KSHV vGPCR: A VIRAL ONCOPROTEIN THAT TRIGGERS AUTOPHAGY AND CELLULAR SENESCENCE

by

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DEDICATIONS

I would like to dedicate this work to my father, Patrick Joseph Cyr (1946 – 1987), and to my Nana, Elizabeth Cyr (1923 – 2008), both of whom lost their lives to pancreatic cancer. We love you and miss you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Autophagy (literally to 'self-eat') is an intracellular, catabolic mechanism to degrade and recycle cytoplasmic contents in response to metabolic, oxidative, and genotoxic stresses. Autophagy plays an important role in cellular homeostasis, and dysfunctional autophagic activity has been implicated in an array of human diseases. Importantly, autophagy has recently been identified to function in host defence against intracellular pathogens, including viruses. For this reason, many viruses have evolved strategies to subvert or exploit autophagy and block its antiviral effects. Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi's sarcoma (KS), an AIDS-related cancer of the endothelium. KSHV gene products have evolved to support viral replication and evade immune surveillance. Some of these same gene products impact KS tumourigenesis, but the precise mechanisms have yet to be elucidated. Furthermore, the impact of autophagy on KSHV replication and KS tumourigenesis remains unexplored.

The KSHV viral G-protein-coupled receptor (vGPCR) is a constitutively active signalling molecule that stimulates a number of host regulatory pathways that would be expