C followed by incubation with fluorescent secondary antibodies (Molecular Probes) for 1 h. All fluorescent samples were visualized with either a Zeiss Axiovert 200 microscope with a Hamamatsu Orca camera or a Zeiss LSM 510 META Laser Scanning Confocal Microscope as indicated.

For electron-microscopy (EM) analyses, transduced cells at 7 days post-selection were trypsinized, resuspended in ice-cold full medium and pelleted. Subsequently, cells were fixed at room temperature for 1 hour in 0.1 M Sorensen's phosphate buffer containing 3.5% glutaraldehyde. Samples were post-fixed in phosphate-buffered 1% osmium tetroxide, dehydrated in ethanol, and embedded in epoxy resin for thin sectioning followed by standard staining in uranium and lead salts. Thin sections were observed in a JEOL JEM-1230 transmission electron microscope equipped with a Hamamatsu ORCA-HR. The criteria outlined in Yla-Anttila et al. (2009a) were employed to enumerate the number of autophagic structures per cell section, in 30 cells in two independent experiments. Essentially, the criteria for autophagosomes include: an obvious double-membrane (or more) cytoplasmic vesicle, with contents in its lumen, approximately 0.5 -2.0 μm in diameter, with no cristae (indicative of a 'swollen' mitochondrion); autolysosomes have many of the same features except they often exhibit loss of part of

the double membrane (rendering portions of single membrane within the vesicle) and display the obvious presence of electron-dense hydrolytic enzymes derived from the lysosomal fusion event.

2.6 Fluorescence Microscopy of Autophagic Structures

To visualize autophagic structures for fluorescence microscopy experiments I transfected senescing cells or proliferating controls 5 days after addition of selection with either pDEST-mCherry-EGFP-LC3 or pDEST-EGFP-LC3 (Terje Johansen, University of Tromsø). Cells were transfected with polyethylenimine (PEI) and Lipofectamine LTX, respectively. Medium was refreshed 24 hours post-transfection and 4 hours later cells were fixed in 4% paraformaldehyde for 15 minutes. Cells were either visualized directly for autophagic structures or permeabilized in ice-cold 90% methanol for co-stained fluorescence microscopy. At least 30 cells were painstakingly evaluated in three separate experiments.

2.7 Gene Expression

For quantitative real-time polymerase chain reaction (qRT-PCR), messenger ribonucleic acid (mRNA) was harvested using an RNeasy Extraction kit (Qiagen). mRNA was reverse-transcribed using Superscript II (Invitrogen) and quantitative PCR analysis was carried out using Quantifast SYBR Green PCR kit (Qiagen) on the Mx3000P Multiplex PCR system (Stratagene). Initially, cloned deoxyribonucleic acid (cDNA) was serially diluted to delineate the appropriate range for quantitative amplification. Target amplification was performed at the appropriate dilution with the primer sets found in Table 2.2. Cycle thresholds (Cts) for the various targets were calculated according to the $2^{(-\Delta\Delta ct)}$ method using β-actin as an internal control. Target mRNA quantification was conducted in duplicate for each experiment, and the data represent the ±SEM of three independent experiments.

For immunoblots, cell extracts were prepared by washing cells twice in ice-cold PBS and lysis in-dish using 2x Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS), and sonicated to shear genomic DNA. Protein concentrations were

Table 2.2 Q-RT-PCR Primer Sequences

Target	Forward Primer	Reverse Primer
β-actin	CTTCCAGCAGATGTGGATCA	AAAGCCATGCCAATCTCATC
Ulk1	AAGCACGATTTGGAGGTCGC	TGATTTCCTTCCCCAGCAGC
Ulk3	TGAAGGAGCAGGTCAAGATGAGG	GCTACGAACAGATTCCGACAGTCC
ATG7	GTCGTCTTCCTATTGATGGACACC	CAAAGCAGCATTGATGACCAGC
LC3A	GCGAGTTGGTCAAGATCATCCG	TGGACACACTCACCATGCTGTG
LC3B	ACGCATTTGCCATCACAGTTG	TCTCTTAGGAGTCAGGGACCTTCAG
DRAM	TCAAATATCACCATTGATTTCTGT	GCCACATACGGATGGTCATCTCTG
Sestrin1	CGACCAGGACGAGGAACTT	CCAATGTAGTGACGATAATGTAGG
Sestrin2	CAAGCTCGGAATTAATGTGCC	CTCACACCATTAAGCATGGAG
p21	GACACCACTGGAGGGTGACT	CAGGTCCACATGGTCTTCCT
INK4a	CTGCCCAACGCACCGA	GCTGCCCATCATCATGACCT
cyclin A	GCGTTCACCATTCATGTGGA	CAGGGCATCTTCACGCTCTATT
HMGA2	CAGCCGTCCACATCAGCCCAG	CTTGCGAGGATGTCTCTTCAG
IL1α	GCCAGCCAGAGAGGGAGTC	TGGAACTTTGGCCATCTTGAC
IL1β	GGCCCTAAACAGATGAAGTGCT	TGCCGCCATCCAGAGG
IL6	CCAGGAGCCCAGCTATGAAC	CCCAGGGAGAAGGCAACTG
IL8	TTGGCAGCCTTCCTGATTTC	TCTTTAGCACTCCTTGGCAAAAC

Table 2.3 Antibodies and Dilutions

Target	Company/ Cataolog#	Dilution
p53	Santa Cruz/ sc-126	1:1000
p21	Santa Cruz/ sc-6246	1:500
HMGA2	Santa Cruz/ sc-30223	1:200
Cyclin A	Santa Cruz/ sc-596	1:500
Lamin A/C	Santa Cruz/ sc-20861	1:1000
β-actin	Santa Cruz/ sc-130656	1:2000
Bcl-2	Santa Cruz/ sc-7382	1:200
Rb1	Santa Cruz/ sc-102	1:500
HA-epitope	Santa Cruz/ sc-805	1:1000
LC3	Nanotools/ 2G6	1:200
Sestrin 1	Novus Biologicals/ H00027244-B01P	1:100
Sestrin 2	Novus Biologicals/ H00083667-M03	1:50
DRAM	Abcam/ ab4739-100	1:100
p-AMPK T172	Cell Signaling/ #2535	1:1000
AMPK	Cell Signaling/ # 2603	1:1000
p-p53 S15	Cell Signaling/ #9284	1:1000
p-mTOR S2448	Cell Signaling/ #2971	1:1000
mTOR	Cell Signaling/ #2983	1:1000
4E-BP1	Cell Signaling/ #9452	1:1000
p-4E-BP1 S37/46	Cell Signaling/ #2855	1:1000
p-p70/S6K S389	Cell Signaling/ #9234	1:1000
p-Bcl-2 S70	Cell Signaling/ #2827	1:500
p-AKT T308	Cell Signaling/ #2965	1:1000
p-AKT S473	Cell Signaling/ #4060	1:1000
AKT	Cell Signaling/ #9272	1:1000
ATG5	Cell Signaling/ #2630	1:1000
ATG7	Cell Signaling/ #2631	1:1000
γH2AX	Cell Signaling/ #2577	1:1000
INK4a	Cell Signaling/ #4824	1:1000
myc-epitope	Cell Signaling/ #2276	1:250

determined using a DC Protein Assay Kit (Bio-Rad). Samples were supplemented with bromophenol blue and 2-mercaptoethanol to final concentrations of 0.01% and 5%, respectively, and boiled. Samples were separated on denaturing polyacrylamide gels, blotted and then probed. A full account of the antibodies used for immunoblotting and their respective dilutions can be found in Table 2.3.

2.8 BAC36-derived Recombinant KSHV

Briefly, 293T cells were transfected with BAC36 DNA and selected in 150 μg/mL hygromycin for two weeks. Stable transfectants were treated with 0.3 mM valproic acid for 5 days. Cells were washed to remove residual chemical and medium was replaced. After 3 days medium was harvested, sequabrene added to 8 μg/mL and target cells 'spinfected' at 2000 rpm for 2 hr. Virus medium was replaced and cells were visualized two days later.

2.8 IL-6 and IL-8 ELISAs

The concentration of chemokines released into the supernatant was measured by specific IL-6 or IL-8 ELISA (BD OptEIA) according to manufacturer's instructions. The data were normalized to cell numbers enumerated for each time point and reported as ng per $1x10^6$ cells per day.

2.9 Statistics

Data was analyzed using an unpaired, two-tailed, Student's t-test, with 95% confidence on Prism GraphPad.

CHAPTER 3 - KSHV V-CYCLIN INDUCES SENESCENCE

Note: The majority of the research described in this chapter has been published in Cell Host and Microbe as: Leidal, A. M., Cyr, D. P., Hill, R. J., Lee, P. W., and McCormick, C. (2012). Subversion of Autophagy by Kaposi's Sarcoma-Associated Herpesvirus Impairs Oncogene-Induced Senescence. *Cell Host Microbe* 11, 167-180. © Cell Press. Figures appearing in this chapter that are wholly or partially reproduced from figures appearing in that paper include Figures 3.1, 3.3 and 3.4.

3.1 Introduction

In the Lee and McCormick laboratories we are interested in understanding how viral and cellular oncogenes modulate host-pathogen interactions. Oncoviruses encompass the subset of all viruses that encode oncogenes and predispose infected cells to malignant transformation. Although approximately 12% of all cancers are associated with viral infection (Parkin, 2006), relatively little is known regarding the capacity of viral oncogenes to induce DNA damage or OIS. Furthermore, it is also unknown whether the DDRs and OIS constitute important barriers to the viral life cycle and oncogenesis.

Latent infection of endothelial cells with KSHV induces DDRs and predisposes to development of KS (Koopal et al., 2007). Therefore, KSHV latent-gene products are thought to be instrumental in the process of KSHV-induced tumorigenesis. Extensive characterization has revealed that many KSHV latent-gene products usurp pathways important for proliferation, and thus may be perceived as viral oncogenes. For example, LANA inhibits p53 and Rb pathways (Friborg et al., 1999; Radkov et al., 2000), while v-FLIP inhibits autophagy and is a potent activator of NF-κB (Chaudhary et al., 1999; Lee et al., 2009). Although each KSHV latent-gene product has properties that should render it pro-oncogenic, none is capable of inducing cellular transformation on its own (Mesri et al., 2010).

The v-cyclin protein, amongst KSHV latent gene products, is particularly enigmatic: despite its homology to cellular D-type cyclins and capacity to inactivate Rb,

its expression induces primary cells to stop proliferating (Koopal et al., 2007; Verschuren et al., 2002). Furthermore, v-cyclin, in association with CDK6, stimulates cell-cycle progression by phosphorylating Rb family proteins, and components of the origin-recognition complex and has been shown to render CDK6 refractory to the functions of INK4 and Cip/Kip-family CKIs (Laman et al., 2001; Li et al., 1997; Swanton et al., 1997). How could this protein induce proliferation arrest? Importantly, v-cyclin has also been reported to induce robust DDRs (Koopal et al., 2007; Verschuren et al., 2002). Collectively, these observations led me to reason that deregulated proliferation induced by v-cyclin might promote OIS. Therefore, I attempted to characterize v-cyclin-induced proliferation arrest for hallmarks of senescence.

3.2 Results

3.2.1 v-Cyclin Induces Classical Hallmarks of Senescence

To investigate whether v-cyclin-expressing cells display hallmarks of oncogene-induced senescence I developed a retroviral system to stably express v-cyclin and controls within primary and non-transformed cells. To this end I made minor modifications to the Phoenix Retroviral Expression System developed by G. Nolan (Stanford), replacing the vector's GFP selection marker, downstream of the IRES, with a puromycin resistance gene (pBMN-IRES-puro). Subsequently, I generated distinct vectors encoding the *v-cyclin* open reading frame (ORF), *HRas^{V12}* oncogene or an empty-vector negative control. As noted previously, the *HRas^{V12}* oncogene encodes a potent inducer of senescence that has been well characterized in the literature and therefore served as a positive control and comparator. Furthermore, to facilitate v-cyclin detection and enable discrimination of ectopically expressed HRas^{V12} from endogenous HRas I cloned *v-cyclin* and *HRas^{V12}* ORFs in-frame and carboxy-terminal to sequences encoding the hemagglutinin (HA) epitope tag. Importantly, HA-v-cyclin and HA-HRas^{V12} fusion proteins have been used extensively and in each case the HA-epitope appears not to compromise function (Fiordalisi et al., 2001; Swanton et al., 1997).

To determine if ectopic expression of v-cyclin promotes hallmarks of senescence, I transduced telomerase-immortalized microvascular endothelial cells separately with retroviral vectors encoding HA-v-cyclin, HA-HRas^{V12} or an empty vector control and monitored their morphology. TIME cells grow continuously in culture and maintain many of the features of primary endothelial cells (Venetsanakos et al., 2002). Importantly, these cells can be readily infected by KSHV, and display a predominantly latent infection with a small percentage of cells undergoing lytic reactivation, faithfully recapitulating the behavior of KS spindle cells *in vivo* (Lagunoff et al., 2002). Cells that were not transduced with retroviral vectors were eliminated from the population through a 2-day selection in puromycin. Intriguingly, TIME cells expressing HA-v-cyclin and HA-HRas^{V12} began to flatten, enlarge and become vacuolized approximately 3-4 days post-selection. These qualitative hallmarks of senescence persisted in HA-cyclin and HA-HRas^{V12} cells until they were fixed at 7 days post-selection, and were not observed in empty vector control cells (Figure 3.1A). To more definitively establish if HA-v-cyclin cells were undergoing senescence, I carried out SA β-gal activity assays on the fixed populations. Strikingly, cells expressing HA-v-cyclin exhibited robust positive staining for SA β-gal activity, similar to those expressing HA-HRas^{V12}, while staining of empty-vector control cells was negative (Figure 3.1A). These results compellingly suggest that v-cyclin, similar to Ras oncoprotein, promotes OIS.

Senescent cells frequently harbor extensive DNA damage and demonstrate persistent DNA-damage response signaling (d'Adda di Fagagna, 2008; Di Micco et al., 2006). To determine if v-cyclin induces persistent DNA-damage signaling, I fixed HA-v-cyclin, HA-HRas^{V12} and empty-vector control cells at 7 days post-selection and immunostained with anti-53BP1 or anti-γH2AX antibodies. Typically, cells with DNA damage and activated DDR signaling demonstrate a punctate nuclear staining pattern for both 53BP1 and γH2AX as these two proteins become relocalized to sites of DNA damage to serve as signaling scaffolds. Remarkably, the nuclei of HA-v-cyclin and HA-HRas^{V12} cells were replete with 53BP1 and γH2AX foci, whereas empty-vector controls were largely devoid of foci (Figure 3.1A). Clearly, v-cyclin induces persistent DDRs

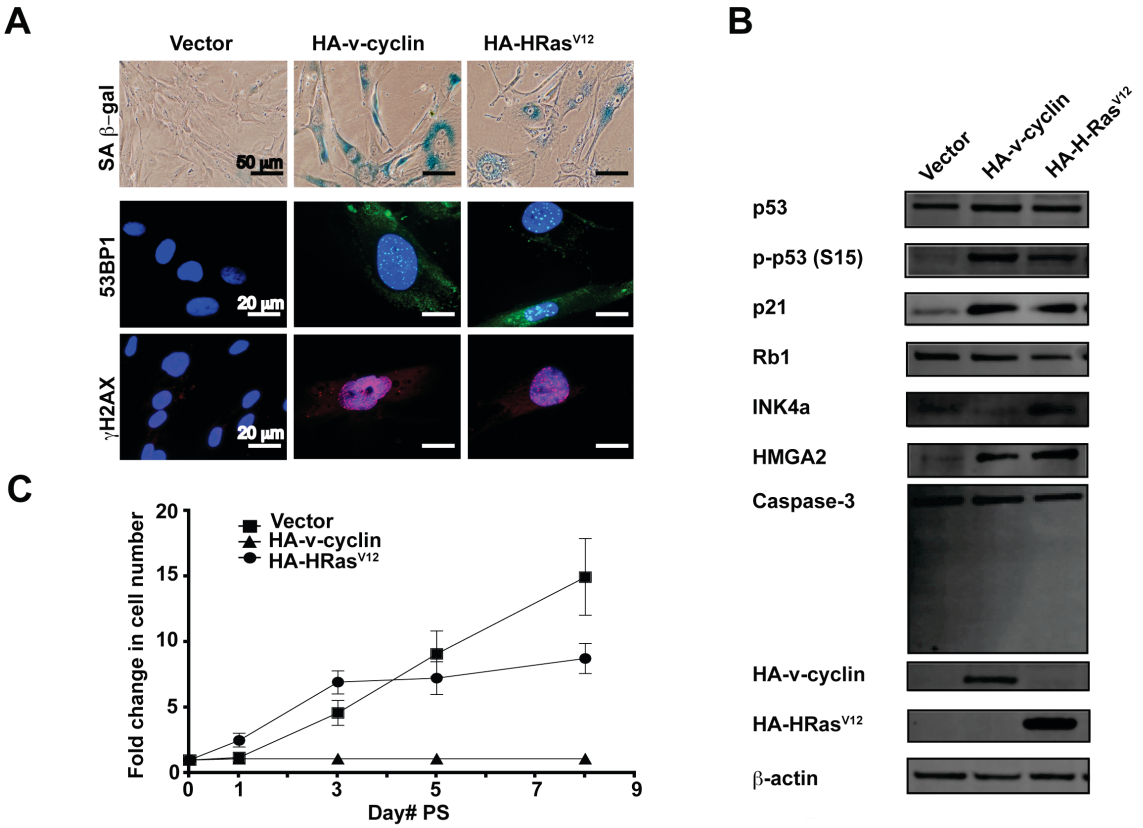

Figure 3.1
KSHV v-Cyclin Induces Classical Hallmarks of Senescence
(A) TIME cells were transduced with retroviral vectors encoding HA-v-cyclin, HA-HRas^{V12} or vector and assayed for SA β-gal activity, 53BP1 foci and γH2AX foci at seven days post-selection. Chromatin was counterstained with DAPI (B) Lysates derived from TIME cells transduced in parallel seven days post-selection were immunoblotted and probed for proteins involved in senescence signaling. (C) Growth curves for cells transduced with HA-v-cyclin, HA-HRas^{V12} and vector post-selection (PS).

consistent with the senescent phenotype.

The p53 and Rb tumor-suppressor pathways are necessary for senescence triggered by diverse oncogenes (Ferbeyre et al., 2002; Lin et al., 1998; Narita et al., 2003; Serrano et al., 1997). To determine if p53 and Rb pathways are involved in v-cyclin OIS, I immuoblotted lysates derived from v-cyclin and Ras senescent cells or proliferating empty-vector controls for markers of activation. v-Cyclin cells, similar to Ras-senescent positive controls, demonstrated robust activation of the p53 tumor-suppressor pathway as indicated by p53 serine 15 phosphorylation and stabilization, and up-regulation of the p53-target gene p21 (Figure 3.1B). The p53 pathway appeared inactive in proliferating cells. Surprisingly, v-cyclin cells failed to activate the INK4a CDK inhibitor, a common marker of Ras-induced senescence and important regulator of the Rb family of pocket proteins. However, the status of Rb1, which is tightly controlled by phosphorylation, remained inconclusive since Rb1 from proliferating cells demonstrated no phosphorylation-associated mobility shifts within SDS-PAGE relative to Rb1 from senescent cells. Intriguingly, immunoblots for high-mobility-group AT-hook protein-2 (HMGA2), a heterochromatin protein and marker of senescence (Narita et al., 2006), revealed sharp up-regulation within v-cyclin and Ras cells, but not proliferating vector controls (Figure 3.1B). Finally, examination of caspase-3, a marker of apoptosis when proteolytically cleaved, revealed no cleaved product within v-cyclin cells. Therefore, in contrast to previous reports v-cyclin induces negligible apoptosis. Together, these data demonstrate v-cyclin triggers two important hallmarks of OIS: activation of the p53 pathway and up-regulation of HMGA2.

Senescence growth arrest is essentially permanent and cannot be reversed by known physiologic stimuli (Campisi and d'Adda di Fagagna, 2007; Kuilman et al., 2010). This feature distinguishes senescence from other transient forms of arrest such as quiescence and conventional cell-cycle arrest. To determine if v-cyclin induces the stable arrest consistent with senescence, I examined the proliferation profile of v-cyclin cells relative to those undergoing Ras-induced senescence or proliferating vector controls. Ras-expressing positive control cells proliferated very rapidly immediately after selection,

indicative of hyper-proliferation, and then ceased dividing after 3-4 days (Figure 3.1B). By contrast, cells expressing v-cyclin failed to divide appreciably after selection and did not appear to go through a period of hyper-proliferation. Empty-vector transduced cells continued to divide throughout the duration of the experiment. Importantly, v-cyclin arrest was found to be very stable and, like Ras senescent cells, v-cyclin cells remained in culture for extended periods (4 weeks) with negligible proliferation (data not shown). Furthermore, v-cyclin cells and Ras cells showed decreased dependence on growth factors for survival relative to proliferating control TIME cells (data not shown). Therefore, the proliferation arrest induced by v-cyclin is consistent with OIS.

3.2.2 v-Cyclin Induces Senescence-Associated Heterochromatin Foci

Significant changes in chromatin frequently occur during transition to the senescent phenotype (Narita et al., 2003; Narita et al., 2006; Zhang et al., 2005). Senescent cells often harbor vast regions of chromatin that are condensed into punctate domains known as senescence-associated heterochromatin foci (SAHF). Genes within regions of chromatin incorporated into SAHF are strongly silenced and frequently encode proteins that function in proliferation (Narita et al., 2003). Intriguingly, HMGA2 is known to be a critical component of SAHF (Narita et al., 2006). The observation that v-cyclin triggers up-regulation of HMGA2 suggests that SAHF are formed within arrested cells. To determine whether v-cyclin induces SAHF, I transduced human foreskin fibroblasts (HFFs) with retroviral vectors encoding HA-v-cyclin or HA-HRas^{V12}, or empty-vector controls, and stained cellular DNA with DAPI at 7 days post-selection. Cells were also co-stained for SA β-gal activity to enable overlay of senescence markers. Remarkably, I observed that v-cyclin, similar to HRas^{V12}, induced discrete DAPI-stained nuclear foci, indicative of SAHF, within cells that were positive for SA β-gal activity (Figure 3.2). In contrast, DAPI-stained proliferating cells failed to demonstrate any nuclear foci and were negative for SA β-gal activity. Together, the formation of SAHF and robust activation of SA β-gal strongly suggest that v-cyclin-induced proliferation arrest corresponds to OIS.

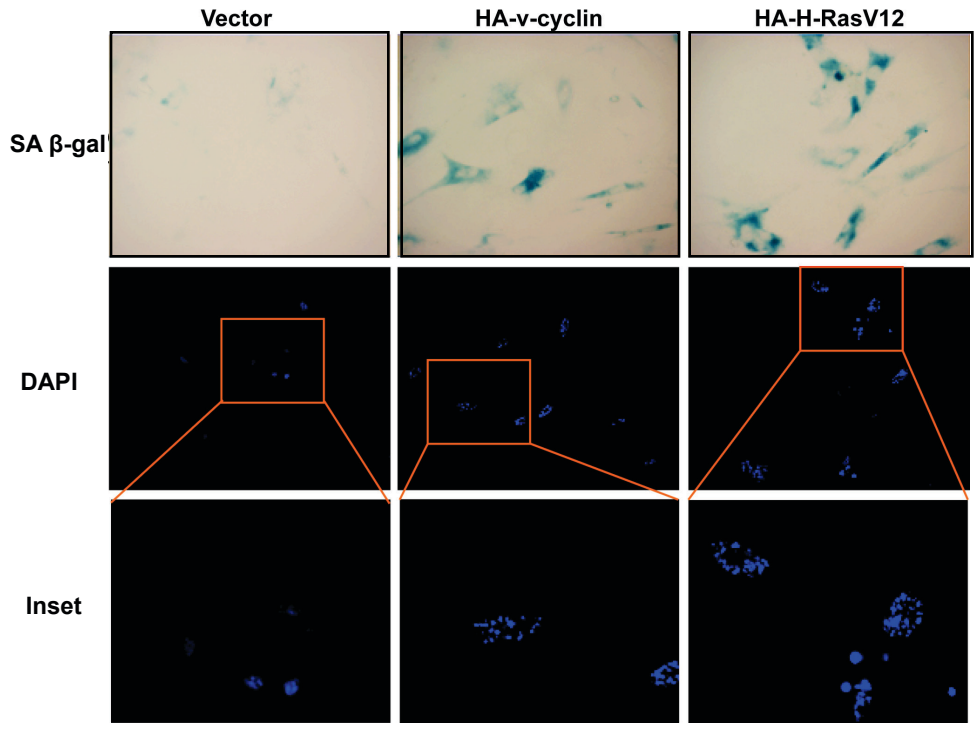

Figure 3.2
KSHV v-Cyclin Induces SAHF
HFFs were transduced with retroviral vectors encoding HA-v-cyclin, HA-HRas^{V12} or vector and assayed for SA β-gal activity and SAHF (using DAPI) at seven days post-selection.

3.2.3 KSHV v-Cyclin Induces the SASP

Previous work has shown that senescent cells frequently express and secrete a plethora of cytokines, including IL-1β, IL6 and IL8 (Acosta et al., 2008; Coppe et al., 2008; Kuilman et al., 2008; Kuilman and Peeper, 2009). Furthermore, some cytokines, such as IL-1α, are trafficked to the cell surface of senescent cells without being secreted, to reinforce autocrine signaling (Orjalo et al., 2009). It is thought that this senescence-associated secretory phenotype (SASP) facilitates immune clearance of senescent cells and tissue repair *in vivo* (Kang et al., 2011; Rakhra et al., 2010; Xue et al., 2007). Furthermore, there is evidence that certain cytokine signaling networks are necessary to enforce senescence (Acosta et al., 2008; Kuilman et al., 2008). Therefore, I was interested to determine whether v-cyclin also induces the SASP. To this end, I transduced HFFs and TIME cells with retroviral vectors encoding v-cyclin, or empty vector controls, and harvested mRNA for quantitative real-time PCR (qRT-PCR) at eight days post-selection. From the two conditions, in each cell type, I quantified the mRNA levels for four cytokines, including IL-1α, IL-1β, IL6 and IL8. Strikingly, I observed robust up-regulation of all cytokines in the presence of v-cyclin, in both cell types (Figure 3.3A). In fact, IL-6 and IL-8 mRNA in HFFs was found to be induced greater than ten fold and seventy fold, respectively.

Despite robust increases in cytokine mRNAs, it was unclear whether v-cyclin also promoted the translation and secretion of cytokines from arrested cells. Therefore, I assayed the conditioned media from v-cyclin-expressing or empty-vector control HFFs and TIME cells for IL-6 and IL-8. Enzyme-linked immunosorbent assays (ELISAs) revealed abundant amounts of IL-6 and IL-8 within the conditioned media of v-cyclin expressing cells, but much reduced levels in vector-control media (Figure 3.3B). v-Cyclin conditioned media contained between eighteen to seventy fold more IL-6 and IL-8 than did media from control cells. Collectively, these results demonstrate that v-cyclin induces the SASP and is a potent activator of OIS.

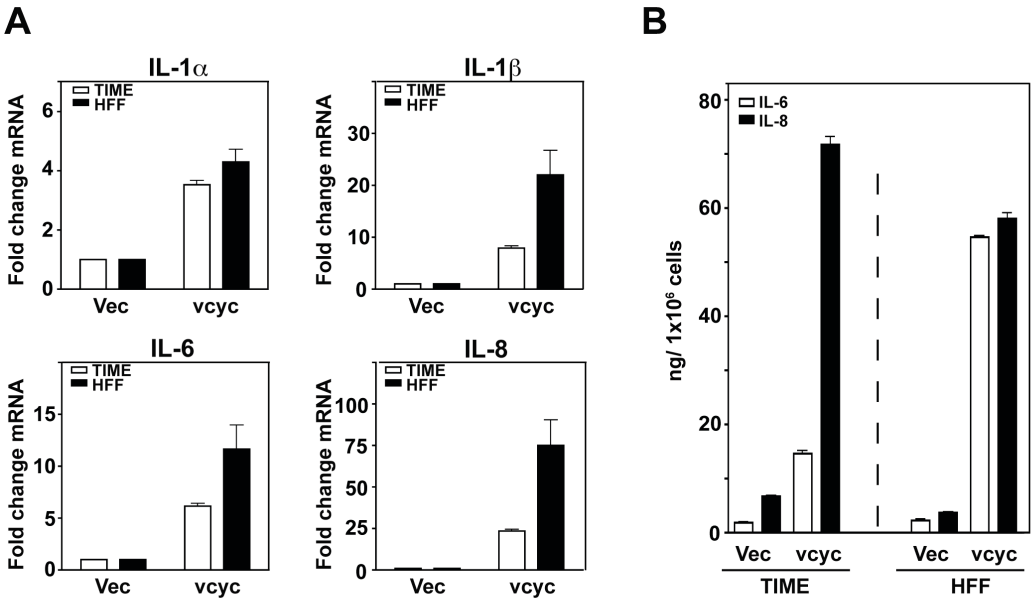

Figure 3.3
KSHV v-Cyclin Induces the SASP
(A) TIME cells or HFFs transduced with HA-v-cyclin retroviral vector or controls at 8 days post-selection were assessed for changes in mRNA levels for IL-1α, IL-β, IL-6 and IL-8 via Q-RT-PCR. Data were normalized to β-actin mRNA levels and analyzed using the $2^{(-\Delta\Delta CT)}$ method. Values are means ± SEM of two separate experiments. (B) Conditioned media from the populations in panel A were analyzed for the levels of IL-6 and IL-8 at eight days post-selection by ELISA. Cytokine levels were normalized using cell number. Values are means ± SEM of two separate experiments.

3.2.4 KSHV v-Cyclin is the Only Latent Protein to Induce Senescence

The unambiguous demonstration of v-cyclin OIS led to questions of whether other KSHV latent-gene products might also induce senescence. Certainly, KSHV latent infection is associated with the activation of host cell DDRs (Koopal et al., 2007). Is v-cyclin the only latent-gene product capable of inducing DDRs within latently infected cells? Functions of LANA in chromatin modulation, v-FLIP in the activation of NF-κB and the Kaposins in regulation of the non-canonical checkpoint kinase MK2 all point to possible roles in DDRs and senescence. To this end, I transduced HFFs, separately, with retroviral vectors encoding each of the KSHV latent proteins, or with empty vector controls, and assayed for SA β-gal activity, 53BP1 DNA-damage foci and SAHF at seven days post-selection. Importantly, I observed that v-cyclin was the only KSHV latent-gene product to induce hallmarks of senescence (Figure 3.4). None of the other KSHV latent-gene products had an impact on senescence markers. Therefore, it is likely that v-cyclin is the only KSHV latent-gene product responsible for the induction of DDRs within latently infected cells.

3.3 Discussion

The KSHV *v-cyclin* latency gene was first identified because of its significant homology to cellular genes encoding the D-type cyclins (Chang et al., 1996). Cyclin D1 and its homologues D2 and D3, have well-described roles in the development of numerous cancers, including melanomas, colorectal, bladder and breast cancers (Malumbres and Barbacid, 2009). This suggested that v-cyclin might also be a potent oncogene that contributes to KSHV-induced tumorigenesis. v-Cyclin characterization revealed that it binds cellular CDK6, and potently stimulates cell-cycle progression through phosphorylation of Rb-family members and components of the origin-recognition complex (Laman et al., 2001; Li et al., 1997). Furthermore, v-cyclin is refractory to the action of the Cip/Kip and INK4 families of CKIs (Swanton et al., 1997). However, despite these seemingly pro-oncogenic properties, v-cyclin robustly induces apoptosis and proliferation arrest upon expression in a variety of cell types, and does not induce

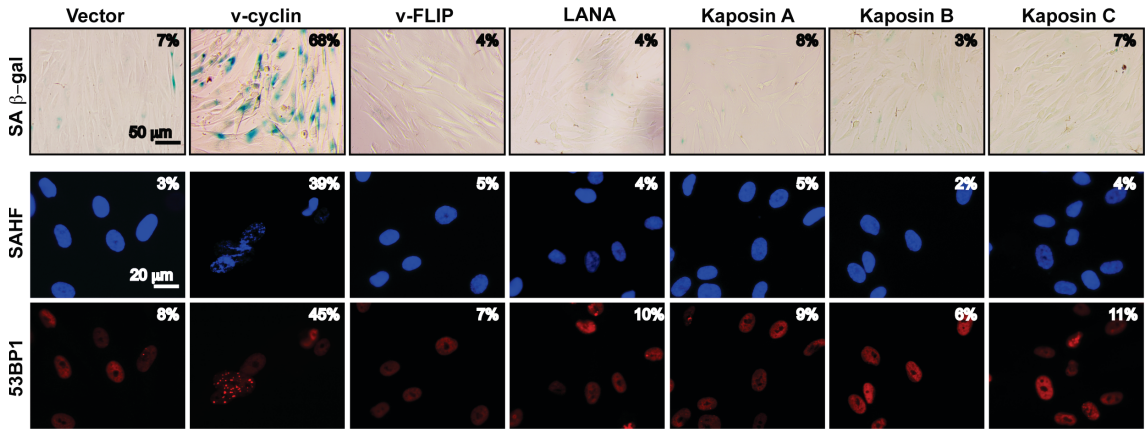

Figure 3.4

KSHV v-Cyclin is the Only Latent Protein to Induce Senescence

HFFs were transduced with retroviral vectors encoding KSHV latent proteins or controls and selected for two days with puromycin. At seven days post-selection, cells expressing KSHV latent proteins were assayed for SA β-gal activity, SAHF and 53BP1 foci. v-Cyclin was the only KSHV latent gene to activate DDRs and OIS markers. Representative images of this experiment are shown and the percentage of cells positive for the indicated markers is reported in the top right corner of each panel.

cellular transformation (Koopal et al., 2007; Ojala et al., 2000; Verschuren et al., 2002). My studies show that v-cyclin expression triggers hallmarks of OIS, including robust increases in SA β-gal activity, DDRs, SAHF and SASP components, suggesting that the proliferative functions of this cell-cycle subverter are kept in check by host-cell stress pathways.

Does KSHV v-cyclin induce senescence of latently infected cells? To date, there have been no reports of KSHV-induced senescence in infected cells. It is, however, well documented that latently infected cells display activated DDRs, and KS spindle cells exhibit only modest levels of proliferation relative to other tumor cells (Koopal et al., 2007; Verschuren et al., 2002). This suggests that v-cyclin-induced senescence is blocked or impaired during latency. Mechanistically, this might be achieved through the action of other latent-gene products, including LANA modulation of p53, Rb1 and chromatin (Friborg et al., 1999; Radkov et al., 2000), and v-FLIP regulation of NF-κB and autophagy (Chaudhary et al., 1999; Lee et al., 2009). Furthermore, it is important to consider that KSHV infection is not sufficient to induce KS, but rather is a necessary step that predisposes to tumorigenesis (Mesri et al., 2010). Therefore, stochastic inactivation of critical tumor-suppressor pathways may also contribute to impaired senescence with latently infected cells.

Why would the KSHV latency program encode a protein that robustly induces OIS? It is known that replication stress is a common feature of many cancer cells, and may be an important step in tumorigenesis. Activated oncogenes that induce stalling and collapse of replication forks promote DNA breaks that may contribute to the genomic instability that characterizes cancer (Halazonetis et al., 2008). Considering that OIS has not been observed in latently infected cells, it is possible that KSHV encodes v-cyclin to, intentionally promote tumorigenesis. The genesis of an immortal cell that could indefinitely carry and replicate the KSHV episome would be of some benefit. This scenario, however, seems unlikely considering that most KHSV-induced cancers demonstrate high clonality. This would suggest that genomic instability does not play a large role in KSHV-induced tumorigenesis.

Cells undergoing oncogene-induced replication stress also elaborate many pro-inflammatory cytokines as part of the SASP (Kuilman et al., 2008). Importantly, the SASP is largely induced by DDRs and remains active even under circumstances of senescence bypass (Coppe et al., 2008; Rodier et al., 2009; Young et al., 2009). Perhaps v-cyclin-induced replication stress contributes to lytic replication 'in *trans*' by promoting the expression of growth factors and pro-inflammatory cytokines from latently infected cells. In fact, many of the growth factors and cytokines secreted from infected cells overlap with components of the SASP, including IL-6, IL-8 and VEGF (Bais et al., 1998; Schwarz and Murphy, 2001; Shepard et al., 2001; Sodhi et al., 2000). Nevertheless, we still remain deeply in the dark about the functions of v-cyclin within the context of KSHV infection.

How is cell-cycle arrest and senescence achieved by v-cyclin-expressing cells? The very properties that enable v-cyclin to phosphorylate Rb-family proteins and avoid the action of CKIs, including p21, p27 and INK4a (Li et al., 1997; Swanton et al., 1997), should also render it an unstoppable activator of the cell cycle. However, this is not the case: cells expressing v-cyclin efficiently and stably arrest, no different from senescence responses elicited by other oncogenes (Koopal et al., 2007; Verschuren et al., 2002). This suggests that inherent mechanisms within the v-cyclin senescence program attenuate its functions. In fact, negative feedback signaling is an essential feature of all senescence programs (Bardeesy and Sharpless, 2006; Courtois-Cox et al., 2006; Courtois-Cox et al., 2008). For example, Ras-induced senescence involves sharp attenuation of Ras signaling through suppression of Ras guanine-nucleotide exchange factors (RasGEFs), activation of Ras-GTPase activating proteins (RasGAPs) and expression of Sprouty (Courtois-Cox et al., 2006). How is v-cyclin function suppressed? The most likely mechanism involves a combination of inhibition of CDK6 kinase activity and down-regulation of v-cyclin targets that promote cell-cycle progression.

The activity of CDK6 is controlled, in part, by interaction with its cognate cyclins, but also by phosphorylation by the CDK-activating kinase (CAK). Intriguingly, v-cyclin has evolved to activate CDK6 without requirement of CAK phosphorylation (Kaldis et

al., 2001). Therefore, regulation of CDK6 by CAK is not likely to be involved with v-cyclin-induced senescence. However, it has been recently reported that CDK6 is targeted for down-regulation by a number of distinct miRNAs up-regulated by oncogenic stress, including miRNA-22 and miRNA-34 (He et al., 2007; Xu et al., 2011). Intriguingly, miRNA-34 is a p53 target gene and miRNA-22 overexpression induces senescence. Furthermore, my preliminary data suggest CDK6 is down-regulated during v-cyclin OIS (data not shown). Another mechanism that likely contributes to attenuation of v-cyclin function is the down-regulation of v-cyclin /CDK6 targets. Numerous proteins important for cell-cycle progression are repressed or degraded upon activation of DDR, including v-cyclin targets Cdc6 and Orc1 (Avni et al., 2003; Duursma and Agami, 2005; Vaziri et al., 2003). Finally, emerging evidence points to a role for autophagic degradation in proliferation arrest (Young et al., 2009), and is discussed with regard to v-cyclin OIS in the next chapter.

CHAPTER 4 - AUTOPHAGY IS NECESSARY FOR V-CYCLIN-INDUCED SENESCENCE

Note: The majority of the research described in this chapter has been published in Cell Host and Microbe as: Leidal, A. M., Cyr, D. P., Hill, R. J., Lee, P. W., and McCormick, C. (2012). Subversion of Autophagy by Kaposi's Sarcoma-Associated Herpesvirus Impairs Oncogene-Induced Senescence. *Cell Host Microbe* 11, 167-180. © Cell Press. Figures appearing in this chapter that are wholly or partially reproduced from figures appearing in that paper include Figures 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, and 4.9.

4.1 Introduction

Recently, autophagy has been shown to be an effector mechanism of oncogene-induced senescence (Young et al., 2009). Elegant work by Young *et al.* demonstrated that oncogenic *Ras* triggers robust increases in autophagic flux within cells as they transition from hyper-proliferation to the senescent state. In that system, activation of autophagy was correlated with negative feedback on the mTOR pathway and activation of key autophagy genes Ulk1, LC3A, LC3B, ATG5 and ATG7. Remarkably, autophagy was also demonstrated to be necessary for senescence, since cells deficient for the essential autophagy proteins failed to efficiently undergo senescence (Young et al., 2009). Examination of Ras-expressing autophagy-deficient cells revealed that syntheses of SASP cytokines IL6 and IL8 were compromised. Prior work has shown that IL6 and IL8 are necessary for OIS. Therefore, it is speculated that impaired cytokine production by autophagy-defective cells is responsible for their failure to senesce efficiently in response to Ras-induced oncogenic stress.

Similarities between the cellular responses to v-cyclin and oncogenic Ras suggest that autophagy may also regulate v-cyclin OIS. This possibility is further supported by the fact that Bcl-2, a critical repressor of the autophagic machinery (Pattingre et al., 2005), is a reported target of v-cyclin/CDK6. In fact, v-cyclin/CDK6 has been directly implicated in the phosphorylation of Bcl-2 residues that control its association with Beclin 1 (Ojala et al., 2000; Wei et al., 2008). In those studies, Bcl-2 phsophorylation by

v-cyclin /CDK6 was found to promote apoptosis, but autophagy was not explored. These intriguing links between v-cyclin and the autophagy suggest that autophagic degradation may be an important effector mechanism for v-cyclin OIS. Therefore, I attempted to determine the status of the autophagy pathway during v-cyclin OIS and assess its contributions to senescence induction.

4.2 Results

4.2.1 Autophagic Activity Increases during v-Cyclin OIS

To determine whether autophagy is involved in v-cyclin OIS, I transduced TIME cells with vectors encoding HA-v-cyclin or HA-HRas^{V12}, or empty vector controls, and lysates were collected and immunoblotted for markers of autophagy and senescence. Changes in autophagy were assessed by examining the status of the LC3 protein, a commonly used autophagy marker (Mizushima et al., 2010). During autophagosome biogenesis phosphatidylethanolamine (PE) is conjugated to LC3 to facilitate its insertion into maturing autophagosomal membranes. When resolved by denaturing polyacrylamide-gel electrophoresis, the unlipidated LC3 (LC3-I) migrates more slowly is PE-conjugated LC3 (LC3-II). Therefore, the relative levels of LC3-II are reflective of the amount of mature autophagosomal membrane within the cell. Significantly, when I immunoblotted lysate derived from v-cyclin and HRas^{V12} cells, a sharp increases in LC3-II and the senescence marker HMGA2 were observed after 5 days of oncogene expression (Figure 4.1A). By contrast, empty-vector transduced control cells failed to accumulate either LC3-II or HMGA2. Because LC3-II is localized to autophagosomes it is also subject to autophagic degradation and recycling. Thus, increases in LC3-II levels during v-cyclin-induced senescence could either reflect an increase in autophagic flux or an inhibition at the late stages of autophagy, thereby preventing the maturation or function of autolysosomes and, in turn, preventing LC3-II degradation. To distinguish between autophagy activation or inhibition, v-cyclin senescent cells were treated with bafilomycin, a specific inhibitor of lysosomal acidification that impairs autophagic degradation. Similar to observations from

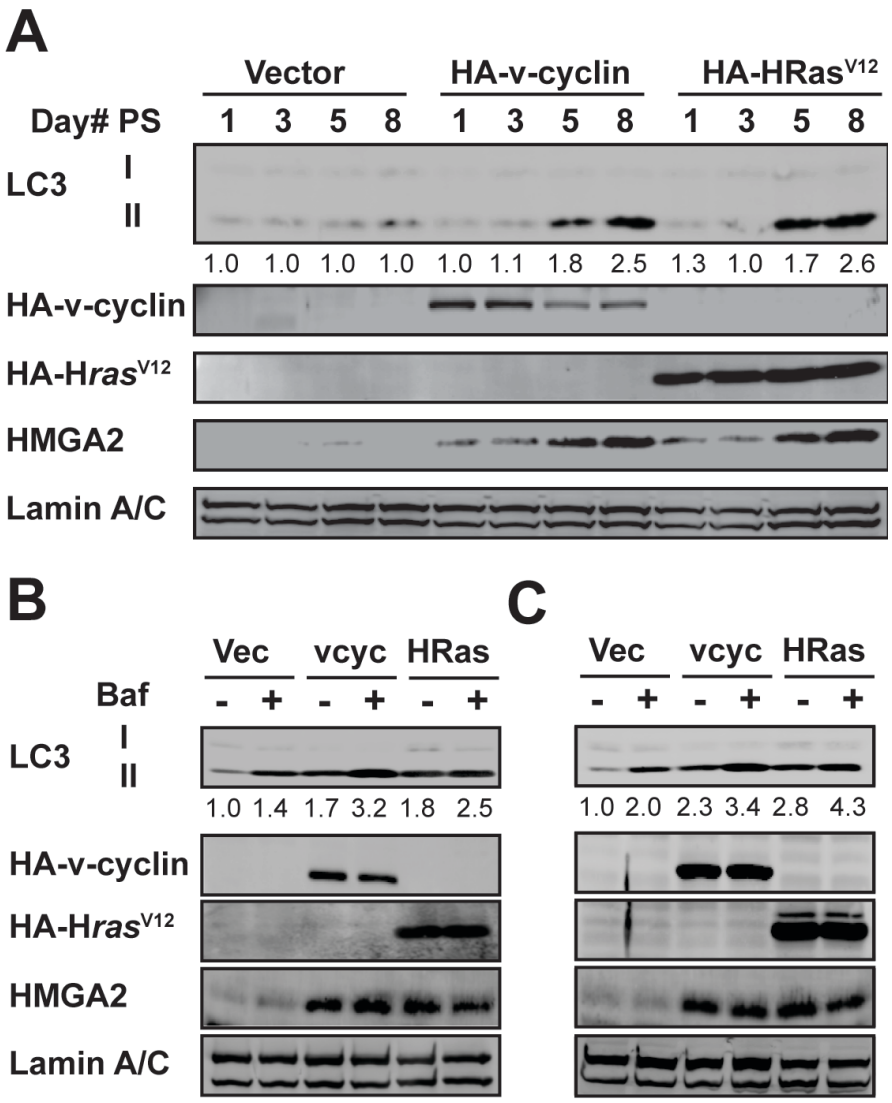

Figure 4.1
KSHV v-Cyclin Induces LC3-II Accumulation During Senescence
(A) Immunoblot analysis of LC3 from TIME cells expressing HA-v-cyclin, HA-HRas^{V12} or vector at various days post-selection; accumulation of HMGA2 was used as a marker of senescence and Lamin A/C served as a loading control. To quantify relative levels of LC3-II, band intensities were normalized to levels of Lamin A/C in each lane and then expressed relative to vector control LC3-II levels. (B) and (C) Immunoblot analysis of LC3 derived from vector control (Vec) cells or senescent HA-v-cyclin (vcyc) and HA-HRas^{V12} (HRas) TIME cells and HFFs, respectively, five days post-selection in the absence or presence of 100 nM Bafilomycin (Baf) for 3 hours prior to lysis. LC3-II levels were quantified as above.

Ras senescent cells, bafilomycin treatment of v-cyclin senescent cells results in accumulation of LC3-II relative to v-cyclin senescent cells treated with vehicle control (Figures 4.1B and 4.1C), thereby confirming that v-cyclin activates autophagy. Furthermore, autophagy induction during senescence was independent of the cell lines employed, since immortal TIME cells and HFFs with limited replicative potential demonstrated similar levels of activation (Figures 4.1B and 4.1C).

To further assess whether autophagy is activated during v-cyclin OIS, I examined the cytoplasm of senescent and proliferating cells for the presence of autophagosomes and autolysosomes, using a transmission electron microscope (TEM). Autophagic structures are easily observed by TEM since they are thought to be the only double-membrane (or more) vesicles within the cytoplasm of eukaryotic cells (Yla-Anttila et al., 2009a). Furthermore, mature autolysosomes are distinguished from autophagosomes by their distinctive electron-dense staining. My studies revealed that v-cyclin cells and Ras senescent cells were replete with autophagosomes and autolysosomes, whereas proliferating empty-vector cells had comparatively few autophagosomes (Figure 4.2A). v-Cyclin cells and Ras senescent cells were found to have approximately 3-fold the number of autophagic structures, inclusive of autophagosomes and autolysosomes, relative to non-senescent empty-vector controls (Figures 4.2B and 4.2C).

Another commonly used technique to estimate relative autophagic flux involves ectopic expression of fluorescent LC3 fusion proteins that upon autophagy induction re-localize to autophagosomes and autolysosomes (Mizushima et al., 2010). I employed a dual mCherry and EGFP fluorescent LC3 fusion protein (mCherry-EGFP-LC3) to determine the abundance of autophagosomes and autolysosomes within v-cyclin senescent cells and controls (Figure 4.3A). This fusion protein allows specific discrimination of autophagosomes (yellow) from mature autolysosomes (red) in which EGFP fluorescence had been quenched in the acidic microenvironment (Pankiv et al., 2007). In this system, v-cyclin expression promoted re-localization of diffuse cytoplasmic mCherry-EGFP-LC3 reporter proteins into discrete cytoplasmic puncta that marked the location of autophagosomes. Similar to what was seen for Ras senescent cells, I observed

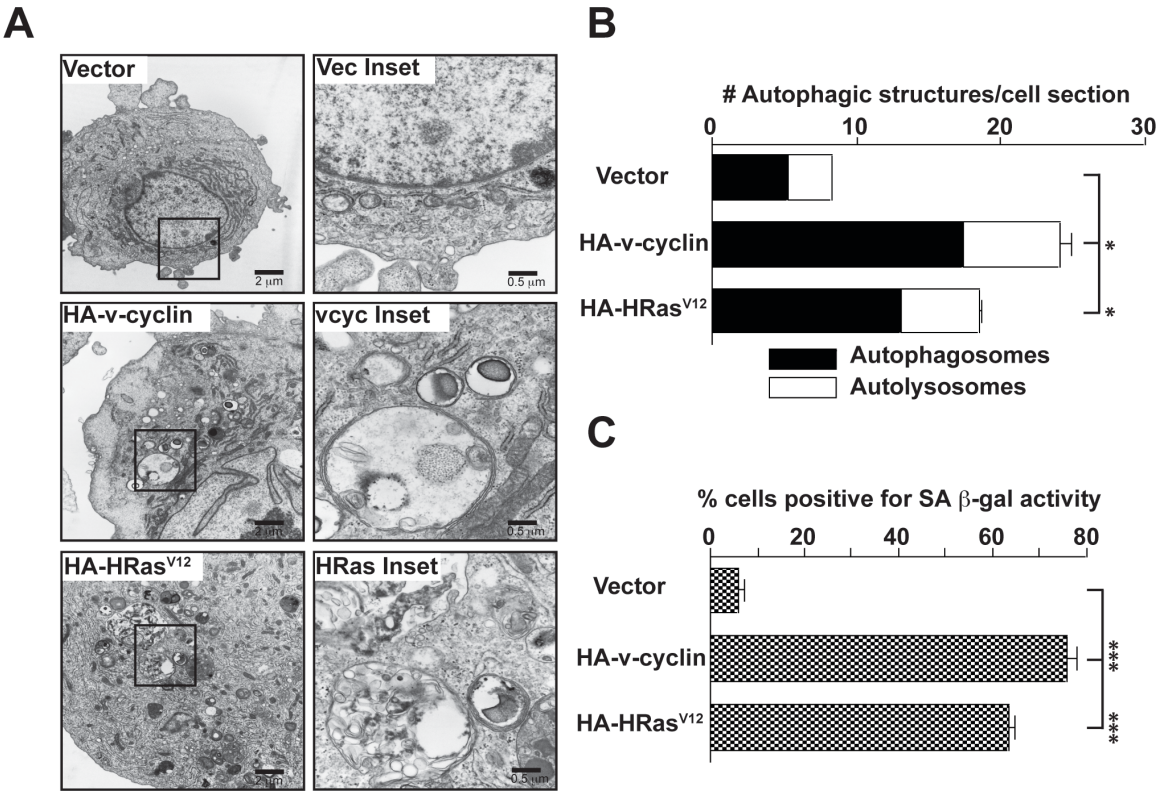

Figure 4.2
KSHV v-Cyclin Senescent Cells are Replete with Autophagosomes
(A) Electron microscopy (EM) of senescent HA-v-cyclin and HA-HRas^{V12} TIME cells or proliferating controls seven days post-selection. (B) Quantification of autophagic structures per cell section in panel B. (C) SA β-gal activity within the populations used for EM analysis. Values are means ± SEM of two independent experiments. * $P < 0.05$ and *** $P < 0.005$ for the indicated parameters analyzed.

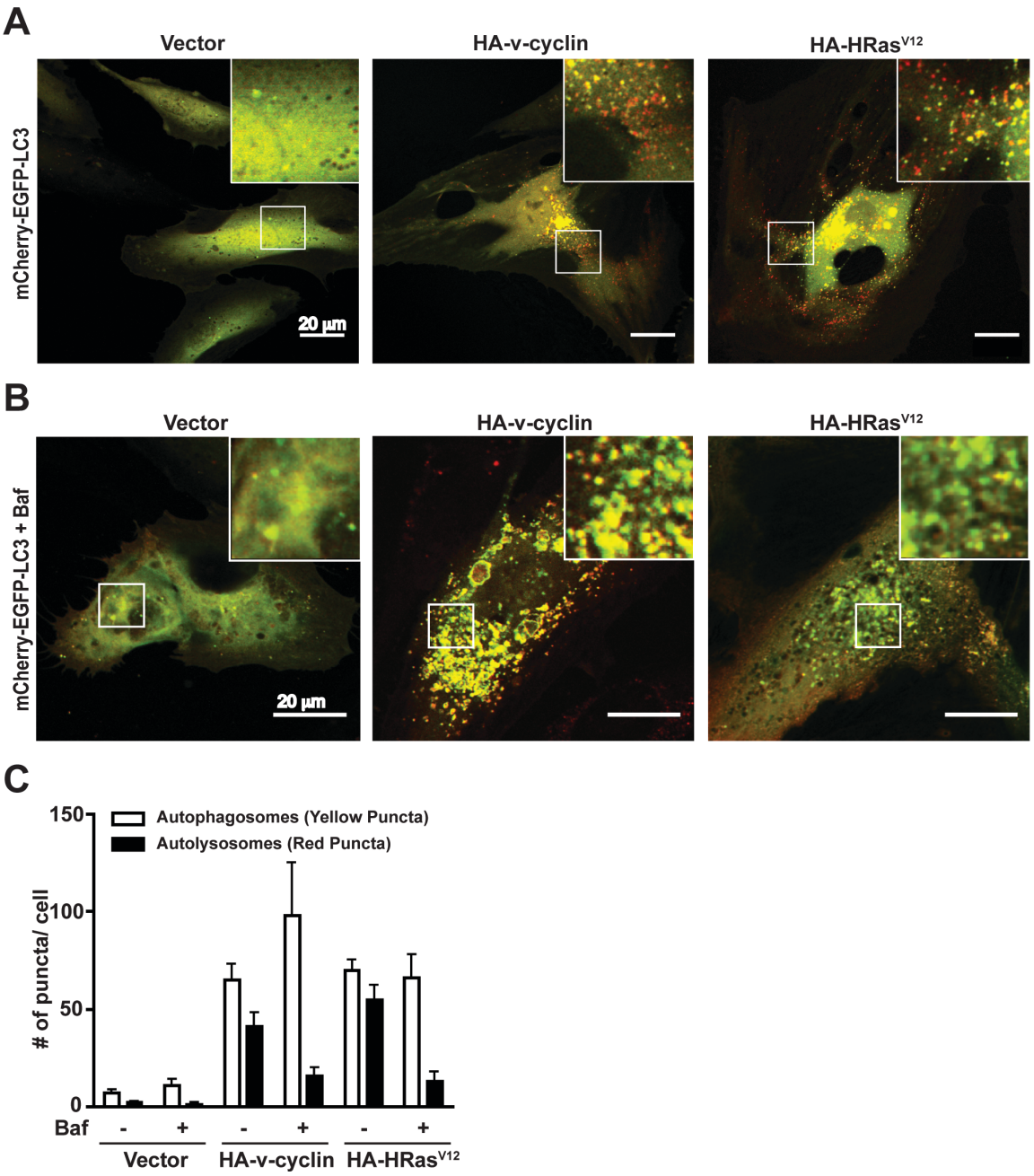

Figure 4.3
KSHV v-Cyclin Senescent Sells Display Abundant Autophagic Puncta
(A) Confocal images of senescent HA-v-cyclin and HA-HRas^{V12} TIME cells or controls transfected with mCherry-EGFP-LC3 for the visualization of autophagosomes (yellow puncta) and autolysosomes (red puncta). (B) Confocal images of senescent HA-v-cyclin and HA-HRas^{V12} TIME cells or controls in the presence of 100 nM bafilomycin, transfected with mCherry-EGFP-LC3 for the visualization of autophagosomes (yellow puncta) and autolysosomes (red puncta). (C) Yellow and red puncta were enumerated from representative images. Values in panels C are ± SEM of three independent experiments.

a significant proportion of mature autolysosomes in v-cyclin cells, and further accumulation of autolysosomes in bafilomycin-treated cells, indicating an increased autophagic flux (Figures 4.3A and 4.3B). By contrast, the mCherry-EGFP-LC3 reporter exhibited diffuse cytoplasmic staining in proliferating control cells. Quantification of yellow autophagic and red autolysosome puncta within senescent and proliferating cells further reinforced compelling images, showing dramatically increased numbers of autophagic structures during senescence (Figures 4.3C). Taken together, these findings confirm that autophagy is activated during v-cyclin-induced senescence.

4.2.2 mTOR Signaling is Inhibited During v-Cyclin OIS

To determine the status of signaling pathways that may contribute to autophagy activation in v-cyclin-expressing cells, I developed a tetracycline-responsive expression system in TIME cells. These cells induce HA-tagged v-cyclin expression upon removal of the tetracycline analog doxycycline. Following induction of v-cyclin expression, the percentage of proliferating and senescent cells over an 8-day period was assessed via BrdU incorporation and SA β-gal staining, respectively. In this system, v-cyclin induced a modest increase in the percentage of cells that incorporated BrdU after 1 day of expression, followed by a sharp decrease over the remainder of the experiment (Figure 4.4). SA β-gal activity after induction of v-cyclin showed a reciprocal pattern to that of BrdU incorporation, with few positive cells prior to day-3 and a robust increase in days following. These results suggested that v-cyclin, like other senescence-inducing oncogenes, initially promotes a period of hyper-proliferation followed by transition into senescence. Immunoblots of lysates derived from this time course show that v-cyclin rapidly promoted accumulation of γH2AX after only one day of expression (Figure 4.4). Approximately two-to-three days after v-cyclin induction, LC3-II and HMGA2 increased synchronously and persisted throughout the remainder of the experiment. The levels of cyclin A, a marker of cell proliferation, decreased dramatically after induction of v-cyclin and were undetectable by day 3. Furthermore, I found a negligible increase in the levels of INK4a during v-cyclin-induced senescence in the inducible TIME cells, consistent with previous observations employing a retroviral ectopic expression system (Figure 4.4).

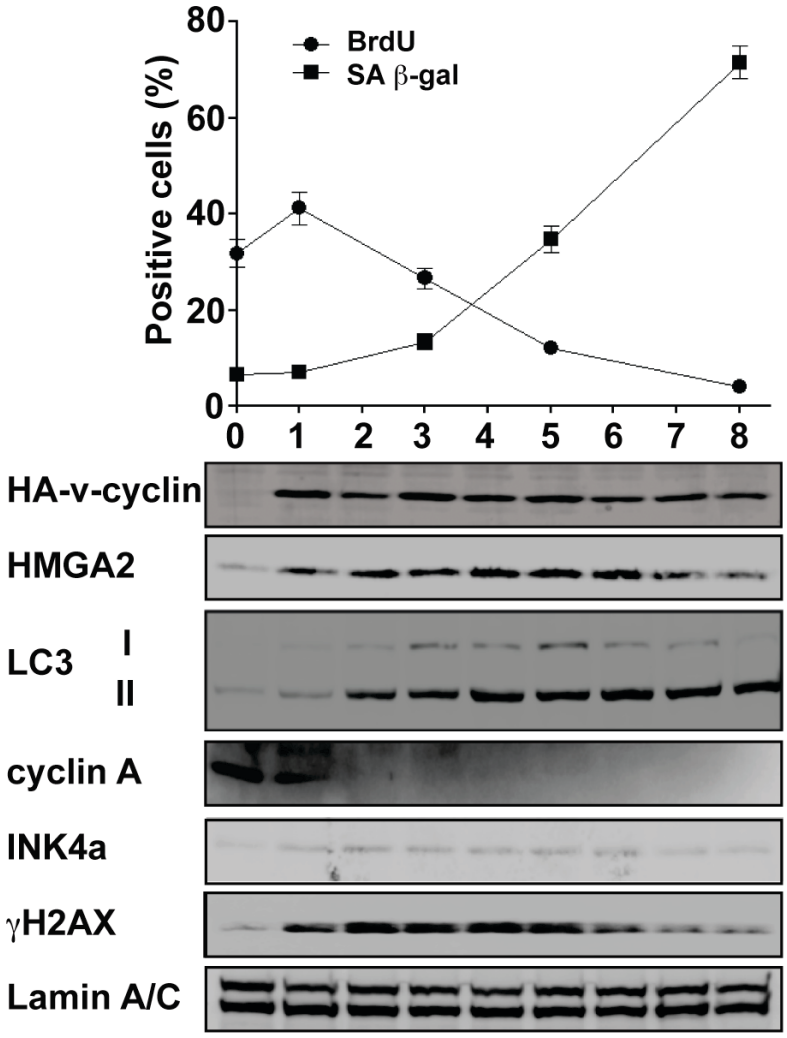

Figure 4.4
Development of an KSHV v-Cyclin Inducible Cell Line
Clonal Tet-off HA-v-cyclin inducible TIME cells were assayed for BrdU incorporation, SA β-gal activity and protein expression at various days after removal of doxycycline. Values are means ± SEM of three separate fields (~100 cells).

Together, these results suggest that the inducible v-cyclin expression system I developed faithfully recapitulates features of v-cyclin OIS, including triggering DNA damage and autophagy prior to the full onset of senescence.

Activation of autophagy during Ras-induced senescence correlates with negative feedback on mTOR signaling (Young et al., 2009). Although the cellular genes that function in v-cyclin-induced senescence remain largely undefined, p53 is strongly activated in cells expressing v-cyclin and is necessary for proliferation arrest (Koopal et al., 2007; Verschuren et al., 2002). Intriguingly, p53 is known to regulate the expression of several genes that have been linked to autophagy and OIS, including Damage-Regulated Autophagy Modulator (DRAM), Sestrin1 and Sestrin2 (Budanov and Karin, 2008; Crighton et al., 2006). To determine if DRAM, Sestrin1 or Sestrin2 is activated during v-cyclin senescence, I conducted quantitative real-time PCR (qRT-PCR) on cellular mRNAs expressed in v-cyclin senescent cells. I observed that v-cyclin senescent cells strongly up-regulate DRAM and Sestrin1 expression, whereas Ras senescent cells display increases in both Sestrin 1 and Sestrin 2 (Figure 4.5A). Importantly, cells subjected to serum and amino acid starvation activated autophagy (Figures 4.5B and 4.5C) but failed to up-regulate DRAM, Sestrin 1 or Sestrin 2. This indicates that up-regulation of DRAM, Sestrin 1 or Sestrin 2 is not a universal feature of autophagy, but instead may be unique to OIS. Importantly, I also observed potent up-regulation of p21 and HMGA2 and concomitant down-regulation of cyclin A in v-cyclin cells and Ras cells, confirming the activation of p53 and OIS. In agreement with previous experiments, I found that INK4a was exclusively up-regulated in Ras senescent cells and not v-cyclin senescent TIME cells (Figure 4.5A). Furthermore, this experiment confirmed that autophagy is a general feature of OIS; both v-cyclin and Ras up-regulated the autophagy genes ULK1, ULK3, ATG7, LC3A and LC3B (Figure 4.5A). Collectively, these findings indicate that v-cyclin triggers the induction of a discrete set of autophagy-regulating genes that set the stage for OIS.

The up-regulation of DRAM and Sestrin1 during v-cyclin OIS suggests these proteins may contribute to the activation of autophagy. Although DNA damage can trigger autophagy through p53-dependent expression of DRAM, the precise function of this lysosomal protein remains unknown (Crighton et al., 2006). By contrast, Sestrins

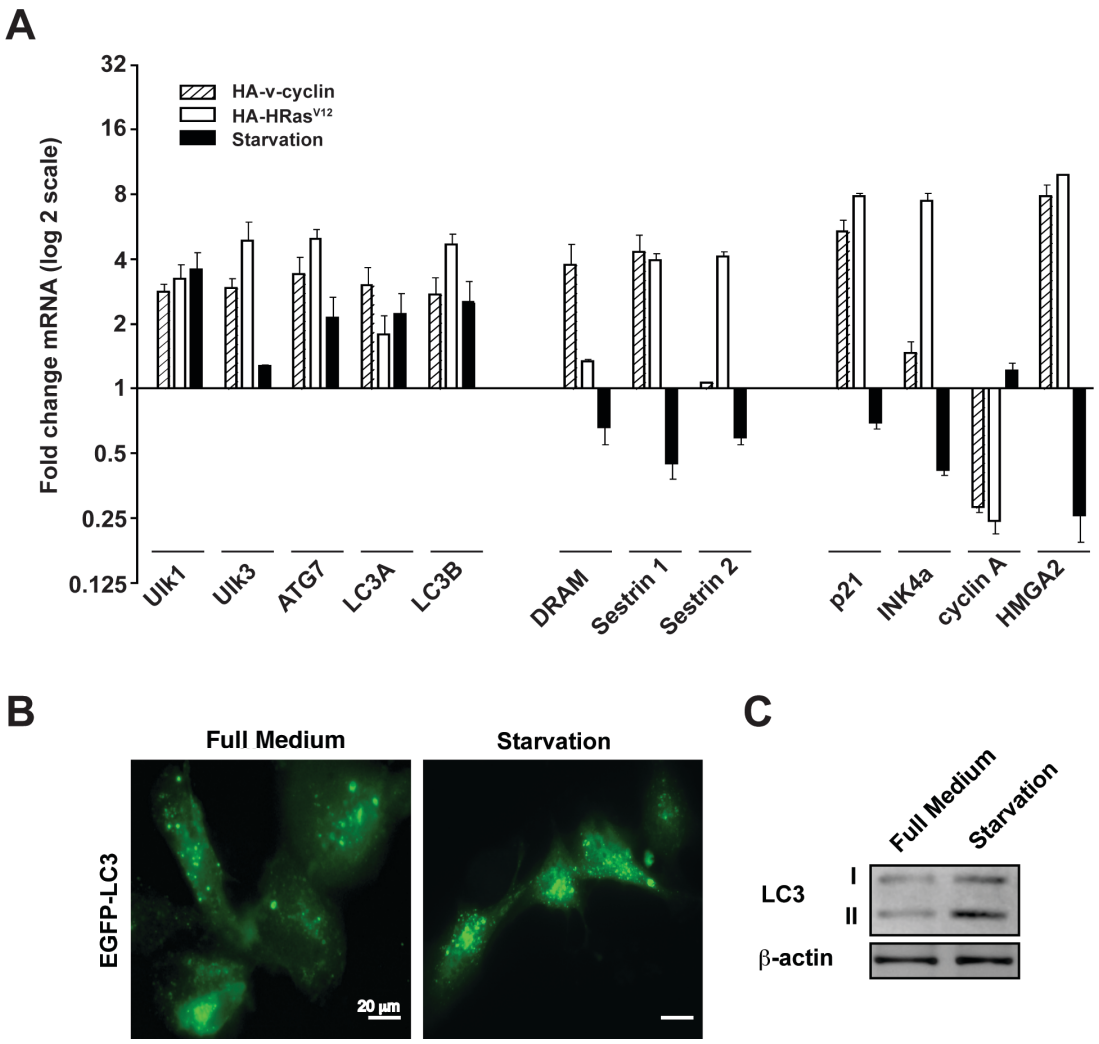

Figure 4.5
Transcripts of Autophagy and mTOR Regulators Increase During OIS
(A) HFFs transduced with the indicated retroviral vectors seven days post-selection, or starved in EBSS for 4 hours, were assessed for changes in mRNA levels by Q-RT-PCR. Data were normalized to actin values and analyzed using the $2^{(-\Delta\Delta CT)}$ method. Values are means ± SEM of three separate experiments. (B) To demonstrate the activation of autophagy under starvation conditions, HFFs transfected with a vector encoding EGFP-LC3 were either left untreated in full medium or starved for 4 hours in EBSS, fixed and examined using fluorescence microscopy for autophagosomes (green puncta). (C) Lysate derived from cells in full medium or starved for 1 hour in EBSS were blotted for LC3.

activate autophagy by AMPK, which phosphorylates the TSC2 subunit of TSC1:TSC2 complexes (Budanov and Karin, 2008; Maiuri et al., 2009a). TSC2 functions as a GAP for the small GTPase Rheb, a direct activator of mTOR (Inoki et al., 2003). Thus, Sestrin-directed AMPK phosphorylation of TSC2 stimulates its GAP activity, decreasing Rheb-GTP levels and inhibiting mTOR signaling. Subsequently, AMPK phosphorylates the ULK complex to trigger autophagy initiation directly (Kim et al., 2011; Egan et al., 2011). To determine the involvement of this signaling cascade in v-cyclin-triggered autophagy and OIS, I immunoblotted protein lysates from a five-day expression time course. I observed early activation of p53, up-regulation of DRAM and Sestrin1, and AMPK Thr172 phosphorylation (Figure 4.6). By day three, mTOR was inactivated, as measured by loss of Ser2448 phosphorylation and simultaneous loss of phosphorylation of mTOR target proteins S6K and 4EBP1. Although these events suggest a role for Sestrin1 in inhibiting mTOR by AMPK, I also observed a loss of Akt Thr308 and Ser473 phosphorylation, suggesting that redundant mechanisms may facilitate mTOR inhibition. Furthermore, the loss of mTOR activity correlated with strong accumulation of LC3-II and HMGA2. Experiments performed on an additional clonal isolate of inducible TIME v-cyclin cells confirmed these findings (Figure 4.7A). By contrast, Ras-inducible cells up-regulated Sestrin 1 and Sestrin 2 protein expression during senescence, but failed to up-regulate DRAM, suggesting that these oncogenes may inactivate mTOR by similar but distinct mechanisms (Figure 4.7B). Together, these data strongly suggest that v-cyclin triggers autophagy by activating a DNA-damage-sensing pathway that results in mTOR inhibition.

v-cyclin-CDK6 complexes phosphorylate a broad range of host proteins, including Bcl-2 (Ojala et al., 1999), a well-described anti-apoptotic protein that also has import roles in repression of autophagy (Pattingre et al., 2005). Phosphorylation of Bcl-2 relieves its repressive effect on Beclin-1, allowing the formation of a class III PI3K complex that is necessary to nucleate autophagosomal membranes (Wei et al., 2008). Surprisingly, I was unable to detect p-Ser70 Bcl-2 in v-cyclin cells. By contrast, control docetaxel-treated cells displayed a marked increase in p-Ser70 Bcl-2 levels (Figure 4.6). The absence of detectable Bcl-2 phosphorylation, coupled with pronounced loss of mTOR activity during the period of autophagy activation, suggest that autophagy is

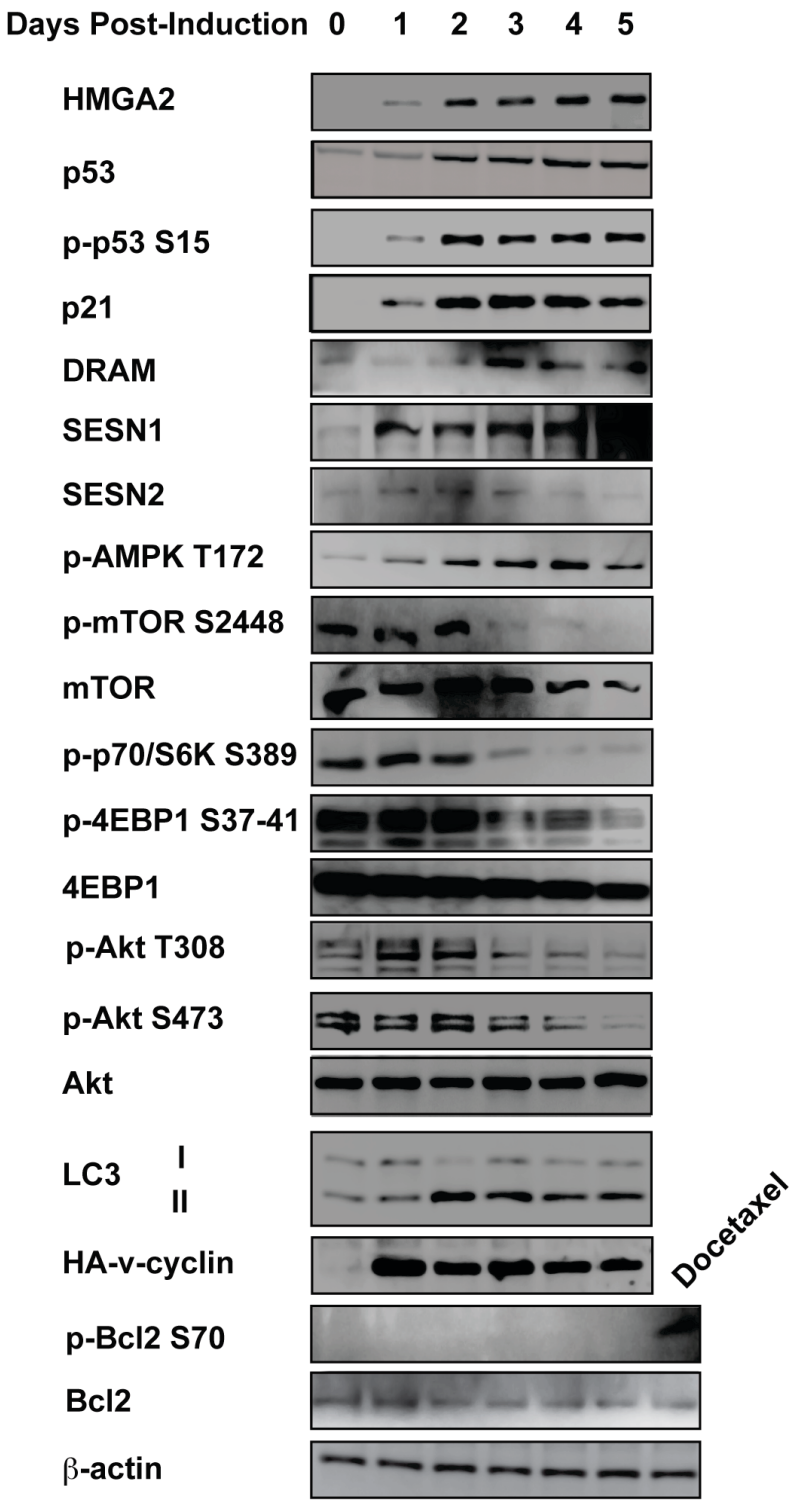

Figure 4.6
Negative Feedback on mTOR Signaling During v-Cyclin OIS
Immunoblotting for mTOR-pathway proteins and downstream effectors in lysates derived from v-cyclin-inducible TIME cells at various days after removal of doxycycline.

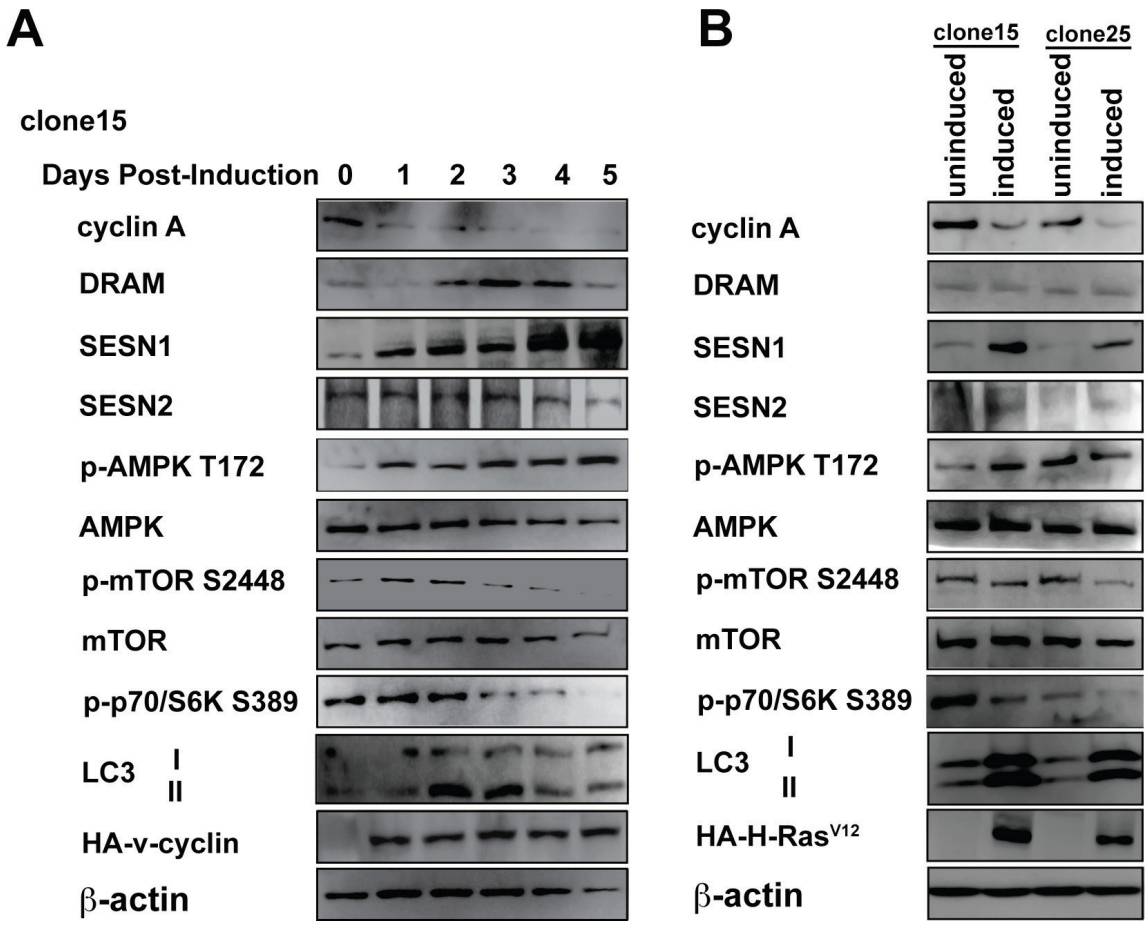

Figure 4.7
mTOR Inhibition is a General Feature of OIS
(A) A time course using an alternate HA-v-cyclin-inducible clone (#15) was conducted and lysates derived from cells at various days after removal of doxycycline were immunoblotted for mTOR-pathway proteins. (B) Lysates derived from two separate HA-HRas^{V12}-inducible clones (#15 and #25) left uninduced or induced for five days were probed for mTOR-pathway proteins.

triggered in v-cyclin-senescing TIME cells by negative feedback on mTOR signaling, and not likely through derepression of Beclin 1.

4.2.3 Autophagy is Necessary for v-Cyclin OIS

To determine whether autophagy is required for efficient execution of v-cyclin-induced senescence, I transduced TERT-HFFs with shRNAs targeting the essential autophagy genes ATG5 and ATG7, as well as the senescence-regulating checkpoint genes p53 and Rb1. In accord with previous reports, v-cyclin-induced senescence was found to be p53-dependent (Figures 4.8A, 4.8B and 4.8D) (Koopal et al., 2007; Verschuren et al., 2002); p53-deficient TERT-HFFs expressing v-cyclin showed dramatically reduced SA β-gal activity and increased BrdU incorporation. Surprisingly, Rb1 deficiency had only a modest impact on SA β-gal activity and BrdU incorporation. Importantly, shRNAs targeting ATG5 or ATG7 strongly diminished SA β-gal activity and increased BrdU incorporation. This demonstrated that autophagic function is required for efficient acquisition of the senescent phenotype. To further address the requirement for p53 and autophagy in v-cyclin OIS, I carried out a colony formation assay. v-Cyclin-expressing cells deficient for p53, Rb1, ATG5 or ATG7, or controls, were permitted to reach senescence over one week and then were seeded at low density and maintained for two additional weeks with only medium refresh, after which colonies, indicative of continued proliferation and delayed or bypassed senescence, were fixed and stained with crystal violet. Remarkably, shRNAs targeting p53 and ATG7 produced dramatic increases in the number of colonies relative to scrambled shRNA control expressing cells (Figure 4.8C); in contrast, shRNAs targeting ATG5 or Rb1 only resulted in modest increases in the number of v-cyclin cells that continued to proliferate (Figure 4.8C). Taken together, these results indicate that the functions of p53 and autophagy are required for the efficient establishment of OIS in response to v-cyclin expression.

Activation of autophagy during Ras-induced senescence is important for the synthesis of IL-6 and IL-8 components of the SASP (Young et al., 2009; Narita et al., 2011). Young *et al.* reasoned that defects in IL-6 and IL-8 production might account for

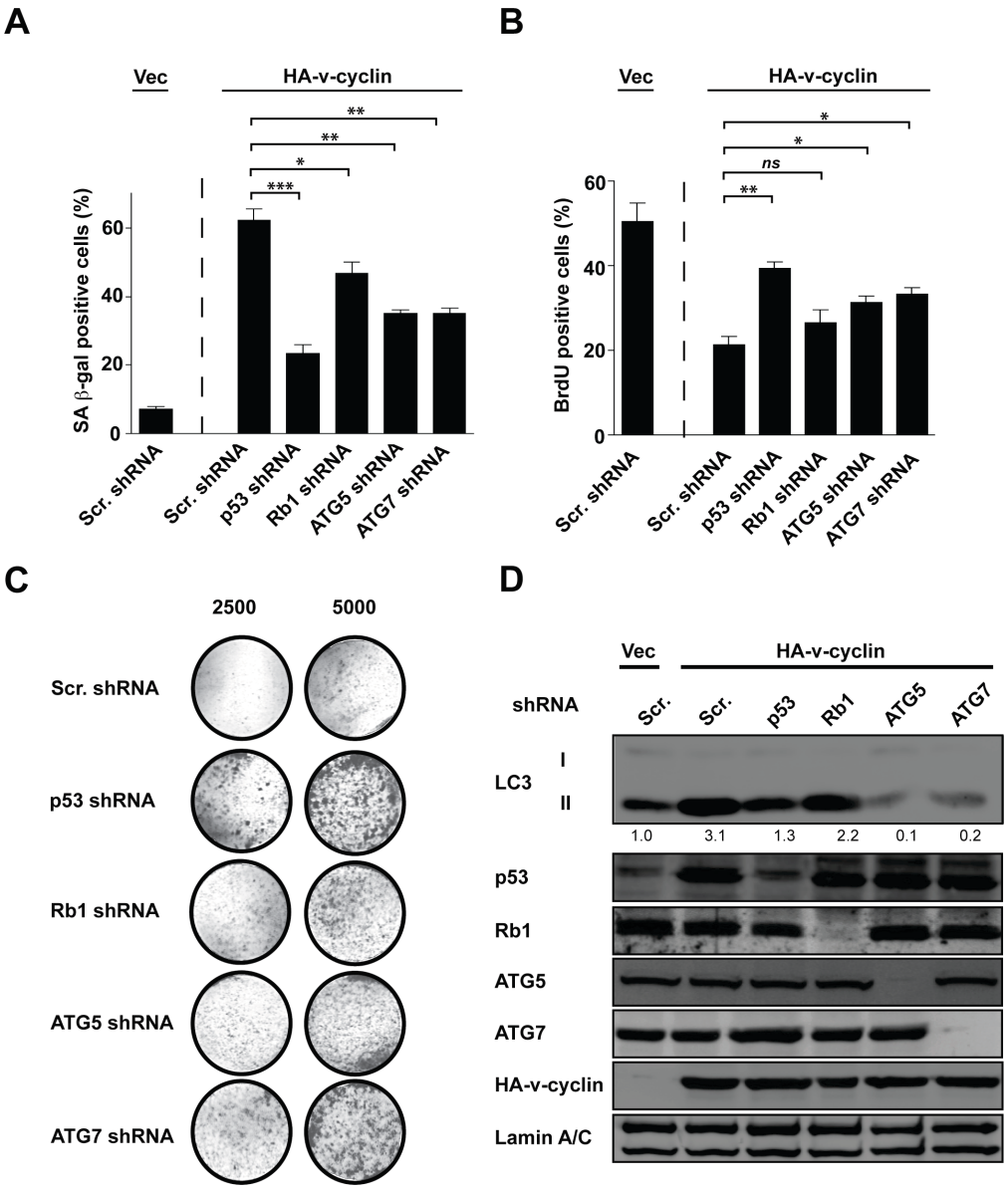

Figure 4.8
Autophagy and p53 are Necessary for v-Cyclin OIS
TERT-HFFs were transduced with retroviral vectors and selected in neomycin for seven days. Subsequently, knockdown cells and scrambled controls (Scr.) were transduced with HA-v-cyclin or vector (Vec) and selected in puromycin. At 4 days post-selection, transduced cells were either assayed for (A) SA β-gal activity and (B) BrdU incorporation. Values are means ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$ for the indicated parameters analyzed. (C) TERT-HFF knockdown cells transduced with HA-v-cyclin were analyzed for colony formation. At eight days post-selection, cells were seeded at the indicated densities and after two weeks were stained with crystal violet to visualize clonal outgrowth. (D) Immunoblots demonstrating silencing of targets from panels A and B, as well as relative LC3 II levels, which were quantified as in Figure 4.1.

the impaired OIS observed in autophagy-defective cells. Accumulating evidence suggests that autophagy has important roles in both the mobilization of amino acids and the secretion of numerous soluble factors. To determine if autophagy regulates the SASP during v-cyclin-induced senescence, I assayed for IL-6 and IL-8 within the conditioned media of autophagy-impaired or competent cells expressing v-cyclin over a 6-day time course. Similar to what was seen in previous experiments, autophagy was attenuated by expressing shRNAs targeting ATG5 or ATG7. Remarkably, v-cyclin-expressing cells deficient for ATG5 or ATG7 showed delayed accumulation of IL-6 and IL-8 relative to controls (Figure 4.9A and 4.9B). Furthermore, v-cyclin was required for the induction of IL-6 and IL-8. Collectively, these data demonstrate that autophagy is necessary for the efficient production of IL-6 and IL-8 during v-cyclin OIS.

4.3 Discussion

Autophagy has recently been demonstrated to be an important effector mechanism of Ras-induced senescence (Young et al., 2009). However, it remains unclear whether Ras-induced autophagy and senescence are a unique circumstance or a universal paradigm of OIS. In my study, I characterized v-cyclin as a viral oncogene that, similar to Ras, induces autophagy and senescence. v-Cyclin potently induced markers of autophagy, such as lipidation and accretion of LC3-II, during transition to the senescent phenotype. Furthermore, induction of autophagy in v-cyclin senescent cells was correlated with the up-regulation of p53-target genes DRAM and Sestrin 1, and negative feedback on mTOR signaling. Finally, I observed that autophagy is required for v-cyclin-induced senescence and is an important regulator of the v-cyclin SASP. Together, these observations strongly suggest that activation of autophagy is not unique to Ras-induced senescence, and that autophagy performs important common functions that help cells transition to the senescence phenotype. Furthermore, v-cyclin-triggered DDRs, autophagy and senescence highlight the complex integrated networks that facilitate cellular adaptations to stress.

How is autophagy triggered during OIS? In an attempt to understand how autophagy is induced during v-cyclin OIS, it is first useful to consider the precise stimuli

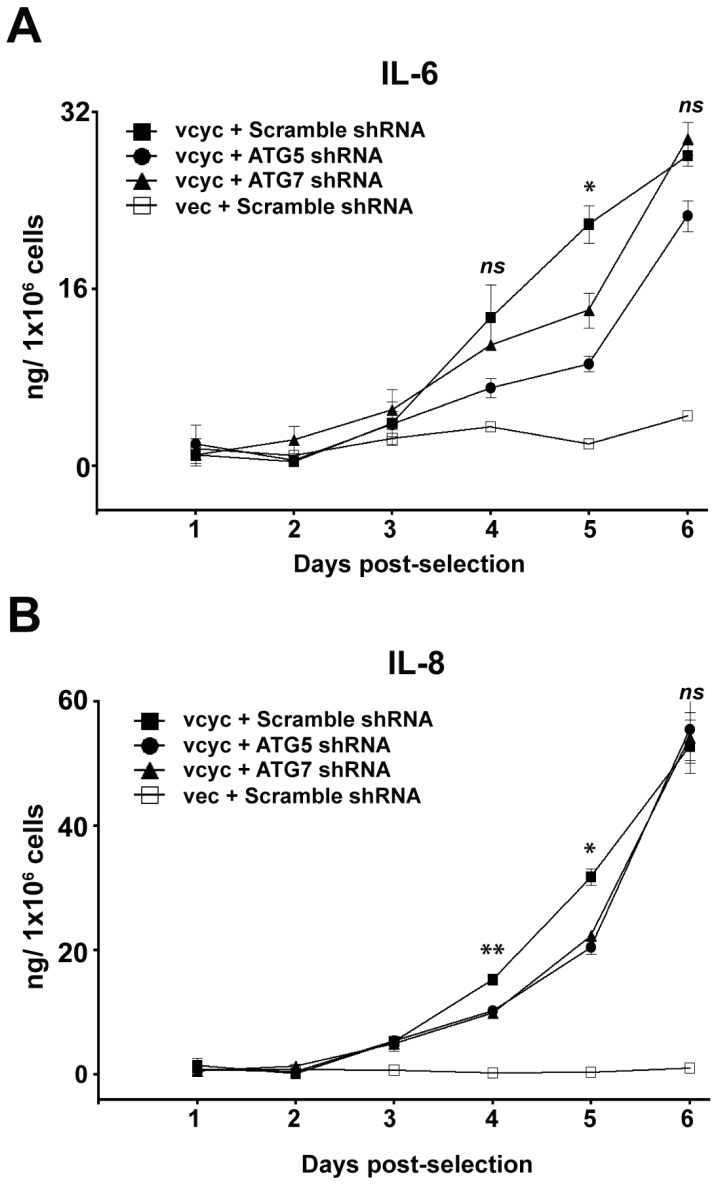

Figure 4.9
Autophagy is Important for v-Cyclin SASP
To examine the impact of autophagy on chemokine secretion, HFF knockdown cells transduced with HA-v-cyclin or control vector were analyzed for IL6 (A) or IL8 (B) by ELISA at various days post-selection. Values were normalized as in panel B and are means ± SEM of two independent experiments. ns > 0.05, * $P < 0.05$ and ** $P < 0.01$ the indicated parameters analyzed.

that activate autophagic responses during senescence. Autophagy is stimulated by multiple forms of stress, including nutrient or growth-factor deprivation, hypoxia, protein aggregates, intracellular pathogens, reactive oxygen species and DNA damage (Kroemer et al., 2010). Furthermore, these stresses promote signaling cascades that converge upon the four complexes that constitute the core autophagic machinery. Considering that DNA damage is a critical component of OIS and also induces autophagy, it seems most reasonable to believe that DDRs initiate signaling that culminates in autophagy activation. Furthermore, the observation that DDRs always slightly precede the activation of autophagy during OIS suggests that signals generated by genomic damage are at the root of this response.

Although DDRs trigger autophagy, the precise signaling mechanisms involved with activation remain poorly defined. Presently, it is postulated that DDRs activate autophagy through two distinct means. First, it has been shown that ATM, a critical kinase at the apex of DDR signaling, activates the tumor suppressor TSC2 to repress mTOR signaling (Alexander et al., 2010). Second, the tumor suppressor p53, a downstream target of DDRs, up-regulates a number of gene products, including PTEN and TSC2, that help repress mTOR signaling (Feng et al., 2005). Noting that both DNA-damage signaling cascades associated with autophagy converge upon the mTOR kinase, and that autophagy activation in the context of Ras-induced senescence coincided with repression of mTOR, I explored the possibility that all these observations were linked. Remarkably, I found that induction of autophagy within v-cyclin senescent cells correlated with p53 activation, up-regulation of the p53-target gene Sestrin1, robust activation of AMPK and repression of mTOR signaling. Up-regulation of the Sestrins and AMPK was not limited to v-cyclin-induced senescence: Ras-induced senescence demonstrated increases in both Sestrin1 and Sestrin2, suggesting conserved functions for the Sestrins and AMPK in repression of mTOR during OIS.

Numerous lines of evidence support a role for the AMPK/Sestrin pathway in suppression of mTOR signaling and in autophagy activation during OIS. In cultured cells, ectopic expression of Sestrin 2 has been shown to potently induce autophagy and

proliferation arrest (Budanov and Karin, 2008; Maiuri et al., 2009a). Futhermore, Sestrin1 and Sestrin2 are necessary for p53-dependent arrest of mTOR signaling and cell growth in mouse embryo fibroblasts and liver cells . Interestingly, the *Sestrin1* (6q21) *Sestrin2* (6q21) loci are frequently deleted in a variety of human cancers (Ragnarsson et al., 1999; Schwab et al., 1996; Velasco-Miguel et al., 1999), implicating loss of Sestrins in tumorigenesis. In line with these observations, activation of AMPK, and related kinases, has also been shown to be characteristic of Ras-induced and replicative senescence (Humbert et al., 2010; Moiseeva et al., 2009). These notions are further confirmed by recent discovery of the involvement of AMPK in autophagy and senescence induced by a proteolytic fragment of cyclin E (Singh et al., 2012). Collectively, this suggests an important role for the Sestrin-AMPK axis within the senescence program, possibly through activation of autophagy.

Additional mechanisms are also likely to contribute to activation of autophagy during senescence. I cannot exclude a role for DRAM, which was up-regulated during v-cyclin OIS, albeit with delayed kinetics relative to Sestrin1 expression and increases in cellular autophagy. Furthermore, it has recently been shown that cyclin B/CDK1-dependent phosphorylation of the Beclin 1/ class III PI3K complex represses autophagy in proliferating cells, while inhibition of cellular CDK activity facilitates autophagy induction (Furuya et al., 2010). Therefore, robust activation of DDRs that induce cell-cycle arrest through p53 and Rb pathways may also function to activate autophagy. Finally, emerging evidence suggests that ATM, under specific conditions, can phosphorylate and activate AMPK, perhaps promoting autophagy more directly (Fu et al., 2008; Sun et al., 2007).

Interestingly, Bcl-2 phosphorylation, and thus derepression of Beclin 1, appears not to contribute to activation of autophagy during v-cyclin OIS. v-Cyclin, in association with CDK6, has been reported to phosphorylate Bcl-2 on serine 70 (Ojala et al., 2000). This same residue has also been shown to regulate Bcl-2 interactions with Beclin 1 during starvation induced autophagy (Wei et al., 2008). In my system, failure of v-cyclin to stimulate Bcl-2 phosphorylation may be due to cell-type differences and different

levels of v-cyclin expression. It has been noted that different cell types can express dramatically different levels of Bcl-2-family proteins; endothelial cells are generally regarded as expressing low levels of Bcl-2 relative to other Bcl-2 family proteins (Simonart et al., 1998). Therefore, other Bcl-2 proteins, such as Bcl-XL and Mcl-1, may be more important for the regulation of the Beclin 1/class III PI3K complex in endothelial cells. It is not known whether v-cyclin can phosphorylate other Bcl-2-family proteins. Nevertheless, delayed activation of autophagy in v-cyclin-expressing cells suggests that v-cyclin does not directly subvert the autophagy machinery, but rather activates autophagy indirectly through DDRs.

How does autophagy promote transition to the senescent phenotype? Defects in senescence and SASP cytokine production from v-cyclin-expressing ATG5- or ATG7-deficient cells points to an important role for autophagy in v-cyclin OIS. Autophagy has been proposed to affect senescence through facilitation of the SASP (Young et al., 2009); because IL-6 and IL-8 of the SASP appear necessary for senescence, reduced cytokine synthesis in autophagy-defective cells would lead to delayed or permanently impaired OIS (Acosta et al., 2008; Kuilman et al., 2008). Importantly, the reduced secretion of IL-6 and IL-8 observed in autophagy-defective cells correlates with impaired protein synthesis, rather than differences in the levels of cytokine mRNAs (Young et al., 2009). Therefore, autophagic degradation appears, paradoxically to be important for protein synthesis.

Autophagy is increasingly acknowledged as an important mechanism for mobilizing amino acids to facilitate massive protein synthesis required by cells undergoing differentiation or elaborating cytokines during immune activation. For example, at the time of oocyte fertilization, autophagy is strongly induced, and necessary for ‘reprogramming’ of the highly differentiated oocyte into an undifferentiated state (Tsukamoto et al., 2008b; Tsukamoto et al., 2008a). This process involves degradation of maternal proteins and mRNA and the synthesis of enormous amounts of macromolecules that direct future development. Similarly, autophagy is required by osteoclasts during the process of bone resorption for the secretion of cathepsin K, the major protease in bone

breakdown (DeSelm et al., 2011). Finally, autophagy is required during monocyte-to-macrophage differentiation (Zhang et al., 2012), and may be responsible for the synthesis of soluble factors that remodel the extracellular matrix (Chang et al., 2012; Martinet et al., 2012).

To facilitate 'reprogramming' to the senescent state, and the massive synthesis of IL-6 and IL-8 required, cells appear to spatially couple protein degradation and protein synthesis (Narita et al., 2011). Remarkably, senescing cells relocalize mTOR and autolysosomes to the *trans* side of the Golgi apparatus, where amino acids released from autolysomes activate mTOR and are readily available for protein synthesis (Narita et al., 2011). This process of catabolic and anabolic coupling may also play roles in macrophage differentiation and secretion, as well as podocyte secretion of VEGF (Narita et al., 2011).

It is important to also consider other roles for autophagy in the facilitation of OIS. Recently, it has been shown that autophagy participates in the process of non-canonical secretion (Dupont et al., 2011). Interestingly, IL-1α and IL-1β are two cytokines that lack secretion signals and must be secreted through non-canonical pathways (Harris, 2011; Prudovsky et al., 2003). Given that IL-1α and IL-1β are important for senescence and up-regulated by v-cyclin, it remains possible that impaired secretion of these cytokines by autophagy-defective cells contributes to delayed senescence (Kuilman et al., 2008; Maier et al., 1990; Orjalo et al., 2009). Alternatively, and largely ignored to this point, is a role for autophagic clearance of cellular proteins to facilitate the induction and maintenance of the senescent phenotype. Close cooperation between the autophagosome and proteasome in degradation of ubiquitinated proteins might facilitate the rapid degradation of proteins to arrest the cell cycle (Kirkin et al., 2009). Furthermore, clearance of damaged proteins and organelles, such as dysfunctional mitochondria (Moiseeva et al., 2009), may be important for maintenance of senescent cells and controlled clearance by the immune system.

CHAPTER 5 - KSHV V-FLIP IMPAIRS V-CYCLIN-INDUCED SENESCENCE

Note: The majority of the research described in this chapter has been published in Cell Host and Microbe as: Leidal, A. M., Cyr, D. P., Hill, R. J., Lee, P. W., and McCormick, C. (2012). Subversion of Autophagy by Kaposi's Sarcoma-Associated Herpesvirus Impairs Oncogene-Induced Senescence. *Cell Host Microbe* 11, 167-180. © Cell Press. Figures appearing in this chapter that are wholly or partially reproduced from figures appearing in that paper include Figures 5.1, 5.2, 5.3 and 5.4.

5.1 Introduction

Latently infected 'spindle cells' are the primary proliferating cells within KS lesions. Consequently, gene products encoded by the KSHV latency program are presumed to be instrumental in KS pathogenesis (Ganem, 2010; Mesri et al., 2010); however, it is not yet known precisely how these viral gene products collaborate to drive spindle-cell proliferation. The oncogenic potential of each of the latent-gene products has been explored and, surprisingly, not one is capable of inducing cellular transformation (Mesri et al., 2010). Studies have, however, revealed that transgenic mice engineered to express KSHV LANA, v-cyclin or v-FLIP latent proteins develop lymphoid malignancies with low frequency and after long latency (Chugh et al., 2005; Fakhari and Dittmer, 2002; Verschuren et al., 2002). For these reasons, the group of ORFs encoding LANA, v-cyclin and v-FLIP have been coined KSHV's 'oncogenic cluster' and have been the focus of intensive investigation.

Expression of LANA, v-cyclin and v-FLIP within the KSHV latency program is regulated from a single promoter (Dittmer et al., 1998). This promoter is located proximal to LANA and generates three alternatively spliced transcripts from which the viral proteins are made. LANA and v-Cyclin are translated in a cap-dependent manner, whereas v-FLIP is expressed from an IRES downstream of the v-cyclin ORF (Grundhoff and Ganem, 2001). Evolution of this gene expression arrangement strongly suggests that the host-cell functions of LANA, v-cyclin and v-FLIP are interdependent. Intriguingly,

LANA is a documented regulator of p53 and Rb, and modulates chromatin (Friborg et al., 1999; Matsumura et al., 2005; Radkov et al., 2000; Wong and Wilson, 2005), while v-FLIP has been shown to activate NF-κB and represses autophagy (Chaudhary et al., 1999; Lee et al., 2009). Given that v-cyclin induces senescence through a p53- and autophagy-dependent mechanism, I reasoned that LANA or v-FLIP is likely to impair v-cyclin OIS. Therefore, I attempted to determine whether LANA or v-FLIP could inhibit v-cyclin OIS and elucidate the mechanisms that contributed to senescence suppression.

5.2 Results

5.2.1 Latent KSHV Infection Induces Oncogenic Stress

Previous reports suggest that latent KSHV infection triggers cellular DDRs and anti-proliferative checkpoints in host cells (Koopal et al., 2007). My work and that of others demonstrate that the latent v-cyclin protein is a potent inducer of cellular DDRs, and when expressed ectopically is capable of triggering senescence (Koopal et al., 2007; Verschuren et al., 2002; Verschuren et al., 2004a). However, it remains unclear whether latent KSHV infection triggers senescence. To address this point directly, I latently infected HFFs with KSHV and stained for markers of DNA damage, senescence and establishment of latency at seven days post-infection. For comparative purposes, I also assayed senescence and DNA-damage markers in HFFs transduced with a retroviral vector encoding HA-v-cyclin. In agreement with the literature, latently infected cells, which contained obvious LANA puncta, displayed strong accumulation of nuclear γH2AX foci, indicative of DDR activation (Figures 5.1A and 5.1B) (Koopal et al., 2007). Approximately 40% of cells latently infected with KSHV had obvious γH2AX foci, as compared to less than 10% of mock-infected controls. As anticipated, nearly 80% of cells expressing v-cyclin were found to have γH2AX foci. Despite significant increases in γH2AX foci within KSHV-infected cells, I was surprised to find that only 17% stained positive for SA β-gal activity (Figure 5.1A and 5.1B). By contrast, nearly 80% of cells expressing v-cyclin were positive for SA β-gal activity. Together, these striking results

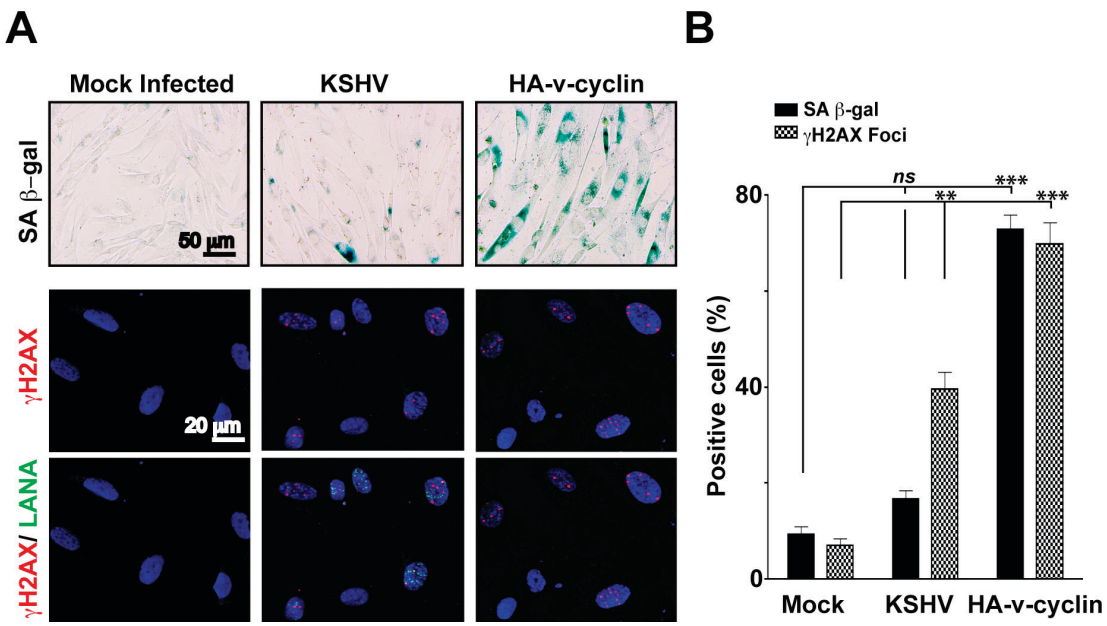

Figure 5.1
Latent KHSV Infection Induces Oncogenic Stress
(A) HFFs infected with KSHV, retrovirus encoding HA-v-cyclin or controls at seven days post-infection were assayed for SA β-gal activity (top row), γH2AX foci (red) and LANA (green). DNA was counterstained with TO-PRO (blue). SA β-gal activity was assessed separately due to reagent autofluorescence. (B) Quantification of SA β-gal activity and γH2AX foci from panel A. Values are means ± SEM of three independent experiments. Not significant (ns), ** $P < 0.01$ and *** $P < 0.005$ for the indicated parameters analyzed.

demonstrate that latent KHSV infection, despite potently activating DDRs, fails to effectively trigger the senescence of host cells.

5.2.2 v-FLIP Suppresses v-Cyclin-Induced Senescence

The failure of latently infected cells to senesce led me to hypothesize that certain KSHV latent-gene products may interfere with the host senescence program. v-Cyclin is expressed as part of a common transcription unit with LANA and v-FLIP during latency (Dittmer et al., 1998). Perhaps these co-regulated latent-gene products modulate v-cyclin-induced senescence. To determine the effects of LANA and v-FLIP on v-cyclin OIS, I co-expressed these latent-gene products with v-cyclin in HFFs and assayed for DNA replication, DNA-damage foci and senescence. Surprisingly, despite its reported role in regulating p53 and Rb (Friborg et al., 1999; Radkov et al., 2000), I observed no significant impact of LANA expression on v-cyclin-induced DDRs, proliferation arrest or the proportion of cells positive for SA β-gal activity and SAHF (Figures 5.2A-5.2C). In sharp contrast, co-expression of v-FLIP with v-cyclin dramatically reduced the proportion of SA β-gal-positive cells and the incidence of SAHF (Figures 5.2A-5.2C). In fact, the proportion of v-cyclin-expressing cells positive for SA β-gal activity and SAHF was more than halved by v-FLIP co-expression. v-FLIP also reversed the senescence-mediated inhibition of DNA replication as measured by BrdU incorporation. Importantly, despite suppressing v-cyclin OIS, v-FLIP had no obvious impact on v-cyclin-induced 53BP1 DNA-damage foci, suggesting that v-FLIP subverts senescence downstream of DDRs (Figures 5.2A-5.2C). Together, these surprising findings suggest that v-FLIP limits the anti-proliferative effects of host DNA-damage responses triggered by v-cyclin, thereby facilitating continued proliferation of latently infected cells.

5.2.3 v-FLIP Suppresses $HRas^{V12}$-Induced Senescence

The efficient suppression of v-cyclin OIS by v-FLIP led me to hypothesize that v-FLIP could also inhibit Ras-induced senescence. For the most part, v-cyclin and Ras OIS share many of the same pathways (Koopal et al., 2007; Leidal et al., 2012), and therefore, v-

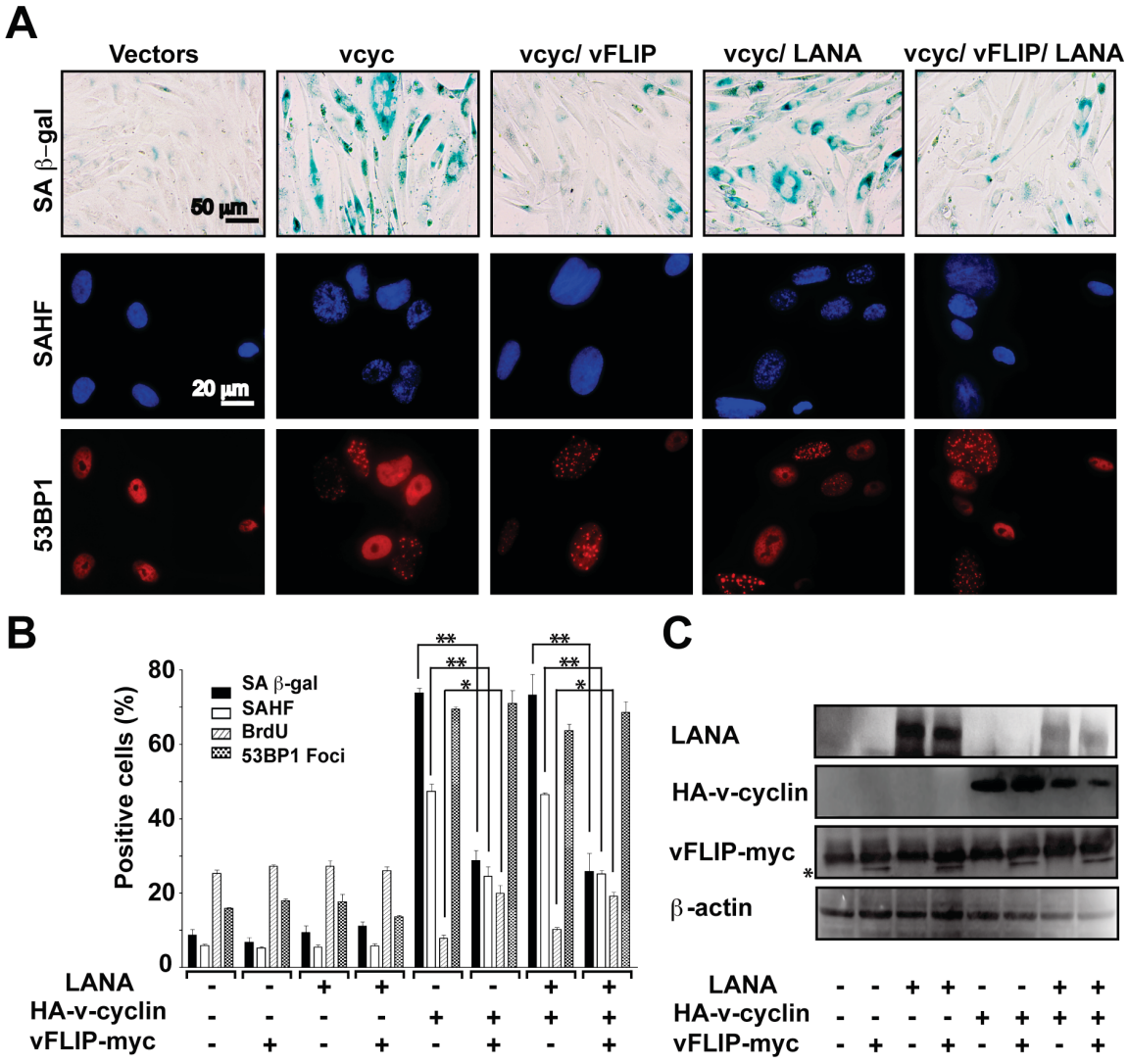

Figure 5.2
v-FLIP Suppresses v-Cyclin-Induced Senescence
(A) HFFs were simultaneously transduced with the appropriate combinations of empty retroviral vectors, and vectors encoding HA-v-cyclin-IRES-blast (vcyc), v-FLIP-myc-IRES-neo (v-FLIP), and/or LANA-IRES-puro (LANA), and after five days of selection were stained for SA β-gal activity (top row), SAHF (blue) and 53BP1 foci (red). (B) Quantification of SA β-gal activity, SAHF, BrdU incorporation and 53BP1 foci from panel A. Values are means ± SEM of three independent experiments. Not significant (ns), * $P < 0.05$, ** and $P < 0.01$ for the indicated parameters analyzed. (C) Immunoblots for LANA, HA-v-cyclin and v-FLIP-myc (indicated by an *) in the expression matrix employed in panels A and B.

FLIP should also facilitate senescence bypass. To determine whether v-FLIP can modulate Ras OIS, I co-expressed this KSHV latent-gene product with $HRas^{V12}$ in HHFs using retroviral vectors and assayed for SA β-gal activity, SAHF and 53BP1 DNA-damage foci after 7 days of selection. Remarkably, v-FLIP co-expression also suppressed the development of senescence markers induced by oncogenic Ras (Figures 5.3A and 5.3B). I observed modest reductions in the percentages of SA β-gal- and SAHF-positive cells within populations co-expressing v-FLIP. Intriguingly, v-FLIP suppression of Ras-induced senescence appeared to be more modest than suppression of v-cyclin-induced effects. It is unclear why this would be the case. Importantly, v-FLIP showed negligible impact on Ras-induced DNA-damage foci (Figures 5.3A and 5.3B), similar to the situation with v-cyclin OIS, suggesting suppression is independent of DDRs. Collectively, these results demonstrate that v-FLIP suppresses Ras-induced senescence, and suggest that v-FLIP likely regulates senescence through a pathway common to Ras and v-cyclin OIS.

5.2.4 KSHV v-FLIP Subverts Autophagy to Impair v-Cyclin OIS

The latent v-FLIP protein encoded by KSHV, in addition to regulating NF-κB signaling (Chaudhary et al., 1999), has been reported to inhibit autophagy. v-FLIP was found to directly interact with host cell Atg3 through two α-helicies within its death-effector domains, thus blocking LC3 lipidation (Lee et al., 2009). Given that autophagy is necessary for the efficient execution of v-cyclin-induced senescence, and v-FLIP co-expression suppresses OIS, I hypothesized that v-FLIP subversion of autophagy is responsible for impaired v-cyclin OIS. To determine whether v-FLIP blocks v-cyclin OIS through suppression of autophagy, HFF cells were simultaneously transduced with retroviral vectors encoding v-cyclin and v-FLIP or mutant v-FLIP, and then transiently transfected with an EGFP-LC3 reporter. v-FLIP mutants defective for NF-κB activation (v-FLIP 58AAA), or with compound defects in both NF-κB activation and Atg3 binding (v-FLIP Δα2α4), were employed to specifically dissect v-FLIP functions that modulate v-cyclin OIS (Lee et al., 2009). As anticipated, v-cyclin-expressing cells exhibited strong

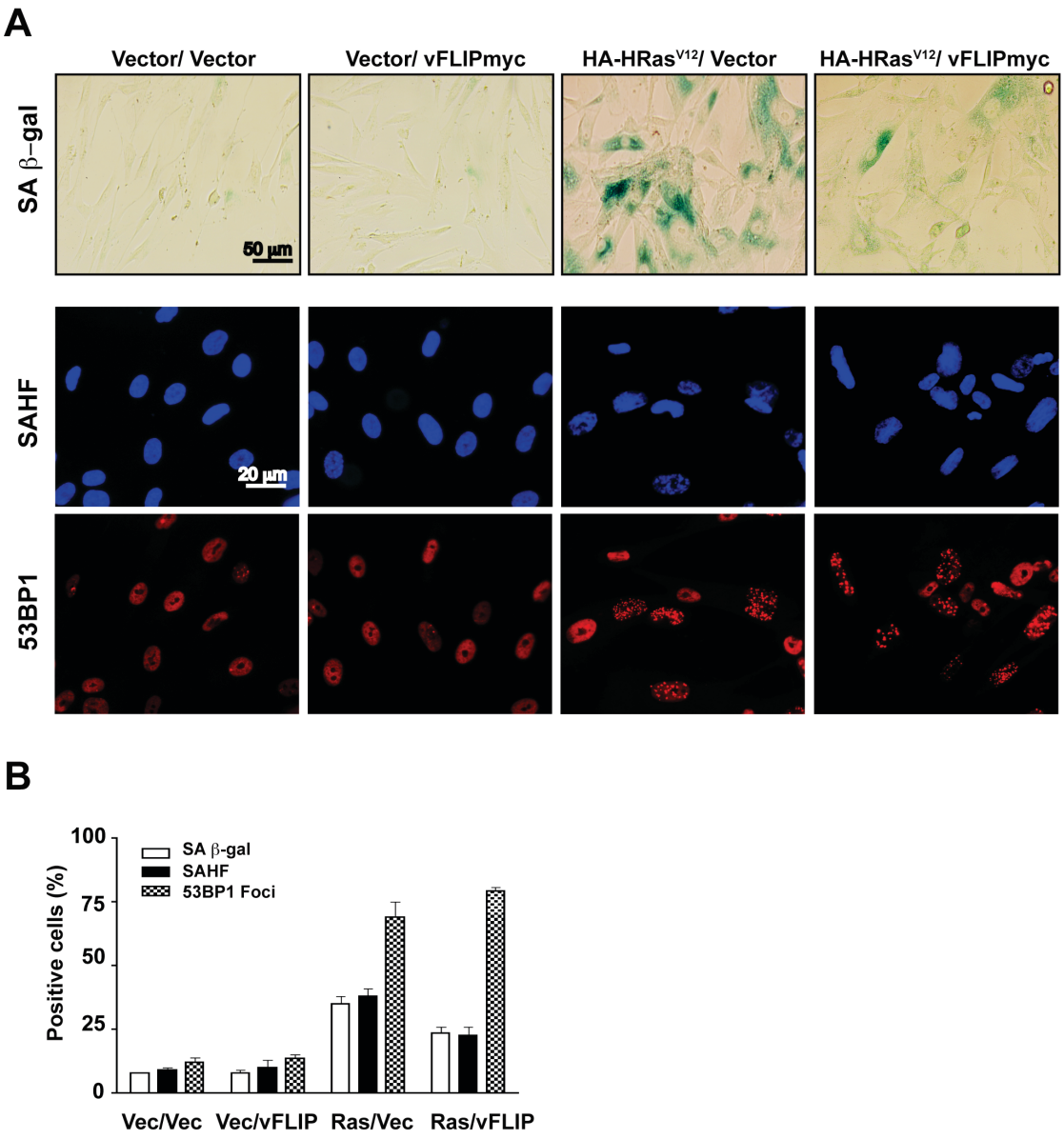

Figure 5.3
v-FLIP Suppresses HRas^{V12}-Induced Senescence
(A) Representative images of HFFs that were co-transduced with retroviral vectors encoding HRas^{V12} and v-FLIP-myc or appropriate controls and assayed for SA β-gal activity, SAHF and 53BP1 foci after 7 days of selection. (B) The percent of cells staining for senescence markers and DDR activation is shown; Vector (Vec); HRas^{V12} (Ras); v-FLIP-myc (vFLIP).Values are ± SEM of 2 independent experiments.

53BP1 puncta, EGFP-LC3 puncta and SA β-gal activity (Figures 5.4A, 5.4B and 5.4C). Specifically, I found that for cells expressing v-cyclin and the EGFP-LC3 reporter, approximately 75% contained greater than 30 autophagic puncta per cell, and 76% displayed SA β-gal activity. Co-transduction of v-FLIP with v-cyclin did not affect DDRs, but markedly reduced the number of cells with greater than 30 cytoplasmic EGFP-LC3 puncta (53%) and SA β-gal activity (31%). Similar to cells co-expressing wild-type v-FLIP, NF-κB-signaling-defective v-FLIP 58AAA and v-cyclin co-transductants exhibited, on average, relatively few EGFP-LC3 puncta per cell (41%) and demonstrated a small but significant increase in the proportion of cells with SA β-gal activity (56%). Importantly, cells co-transduced with v-cyclin and v-FLIP Δα2α4 rendered high proportions of cells with greater than 30 autophagic puncta per cell (79%) and SA β-gal staining (85%) (Figures 5.4A, 5.4B and 5.4C). These results demonstrate that v-FLIP-mediated suppression of autophagy is sufficient to suppress v-cyclin OIS, and suggest an intriguing auxiliary role for NF-κB signaling.

5.2.5 v-FLIP Fails to Suppress IL-6 and IL-8 Secretion During OIS

Autophagy is necessary for v-cyclin-induced senescence, and contributes to the efficient production of IL-6 and IL-8. Furthermore, my studies demonstrate that KSHV v-FLIP is a potent inhibitor of autophagy and efficiently blocks v-cyclin OIS. Intriguingly, v-FLIP has also been shown to potently stimulate the NF-κB signal-transduction pathway and promotes secretion of a plethora of cytokines (Chaudhary et al., 1999; Liu et al., 2002; Chugh et al., 2005; Grossmann et al., 2006). Therefore, it remains unclear if v-FLIP inhibition of v-cyclin-induced autophagy and senescence correlates with reduced interleukin production. To determine the status of IL-6 and IL-8 secretion during v-FLIP suppression of v-cyclin OIS, I simultaneously transduced HFFs with retroviral vectors encoding v-cyclin and v-FLIP or v-FLIP mutants and assayed the conditioned media for interleukin levels. Surprisingly, I observed that IL-6 and IL-8 secretion from wild-type v-FLIP and v-cyclin co-expressing cells was far greater than secretion from v-cyclin and v-FLIP mutants or vector control cells (Figure 5.5); clearly v-FLIP does inhibit IL-6 and

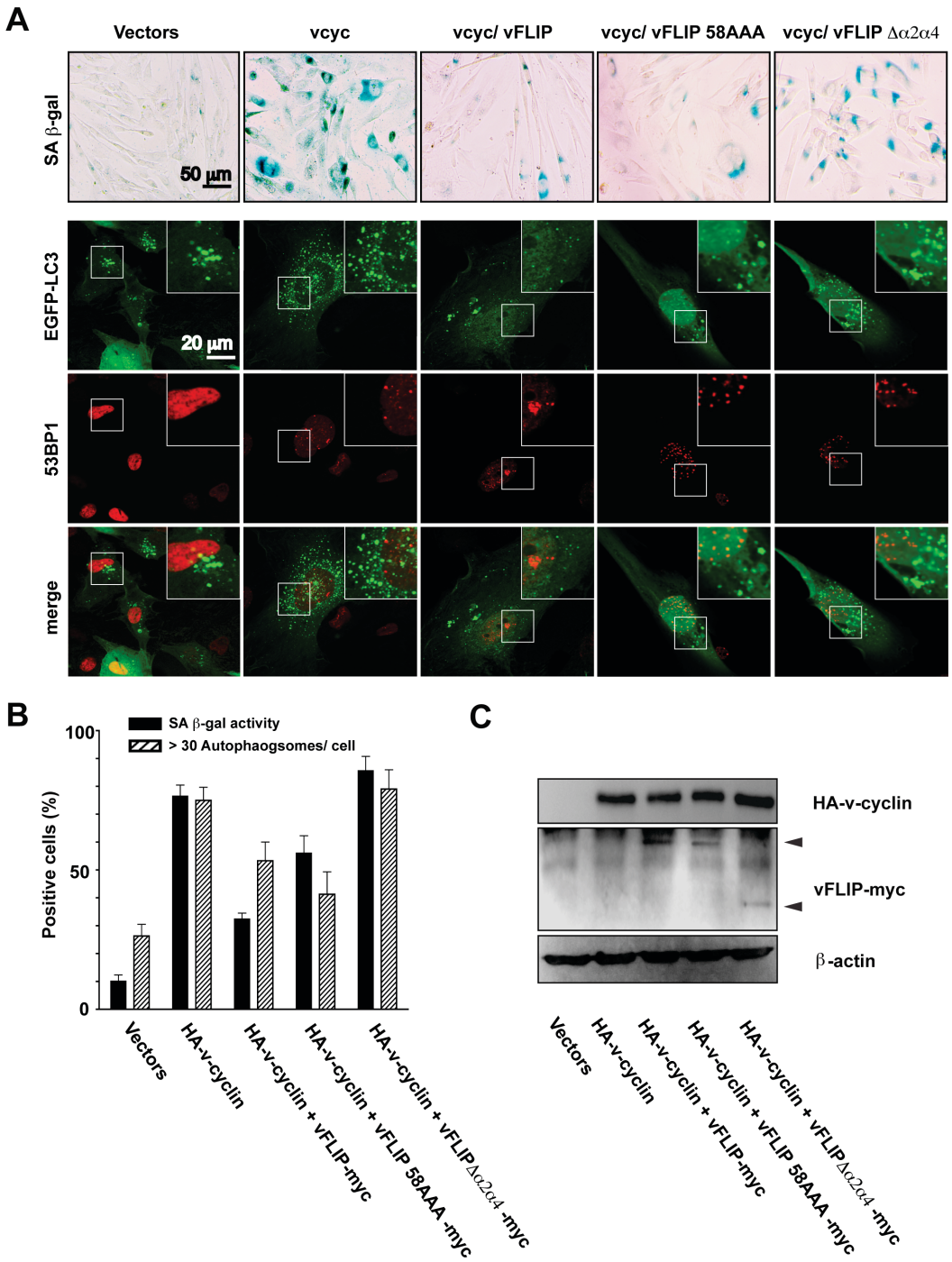

Figure 5.4
KSHV v-FLIP Subverts Autophagy to Impair v-Cyclin OIS
(A) HFFs transduced with the appropriate combinations of empty retroviral vectors (Vec), and/or vectors encoding HA-v-cyclin-IRES-blast (vcyc) and v-FLIP-myc-IRES-neo (v-FLIP) or v-FLIP mutant constructs were assayed at seven days for SA β-gal activity post-infection (top row), EGFP-LC3 autophagic puncta (green) and 53BP1 DNA damage foci (red). (B) Quantification of cells that stain positive for SA β-gal and have > 30 autophagosomes from panel A. Values are means ± SEM of three independent experiments. (C) Immunoblots for HA-v-cyclin, v-FLIP-myc and actin from lysates in panel A.

IL-8 in its suppression of v-cyclin-induced autophagy and OIS. Furthermore, v-FLIP mutants impaired for NF-κB stimulation (v-FLIP 58 AAA) or harboring compound defects in NF-κB and autophagy regulation (v-FLIP Δα2α4) demonstrated relatively modest impacts on v-cyclin-induced IL-6 and IL-8 secretion. Nevertheless, a small reduction in interleukin production was observed in conditioned medium from cells co-expressing the v-FLIP NF-κB mutant (v-FLIP 58 AAA) relative to the v-FLIP compound mutant (v-FLIP Δα2α4) or vector control (Figure 5.5). Taken together, these results demonstrate that v-FLIP subversion of v-cyclin-induced autophagy and senescence does not correlate with impaired IL-6 and IL-8 production. Clearly, more work is required to elucidate the precise functions of v-FLIP in autophagy and senescence.

5.3 Discussion

These studies demonstrate that v-FLIP subversion of autophagy functionally suppresses v-cyclin OIS. Ectopic co-expression of v-FLIP was found to potently suppress v-cyclin-induced SA β-gal activity, SAHF and autophagy, and increased BrdU incorporation relative to senescent controls. v-FLIP is known to both activate the NF-κB pathway and inhibit autophagy by binding directly to Atg3. Co-expression of v-FLIP mutants deficient for NF-κB, or compound NF-κB and autophagy mutants, confirmed roles for both v-FLIP regulatory functions toward the impairment of v-cyclin OIS. Interestingly, v-FLIP subversion of autophagy and senescence was found to be independent of IL-6 and IL-8 secretion from v-cyclin-expressing cells. Studies also determined that KSHV latent infection induces DDRs characteristic of oncogenic stress, but infected cells fail to senesce, suggesting OIS is also suppressed in the context of infection.

Why does LANA fail to suppress v-cyclin OIS? KS tumorigenesis represents the outcome of complex interplay between KSHV latent gene products and the host-cell responses that they elicit. Unfortunately, our understanding of both the latency program and host responses remains fragmented. My work has clearly demonstrated that p53 is robustly activated during v-cyclin OIS, and is required for efficient induction of autophagy and senescence. Therefore, it is surprising that KSHV LANA, a p53- and Rb-

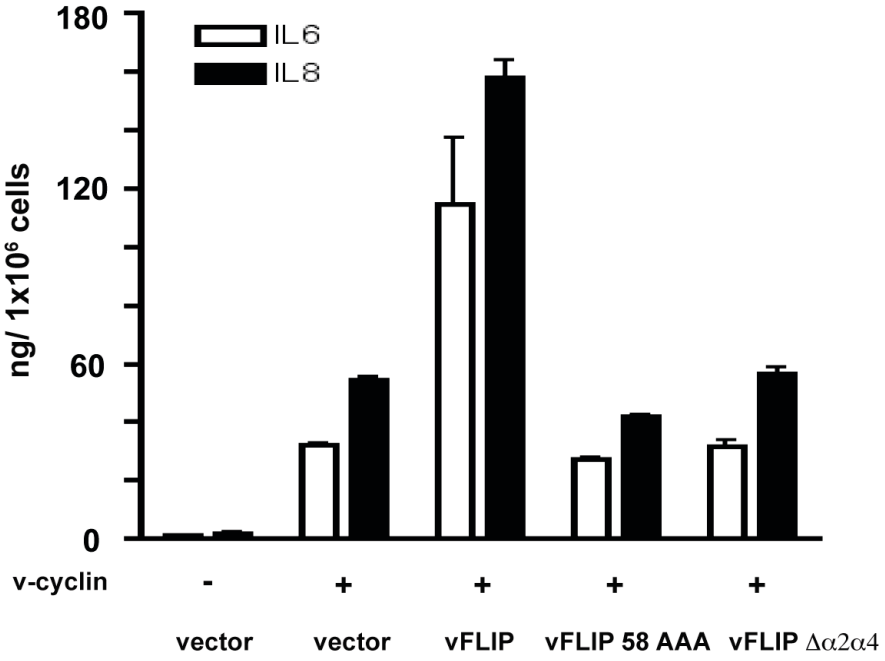

Figure 5.5
v-FLIP Fails to Suppress v-Cyclin-Induced IL6 and IL8 Secretion
Conditioned media derived from HFFs transduced with the appropriate combinations of empty retroviral vectors and/or vectors encoding HA-v-cyclin-IRES-blast (v-cyclin) and v-FLIP-myc-IRES-neo (v-FLIP) or v-FLIP mutant constructs were assayed for the abundance of IL-6 and IL-8 by ELISA. Values are means ± SEM of two independent experiments and were normalized relative to the amount secreted from $1x10^6$ cells.

binding protein demonstrated to stimulate the cell cycle and impair p53-dependent transactivation, had a negligible impact on v-cyclin-induced senescence. KS and PEL tumors have been found to most often possess wild-type p53 and can be effectively treated with Nutlin-3 and the DNA-damaging agent doxorubicin, indicating intact p53 activity in tumor cells (Dittmer and Krown, 2007; Katano et al., 2001; Nador et al., 1996; Petre et al., 2007). Recently it has been shown that LANA only binds a minor fraction of total p53 in KSHV-positive PEL tumor cells, and that p53 Ser15 phosphorylation disrupts this interaction (Chen et al., 2010). This suggests that LANA fails to block v-cyclin OIS because v-cyclin-induced DDRs inhibit the capacity of LANA to bind activated p53.

How does v-FLIP activation of NF-κB contribute to suppression of v-cyclin OIS? In addition to suppressing autophagy, v-FLIP is a robust activator of the NF-κB pathway. To accomplish NF-κB activation, v-FLIP appears to bind and activate the multiprotein IKK complex, thus promoting degradation of IκB proteins and NF-κB-dependent transactivation (Chaudhary et al., 1999; Liu et al., 2002). Importantly, NF-κB signal transduction also regulates autophagy and senescence. Activation of NF-κB signaling has been shown to potently stimulate autophagy (Criollo et al., 2009) and promote OIS (Basseres and Baldwin, 2006; Chien et al., 2011; Rovillain et al., 2011), but there have also been conflicting reports that implicate elevated NF-κB activity and/or secretion of inflammatory mediators in senescence bypass (Acosta et al., 2008; Batsi et al., 2009; Coppe et al., 2008; Groppo and Richter, 2011; Guerra et al., 2011). Furthermore, many studies have shown that tumor cells that bypass senescence have elevated NF-κB activity and/or aberrant cytokine secretion profiles (Grivennikov and Karin, 2010); indeed, my studies show that v-FLIP bypass of v-cyclin OIS occurs in the presence of high levels of secreted IL-6 and IL-8. Therefore, the v-FLIP subversion of autophagy appears to suppress v-cyclin OIS independently of IL-6 and IL-8; however, this relationship remains to be examined. As previously noted, there are alternative roles for autophagy as an effector of OIS, including non-canonical secretion of cytokines necessary for efficient senescence (Dupont et al., 2011; Maier et al., 1990; Orjalo et al., 2009; Prudovsky et al., 2003). Collectively my findings indicate that v-FLIP suppression of autophagy is

sufficient to suppress v-cyclin OIS, but also suggest an intriguing auxiliary role for v-FLIP-mediated dysregulation of the IKK/NF-kB signaling axis in suppressing OIS.

CHAPTER 6 - SUBVERSION OF AUTOPHAGY BY KSHV SUPPRESSES OIS

Note: The majority of the research described in this chapter has been published in Cell Host and Microbe as: Leidal, A. M., Cyr, D. P., Hill, R. J., Lee, P. W., and McCormick, C. (2012). Subversion of Autophagy by Kaposi's Sarcoma-Associated Herpesvirus Impairs Oncogene-Induced Senescence. *Cell Host Microbe* 11, 167-180. © Cell Press. Figures appearing in this chapter that are wholly or partially reproduced from figures appearing in that paper include Figures 6.1, 6.2, 6.3, 6.4, and 6.5.

6.1 Introduction

Oncogenic γ-herpesviruses, including KSHV, establish lifelong latency within lymphoid and endothelial tissues. To accomplish this challenging feat, KSHV has evolved elaborate mechanisms to deal with host defenses (Speck and Ganem, 2010). The v-FLIP protein encoded by KSHV represents a critical virulence factor for latency and tumorigenesis. v-FLIP is a potent stimulator of NF-κB signal transduction and renders latently infected cells resistant to cell death (Guasparri et al., 2004; Sakakibara et al., 2009). Recent work has shown that v-FLIP is also a potent inhibitor of autophagy (Lee et al., 2009). Through an α-helix within each of it's two DEDs, v-FLIP is able to bind Atg3 and impair LC3-dependent autophagosomal membrane elongation. In light of autophagy being an important component of the integrated cellular stress response and a mechanism for remodeling the intracellular environment, v-FLIP subversion of the autophagic machinery likely contributes significantly to establishment of KSHV latent infection.

At present, the status of autophagy in KSHV-infected cells and the roles for v-FLIP autophagy subversion in viral latency and pathogenesis have not been thoroughly examined. Intriguingly, v-FLIP is expressed from a common transcription unit within KSHV that also encodes v-cyclin and LANA (Dittmer et al., 1998). This suggests that v-FLIP actions during latency are likely intertwined with those of v-cyclin and LANA. KSHV v-cyclin is a potent activator of cell-cycle progression and is thought to drive proliferation of latently infected cells (Chang et al., 1996; Laman et al., 2001). However,

the cell-cycle stimulatory functions of v-cyclin are so robust it frequently induces replication stress, DNA damage and OIS (Koopal et al., 2007; Verschuren et al., 2002). Importantly, KSHV-infected cells frequently display activated DDRs, suggestive of v-cyclin-induced replication stress, and yet fail to senesce (Koopal et al., 2007). In light of autophagy being an essential effector mechanism of OIS, I reasoned that coordinated expression of KSHV v-FLIP and v-cyclin would subvert autophagy and suppress v-cyclin OIS within latently infected cells. Therefore, I attempted to determine whether v-FLIP suppresses OIS in latently infected cells through subversion of autophagy.

6.2 Results

6.2.1 Autophagy Appears Suppressed During KSHV Latent Infection

Despite identification of KSHV proteins that can subvert autophagic pathways, the status of autophagy in KSHV-infected cells remains unknown. Robust suppression of autophagic activity by the latent v-FLIP protein in ectopic-expression studies suggested that autophagy is likely to be inactive or suppressed in KSHV infected cells.

To determine whether KSHV latent infection activates autophagy, HFFs were infected *de novo* with KSHV, and then transiently transfected with an EGFP-LC3 autophagy reporter upon the establishment of latency. At the conclusion of the experiment infected cells were co-immunostained for LANA and 53BP1, to ensure latent infection and assess the status of DDRs, respectively. Results showed that latently infected cells had modest levels of EGFP-LC3 puncta, indicative of basal-level autophagy, despite frequently harboring 53BP1 DNA-damage foci (Figure 6.1). Basal levels of autophagy were also observed in mock-infected cells, but 53BP1 DNA-damage foci were absent. Together, these results demonstrate that KSHV fails to activate autophagy even though latently infected cells have extensive DNA damage and appear to have undergone replication stress.

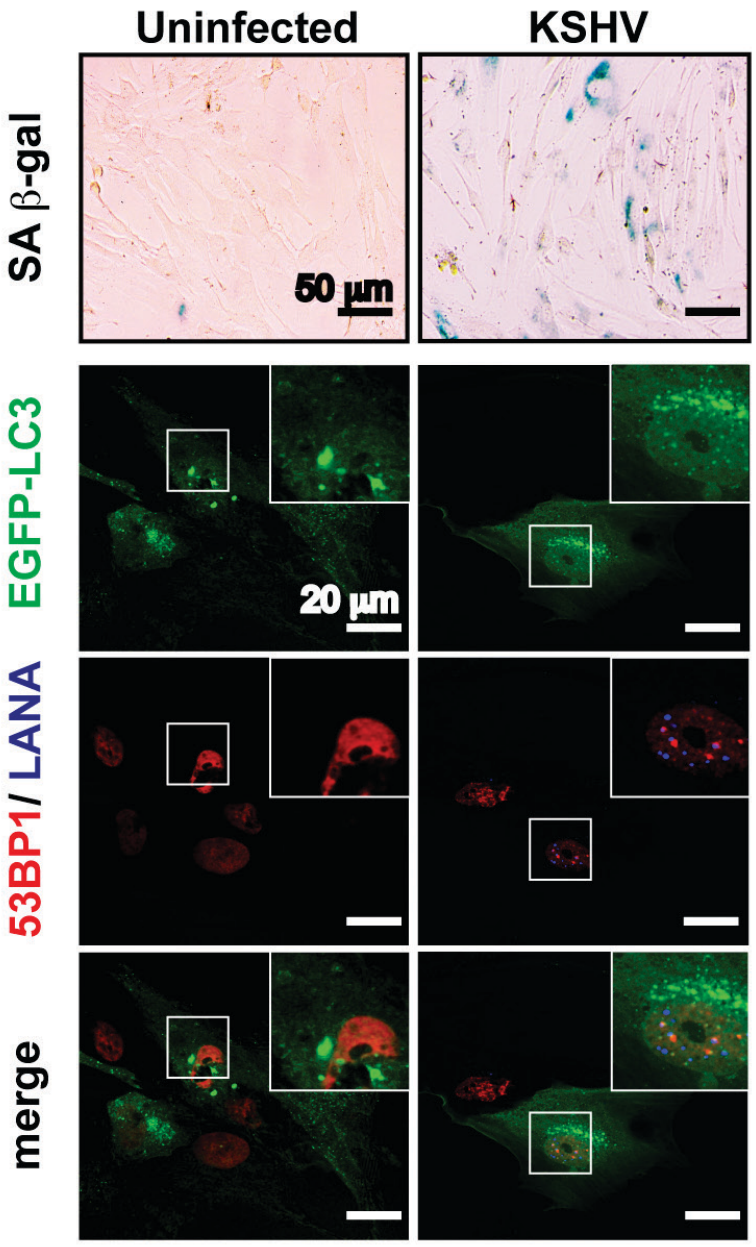

Figure 6.1
Modest Induction of Autophagy During KSHV Latent Infection
HFFs infected with KSHV (or mock infected) were assayed at seven days post-infection for SA β-gal activity (top row), GFP-LC3 autophagic puncta (green), 53BP1 DNA damage foci (red) and LANA (blue).

6.2.2 Autophagy Defects Promote KSHV Latent Infection

To characterize the functions of v-FLIP during KSHV latency, I obtained three bacterial artificial chromosomes (BACs), separately encoding recombinant wild-type KSHV, v-FLIP deficient KSHV or v-FLIP deficient KSHV with v-FLIP artificially restored (Ye et al., 2008). In construction of the recombinant KSHV BACs, the ORFs of GFP and the hygromycin-resistance gene were incorporated into KSHV genomic DNA, enabling fluorescent or antibiotic selection of eukaryotic cells harboring the BAC (Gao et al., 2003; Zhou et al., 2002). Most importantly, eukaryotic cells transiently harboring KSHV BACs have been reported to yield low levels of infectious recombinant KSHV upon treatment with chemicals or proteins that trigger KSHV lytic reactivation. It was my intent to use these recombinant wild-type and mutant viruses to determine whether v-FLIP deficiency affected the levels of autophagy and OIS in virus-infected cells. Furthermore, I had hoped to layer onto this system shRNAs targeting key components of the autophagy machinery to fully assess the role of v-FLIP and autophagy in KSHV latent infection. However, these plans proved premature because I was not able to collect sufficient quantities of v-FLIP-deficient KSHV or v-FLIP-restored KSHV virus for experimental purposes. Nevertheless, I persisted with wild-type recombinant KSHV virus, which I was able to make sufficiently well, and observed intriguing results upon infection of HFFs expressing shRNAs targeting *ATG7* and controls. In particular, I observed that Atg7-deficient HFFs were more readily infected than HFFs expressing scrambled, non-specific shRNAs (Figure 6.2). Taken together, these data demonstrate that autophagy restricts *de novo* KSHV infection, perhaps by xenophagic clearance.

6.2.3 Inhibition of v-FLIP in Latently Infected Cells Promotes Senescence

Failure of the recombinant KSHV BAC system in its original purpose led me to consider other mechanisms that might enable specific ablation of v-FLIP in KSHV-infected cells. Initial characterization of v-FLIP-mediated autophagy suppression revealed two α-helices, one in each of two DEDs, that are essential for v-FLIP anti-autophagy functions (Lee et al., 2009). Independent of each other, v-FLIP DED α-helices bind to Atg3 and

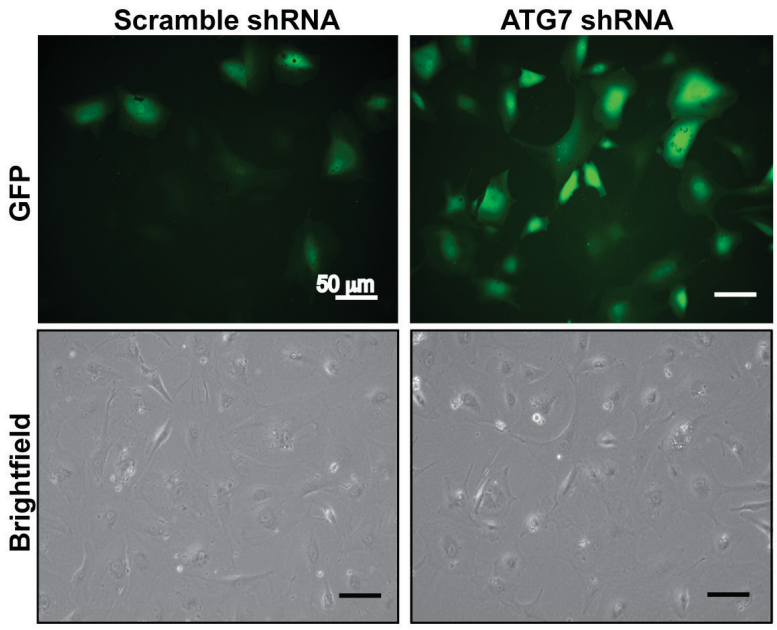

Figure 6.2
Autophagy Defects Promote KSHV Latent Infection
HFFs were transduced with retroviral shRNA constructs targeting either ATG7 or control and subsequently infected with BAC36-derived KSHV, which harbors a GFP reporter gene driven by a constitutive promoter. Cells were visualized at 48 hours post-infection and demonstrated increased KSHV infection of autophagy-deficient HFFs.

prevent elongation of autophagosomal membranes. Remarkably, when each DED α-helix was separately fused to the cell-penetrating peptide of HIV transactivator of transcription (TAT), the resultant fusion proteins were found to function as dominant negatives against v-FLIP, rather than suppressors of cellular autophagy (Lee et al., 2009). In light of these observations, I reasoned that v-FLIP anti-autophagy function during latent infection could be specifically ablated through co-expression of dominant negative v-FLIP DED α-helices. To this end, I generated two retroviral expression vectors that encoded each v-FLIP DED α-helix as a recombinant RFP fusion protein (RFP-α2; RFP-α4). Because of the short length of each v-FLIP α-helix (10-12 amino acids), fusion proteins are hereafter referred to as v-FLIP inhibitory peptides. To determine whether restoration of autophagy in KSHV-infected cells affected senescence or aspects of latency, I simultaneously infected HFFs with KSHV and retrovirus encoding v-FLIP inhibitory peptides or controls. Six days after infections, half of the HFFs for each condition were transfected with EGFP-LC3 reporter constructs to monitor autophagy within infected or control cells. On the seventh day, post infection, all cells were fixed and half were stained for SA β-gal activity, while the other half were examined for autophagy and latent infection. Similar to the findings of previous experiments, KSHV infection alone conferred modest levels of autophagy, as measured by GFP-LC3 puncta, and low levels of senescence (Figures 6.3A and 6.3B). Slightly less than 50% of KSHV-infected control cells had greater than 30 autophagosomes and approximately 20% were positive for SA β-gal activity. By contrast, latently infected cells expressing RFP-α2 or RFP-α4 v-FLIP inhibitory peptides displayed elevated levels of autophagy and senescence. The percentage of infected cells expressing RFP-α2 or RFP-α4 v-FLIP inhibitory peptides with greater than 30 autophagosomes was between 65% and 80% and the percentage of cells positive for SA β-gal activity was nearly 40%. Importantly, RFP-α2 or RFP-α4 v-FLIP inhibitory peptides specifically target v-FLIP function in KSHV-infected cells; I observed no measurable impact on autophagy or senescence in uninfected controls (Figures 6.3B and 6.4). Collectively, these data demonstrate that v-FLIP subversion of autophagy helps to impair senescence of cells latently infected with KSHV.

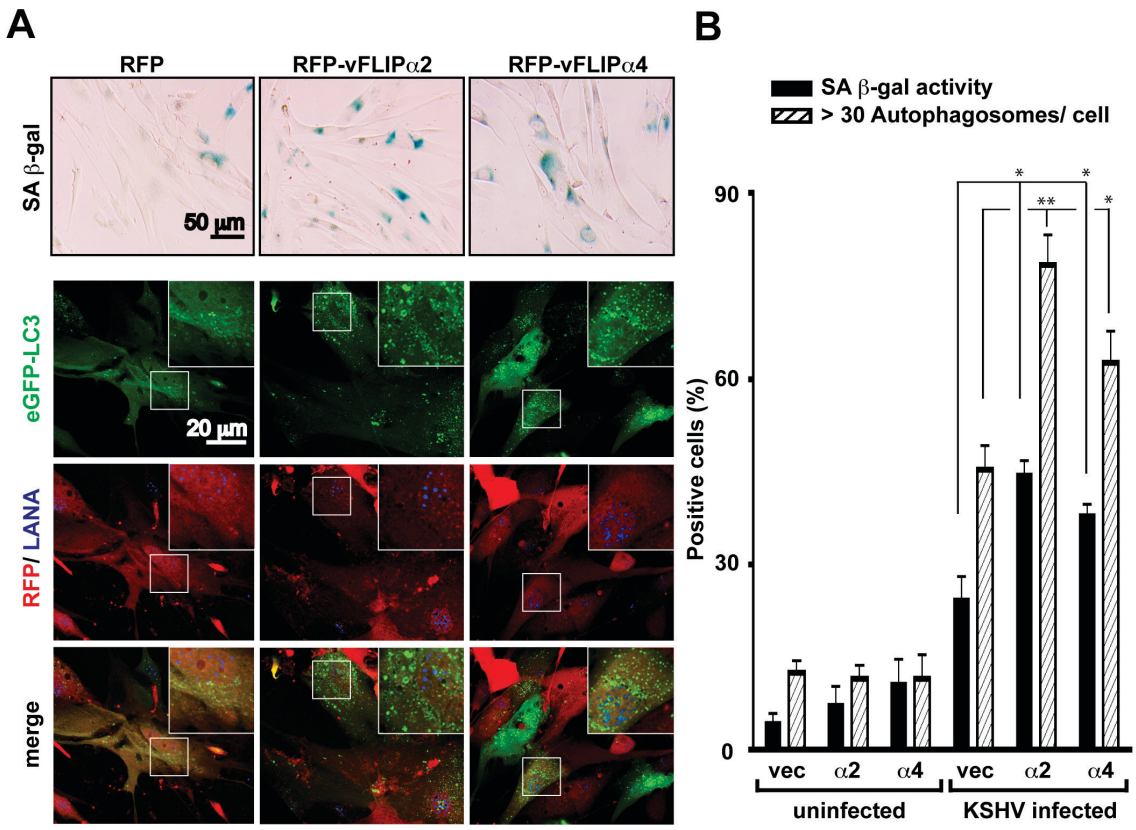

Figure 6.3
Inhibition of v-FLIP in Latently Infected Cells Promotes Senescence
(A) HFFs were transduced with RFP-α2 or RFP-α4 v-FLIP inhibitory peptides or an RFP control vector (red) and subsequently infected with KSHV. We observed that >95% of cells were latently infected by immunofluorescence targeting the LANA protein of KSHV (blue). One day prior to analysis, these cells were transfected with an EGFP-LC3 reporter construct (green) to visualize autophagosomes. (B) Quantification of cells that stain positive for SA β-gal and have > 30 autophagosomes from panel A and uninfected controls. Values are means ± SEM of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ for the indicated parameters analyzed.

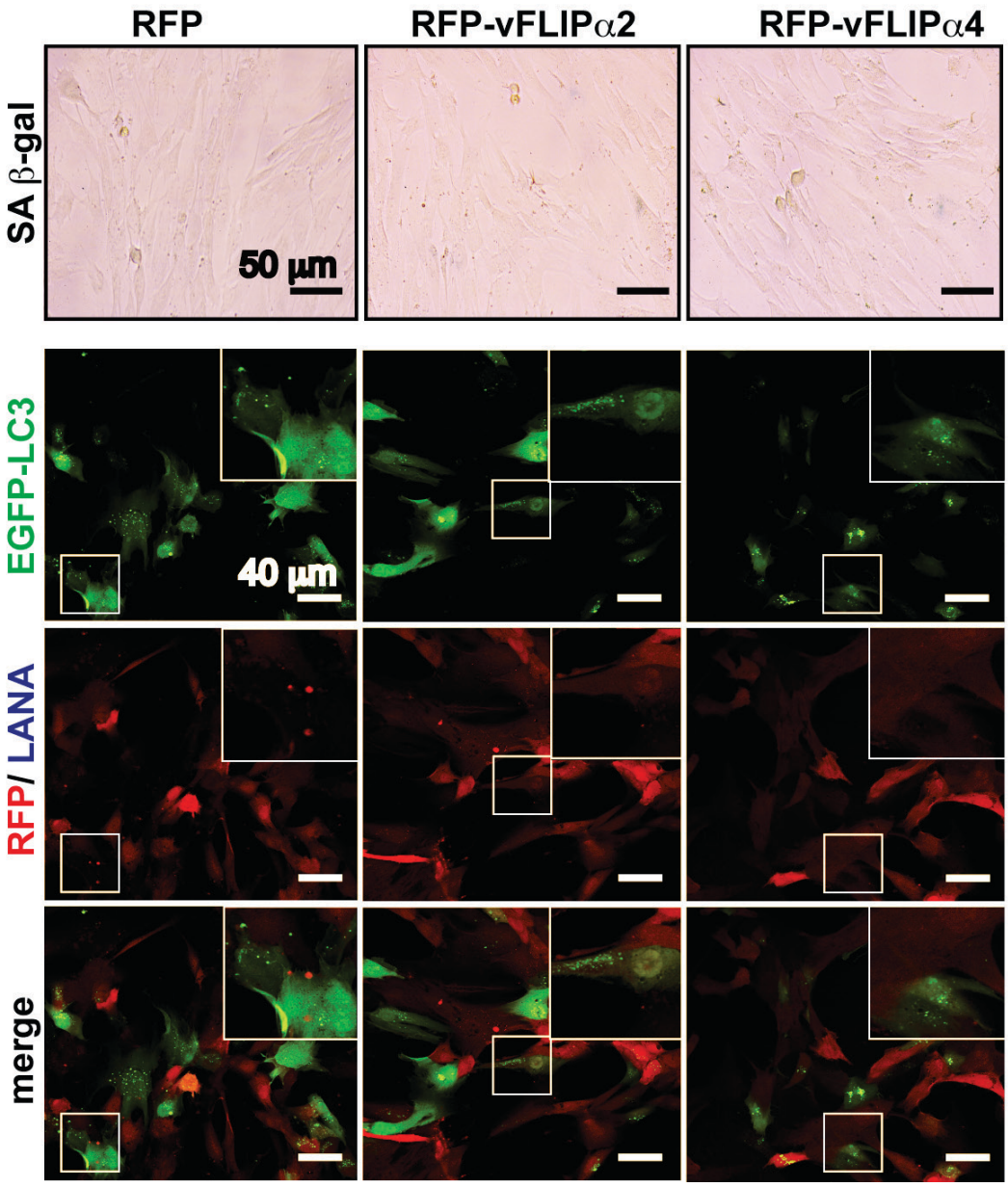

Figure 6.4
v-FLIP Inhibitory Peptides Fail to Induce Autophagy in Uninfected Cells
HFFs were transduced with RFP-α2 or RFP-α4 vFLIP inhibitory peptides or an RFP control vector (red). 1 day prior to analysis, these cells were transfected with an EGFP-LC3 reporter construct (green) to visualize autophagosomes, and monolayers were also stained with anti-LANA antibody (blue) to confirm the absence of KSHV latent infection.

6.2.4 Rapamycin Induces Autophagy and Senescence in KSHV-Infected Cells

Rapamycin is a pharmacological agent frequently used as an immunosuppressant in transplant patients. Recently, this drug has also been demonstrated to be effective in the treatment of numerous cancers, including KS (Kolhe et al., 2006; Stallone et al., 2005). The anti-cancer functions of rapamycin are, in part, linked to its ability to potently inhibit mTOR signaling and activate autophagy (Rubinsztein et al., 2007). Previous reports show that rapamycin treatment of KSHV-infected PEL cells activates autophagy even in the presence of latently expressed v-FLIP (Lee et al., 2009); however, ectopic overexpression of v-FLIP in KSHV-infected PEL cells was able to effectively suppress these effects. This suggested to me that the levels of v-FLIP typically expressed in KSHV-infected cells are insufficient to block the potent effects of rapamycin. For these reasons, I was interested to know whether rapamycin is capable of inducing autophagy and senescence in KSHV-infected cells. To this end, HFFs were infected *de novo* with KSHV and given three days to establish latency; subsequently, KSHV infected cells were treated with 10 nM rapamycin or vehicle control for five days. Autophagy and senescence were monitored in parallel treated cultures by transfecting an EGFP-LC3 reporter or assaying for SA β-gal activity and BrdU incorporation. Results from this experiment showed that rapamycin potently induced EGFP-LC3 puncta in KSHV infected cells, indicative of autophagy activation (Figure 6.5A). Furthermore, latently infected cells treated with rapamycin displayed modest, but significant, increases in SA β-gal activity and decreases in BrdU incorporation (Figures 6.5B and 6.5C), suggesting induction of senescence. Taken together, these results demonstrate that activation of autophagy in KSHV-infected cells promotes OIS, and suggests a mechanism of action for rapamycin that may contribute to its effective treatment of KS.

6.3 Discussion

KSHV v-FLIP was identified as a protein that subverts autophagy and impairs OIS, yet the importance of these functions in KSHV latent infection remain unclear. Here, I show

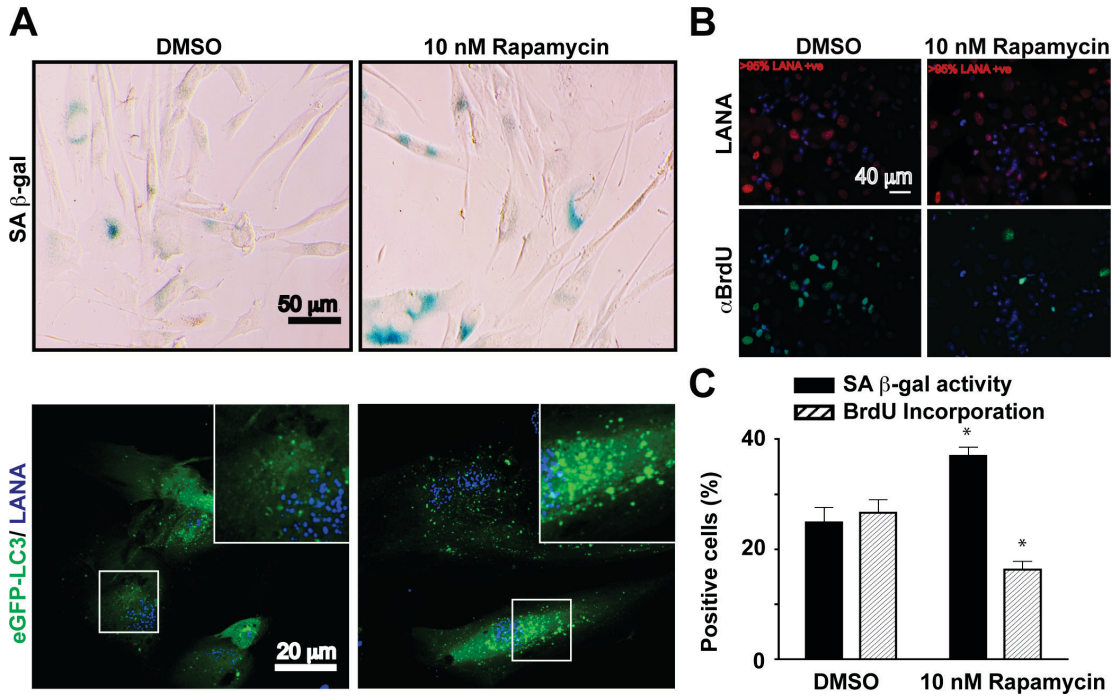

Figure 6.5
Rapamycin Induces Autophagy and Senescence in KSHV Infected Cells
(A) HFFs were infected with KSHV for 3 days to establish latency, and treated with 10 nM rapamycin for five additional days. 1 day prior to SA β-gal staining, a parallel culture of cells was transfected with an EGFP-LC3 reporter construct (green) to visualize autophagosomes. Cells were stained within an anti-LANA antibody (blue) after fixation to demonstrate latent KSHV infection. (B) The proportion of proliferating KSHV infected cells within rapamycin- or vehicle-treated populations was measured by BrdU incorporation assays and anti-LANA co-staining. Representative images are shown. (C) Quantification of cells that stained positive for SA β-gal and incorporated BrdU, from panel B. Values are means ± SEM of two independent experiments whereby 200 cells were enumerated per condition in each experiment. * $P < 0.05$ for the indicated parameters analyzed.

that cells latently infected with KSHV do not activate autophagy; however, specific ablation of v-FLIP anti-autophagy functions rescues underlying host-cell autophagy and reveals strongly activated autophagic responses. These data suggest that one function of v-FLIP during latency is suppression of KSHV-triggered autophagy. Although the precise stresses that stimulate autophagic signaling during latent infection remain unclear, it is highly probable that KSHV-induced DDRs are partly responsible. The notion of KSHV-induced DDRs as the trigger for autophagy is further strengthened by the fact that restoration of autophagy, or super-activation of autophagy (with rapamycin) induced latently infected cells to senesce. Therefore, subversion of autophagy by v-FLIP is an important mechanism for mitigating virus-triggered stress and OIS responses during latent infection.

Is senescence an intrinsic defense against KSHV infection? A cursory survey of the genes encoded within the KSHV latency program strongly suggests that KSHV has evolved to evade or impair host-cell senescence responses; therefore, OIS must represent a host defense against virus spread. Numerous KSHV latent-gene products subvert pathways that are important effectors of senescence. For example, the LANA protein specifically modulates the key senescence effectors p53 and Rb (Friborg et al., 1999; Radkov et al., 2000). Furthermore, LANA degrades GSK-3β, which stabilizes β-catenin and likely impairs senescence induction through the Wnt pathway (Fujimuro et al., 2003; Ye et al., 2007a). Certainly, evolution of LANA toward inhibition of senescence cannot be mere coincidence; its is probable that subversion of these pathways benefits KSHV fitness.

Latent KSHV also subverts the Rb pathway through expression of v-cyclin, a viral homolog of cellular D-type cyclins that complexes with CDK6 (Li et al., 1997). Remarkably, v-cyclin has evolved to activate CDK6 through mechanisms that render this heterodimeric complex refractory to inhibition by Cip/Kip and INK4 family members (Swanton et al., 1997). Recognition that CKIs, including p21 and INK4a, are often only induced by genomic or oncogenic stress suggests that latent KSHV infection elicits these stresses, and that this has shaped the evolution of v-cyclin to maintain CDK6 activity

despite the presence of cell-cycle inhibitors. Intriguingly, v-cyclin is so proficient at promoting host-cell DNA replication that it actually induces OIS (Koopal et al., 2007), the very thing it has evolved to avoid.

In this work, I have shown that expression of KSHV v-FLIP facilitates suppression of autophagy and OIS during latent infection. Although v-FLIP expression, and its associated anti-autophagy functions, likely have numerous benefits for KSHV latency, including immune evasion and alteration of host cell metabolism, I believe that suppression of OIS also contributes to v-FLIP evolution within the latency program. First, v-FLIP is expressed from an IRES within the same transcript that encodes v-cyclin (Grundhoff and Ganem, 2001). Evolution of the unique gene expression arrangement strongly suggests that the functions of v-cyclin and v-FLIP are interdependent; I show that co-expression of v-FLIP with v-cyclin strongly suppresses v-cyclin-induced autophagy and senescence. Second, inspection of the latent gene expression programs of related γ-herpesviruses reveals conservation of the common LANA/v-cyclin/v-FLIP transcription unit in rhesus rhadinoviruses and HVS, but not within the extensively studied murine γ-herpesvirus-68 (MHV-68) (Speck and Ganem, 2010). In fact, MHV-68 encodes LANA and v-cyclin homologs, but does not encode a v-FLIP homolog. Instead, MHV-68 encodes M11, a latently expressed v-Bcl-2 homolog and potent repressor of Beclin 1(Ku et al., 2008; Sinha et al., 2008), in the same genomic location that v-FLIP would be expected to occupy (Speck and Ganem, 2010). Collectively, these observations strongly suggest that OIS is an intrinsic defense against viruses; furthermore, viruses have evolved sophisticated mechanisms to breach host defenses by subverting senescence effectors, including the cellular autophagic machinery.

What are the functions of DDRs within KSHV infection? Intriguingly, DDR pathways have recently been demonstrated to be important for herpesvirus lytic replication. Elegant studies have shown that diverse herpesviruses, including HSV-1, HCMV, EBV, MHV-68 and KSHV, encode kinases that intentionally activate host DDRs to facilitate lytic replication (Li et al., 2011; Tarakanova et al., 2007; Xie and Scully, 2007). For example, ORF36 of MHV-68 encodes a kinase that directly phosphorylates

H2AX (Tarakanova et al., 2007). This event facilitates activation of ATM, which functions to amplify the DDR signal. Importantly, inhibition of this cascade at the level of ORF36, H2AX or ATM dramatically impairs MHV68 replication in murine macrophages (Tarakanova et al., 2007).

Although DDRs have a well-understood role in supporting γ-herpesvirus lytic replication, their impact on latency is unclear. Latent infection elicits prominent DDRs, which may (i) support maintenance of the latent episomal DNA, or (ii) fine-tune the expression of viral gene products. LANA, the primary regulator of KSHV latency, has been reported to interact with the heterochromatin protein HP-1 and histone methyltransferase SUV391H (Sakakibara et al., 2004). Intriguingly, these LANA binding partners have important roles in regulating SAHF during senescence. In response to DNA damage, SUV39H1 has been shown to catalyze H3K9me modifications that demarcate SAHF (Braig et al., 2005), while HP1 helps create the closed chromatin structure that impairs transcription within these nuclear domains (Zhang et al., 2005). Therefore, in the context of latent infection, I would expect these proteins to facilitate repression of KSHV gene expression, perhaps reinforcing latency. Studies have also recently revealed that host-cell Kruppel-associated box domain-associated protein-1 (KAP1 or Tripartite motif-containing 28 [TRIM28]) potently represses lytic-gene expression during KSHV latency (Chang et al., 2009). KAP1 belongs to a family of antiviral proteins (Nisole et al., 2005), that responds to DNA damage and may even have roles in senescence (Li et al., 2007; Mallette et al., 2010). Therefore KAP1 may facilitate fine-tuning of KSHV gene expression. Collectively, these observations suggest that DDRs may have a significant role to play in KSHV latency.

Does autophagy inhibition contribute to other aspects of KSHV latency? Herpesviruses are subject to regulation by autophagy, and thus encode countermeasures to subvert autophagic processes. Several herpesviruses, including KSHV, encode Bcl-2-like proteins that inhibit autophagy by direct interaction with the Beclin 1 autophagy protein (Ku et al., 2008; Liang et al., 2006; Pattingre et al., 2005). These proteins are expressed during the lytic cycle, concurrent with viral assembly, and are thought to

restrict the ability of the autophagic machinery to target newly assembled virions for xenophagic degradation, or inhibit the autophagy-facilitated presentation of viral antigens to the immune system. Although KSHV virions are not made during latency, autophagy may have roles in the degradation of long-lived latent viral proteins. In fact, autophagy has been shown to be required for the efficient presentation of EBV EBNA1 latent antigen, a homolog of KSHV LANA, to CD4 T-cells (Paludan et al., 2005). Therefore, in addition to blocking host anti-proliferative responses to v-cyclin expression, v-FLIP may also antagonize host antiviral immune responses to facilitate viral persistence.

CHAPTER 7 - CONCLUSIONS

7.1 Subversion of Autophagy by KSHV Impairs Senescence

KSHV is the infectious causative agent of KS, a complex neoplasm of the endothelium. The driving force of KS is the latently infected endothelial cell, wherein viral gene products stimulate cell proliferation and reprogram host-gene expression. Although much is known about the functions of individual latent-gene products, it still remains unknown how they collaborate to induce tumorigenesis. Research efforts have focused on three KSHV latent proteins, LANA, v-cyclin and v-FLIP, that are expressed as part of a common transcription unit within the viral genome. Each of these latent proteins has been shown to engage critical proliferation pathways linked to tumorigenesis; however, transgenic mice engineered to express LANA, v-cyclin or v-FLIP only develop lymphoid malignancies with low frequency and after long latency (Chugh et al., 2005; Fakhari et al., 2006; Verschuren et al., 2004a). Furthermore, studies have revealed that ectopic expression of LANA, v-cyclin or v-FLIP in cultured cells does not induce transformation, and v-cyclin actually promotes proliferation arrest. Given the relatively modest effects of KSHV oncogenes *in vitro* and *in vivo* and their unique expression cassette, I reasoned (i) that host cells may restrict the functions of KSHV oncoproteins and (ii) the functions of LANA, v-cyclin and v-FLIP in subversion of cell proliferation pathways may be interdependent.

To test these hypotheses, I first attempted to characterize host-cell responses to individual KSHV latent oncoproteins. My studies revealed that v-cyclin potently induces proliferation arrest and it is the only KSHV latent protein to do so. v-Cyclin arrest was characterized by hallmarks of OIS, including increased SA β-gal activity, activation of DDRs, formation of SAHF and activation of the SASP (Chapter 3). Remarkably, I also demonstrated that autophagy is sharply up-regulated during v-cyclin OIS and necessary for efficient transition to the senescent phenotype (Chapter 4). Activation of autophagy during v-cyclin OIS appeared to involve negative feedback on the mTOR signaling.

Together, these observations demonstrate that host cells restrict the functions of v-cyclin through execution of autophagy and OIS.

Recently KSHV v-FLIP was discovered to be a potent inhibitor of autophagy (Lee et al., 2009). This discovery perplexed many since the KSHV latency program produces no virions. However, I was intrigued by this revelation, since I knew v-cyclin OIS to be autophagy dependent and that v-FLIP is co-expressed with v-cyclin and LANA as part of the KSHV oncogenic cluster. My studies subsequently revealed that co-expression of v-FLIP along with v-cyclin potently inhibits v-cyclin-induced autophagy and senescence (Chapter 5). These results helped to explain observations that KSHV infection induces DDRs, yet latently infected cells fail to senesce. To firmly establish a role for v-FLIP in the suppression of viral OIS, I blocked v-FLIP anti-autophagy functions within latently infected cells, effectively rescuing host-cell autophagy, and observed increased incidence of senescence (Chapter 6). Collectively, my studies reveal that v-FLIP subverts autophagy to impair OIS in cells latently infected with KSHV (Figure 7.1).

7.2 Viral Subversion of Autophagy and OIS: Paradigm or Unique Circumstance?

Microbial pathogens that successfully parasitize eukaryotic cells have evolved in the setting of selective pressures imposed by autophagy, senescence and immune responses. Consequently, it is not surprising that viruses such as KSHV have developed sophisticated strategies to avoid clearance and reinforce host-cell proliferation. The discovery that KSHV subverts autophagy, in part, to impair host-cell senescence responses immediately leads to queries of whether this is a common strategy or unique circumstance.

Unquestionably, KSHV is the first example of an oncovirus that directly inhibits the autophagic machinery to impair host cell OIS (Leidal et al., 2012). However, recent reports suggest that many oncoviruses have evolved to activate mTOR signal transduction, and may promote senescence bypass through this mechanism. For example, the LMP2A latent protein of EBV has been shown to potently trigger mTORC1 activation through the PI3K/Akt pathway (Moody et al., 2005). EBV, similar to KSHV, has been

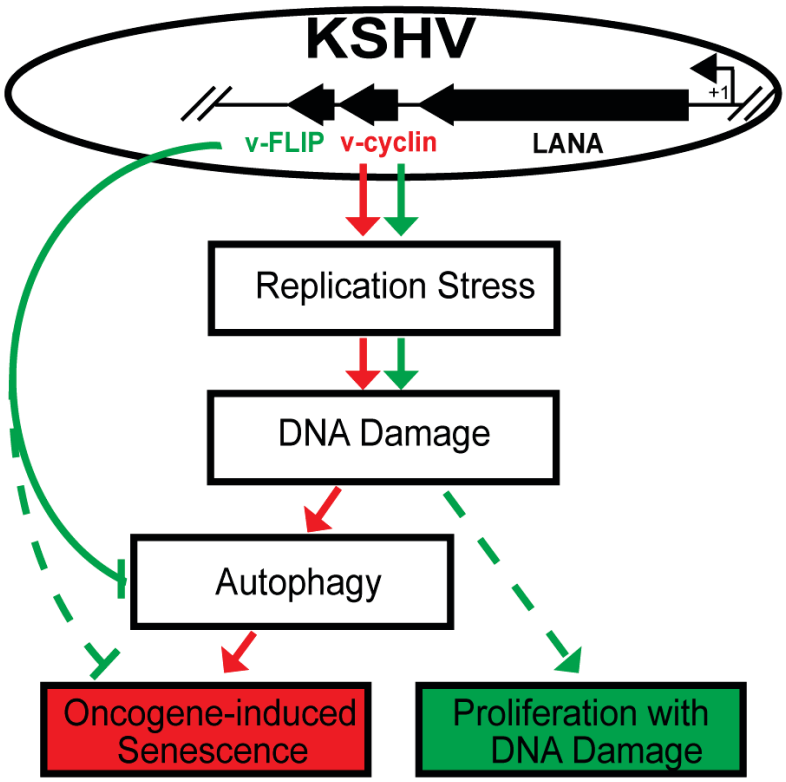

Figure 7.1
Proposed Model for KSHV Subversion of Senescence and Autophagy
v-Cyclin function in latently infected cells induces replication stress and DNA damage. If left unchecked, this would typically induce autophagy and OIS; however, KSHV has evolved to coexpress v-FLIP with v-cyclin, which functions to subvert host-cell autophagic machinery and impair OIS. This scheme ultimately helps latently infected 'spindle cells' avoid OIS and facilitates continued proliferation despite intrinsic genomic damage.

reported to induce oncogenic stress within latently infected B cells, and therefore may employ mechanisms to suppress OIS (Nikitin et al., 2010a). Although the status of autophagy and senescence pathways in cells ectopically expressing LMP2A has not been examined, this potent signaling molecule can transform epithelial cells and inhibit differentiation (Scholle et al., 2000). Taken together, these observations suggest LMP2A may function to suppress autophagy and OIS in cells latently infected with EBV. Another oncovirus that has been shown to trigger host cell DDRs is the human papillomavirus (HPV) (Moody and Laimins, 2009). It has recently been reported that the HPV E6 oncoprotein, which is a well-characterized inhibitor of p53, robustly activates mTORC1 through Akt (Spangle and Munger, 2010). Importantly, the E6 oncoprotein has also been shown to be essential for repression of senescence pathways in HPV-infected cervical carcinoma cells, and ablation of its function in this context promotes OIS (DeFilippis et al., 2003; Horner et al., 2004).

Finally, I have recently begun examining the paradoxical activation of autophagy by the KSHV lytic protein vGPCR. This potent signaling protein has been reported to robustly activate the PI3K/AKT and mTOR signaling pathways in KSHV infected cells undergoing lytic replication (Sodhi et al., 2006); furthermore vGPCR is responsible for elaborating a profound secretory phenotype that contributes to KS pathogenesis (Montaner et al., 2004). Remarkably, vGPCR also induces some hallmarks of senescence when expressed ectopically in TIME cells, including proliferation arrest and up-regulation of SA β-gal (Cyr, Olsthoorn, Leidal and McCormick, unpublished data). Although lytically infected cells are destined to perish, and therefore are unlikely to undergo senescence, the stresses triggered by vGPCR may be detrimental to viral fitness. Intriguingly, the lytic replication program of KSHV also encodes an inhibitor of autophagy, v-Bcl-2 (Pattingre et al., 2005). The functions of v-Bcl-2 during lytic replication may serve to dampen autophagic signaling triggered by vGPCR, thus preventing xenophagy of nascent KSHV virions or premature cell death. Collectively, these observations illustrate that oncoviruses frequently suppress autophagy by mTORC1 activation or other mechanisms, and this likely contributes to impairment of host-cell

anti-proliferative responses, including OIS. Therefore, suppression of autophagic pathways likely represents a paradigm for oncoviruses as they simultaneously attempt to thwart xenophagic clearance, promote host-cell proliferation and evade immune activation.

7.3 Therapeutically Targeting mTOR

Critical roles for mTOR signaling in autophagy regulation and viral-subversion strategies renders this target therapeutically attractive for the treatment of virus-associated malignancies. My work has shown that treatment of KSHV-infected cells with rapamycin induces autophagy and increases the incidence of senescence. Rapamycin has also been demonstrated to be effective in the treatment KS (Kolhe et al., 2006; Stallone et al., 2005). Together, these observations suggest that rapamycin-induced senescence may contribute to the effectiveness of this drug towards KS. It will be interesting to determine whether next-generation mTOR inhibitors, which have greater specificity, are more efficacious at inducing autophagy and senescence induction in KS tissues. The increased specificity of new mTOR inhibitors is rooted in their mode of action (Benjamin et al., 2011). Classically, rapamycin inhibits mTORC1 through association with FKBP12 and allosteric inhibition of the mTOR FRB domain. Because FKBP12-rapamycin does not interact with mTORC2, this complex is not inhibited by rapamycin. The failure of rapamycin to inhibit mTORC2 is a significant drawback, since the complex has important functions in regulating Akt, modulating the cytoskeleton and antagonizing pro-autophagy signaling. Therefore, new ATP-competitive mTOR inhibitors, such as Torin or AZD8055, were developed to target both mTORC1 and mTORC2. These new inhibitors have been found to dramatically induce autophagic activity in trials, and may prove more effective than rapamycin in the treatment of cancer (Benjamin et al., 2011; Vakana et al., 2010).

Virus-associated malignancies are likely to be particularly amenable to treatment with mTOR inhibitors because they frequently harbor intact tumor-suppressor pathways that have only become deregulated through the course of viral subversion. Therefore, attenuation of mTOR subversion has the potential to restore cellular tumor-suppressor

functions, such as the senescence program, and promote clearance of viral effectors through autophagy. Intriguingly, links between the cellular senescence program and innate immune system might enable clearance of KS lesions if latently infected tumor cells can be forced to senesce with new ATP-competitive mTOR inhibitors.

7.4 Prospects for the Future

The results presented in this thesis provide strong evidence that KSHV v-FLIP subverts autophagy pathways to facilitate breach of the host cell OIS program. It is important to emphasize that v-FLIP expression does not completely 'release' v-cyclin from anti-proliferative mechanisms to drive host-cell growth and division. DDRs and other tumor-suppressor mechanisms still remain somewhat intact and act redundantly to restrict deregulated proliferation. This is why KSHV is best regarded as being necessary for KS tumorigenesis, but not sufficient to induce KS tumorigenesis. Therefore, v-FLIP subversion of autophagy represents one of the numerous cellular events that contribute to KSHV-induced malignancies.

Collectively, this work expands our view of autophagy and senescence as tumor-suppressor mechanisms and highlights the sophisticated ways that oncoviruses, such as KSHV, interfere with host defenses. Much work needs to be done to better understand how autophagy promotes senescence and identify viruses that employ subversion strategies similar to KSHV. With this knowledge in hand, we will likely be better equipped to treat the diverse set of malignancies linked to oncoviruses.

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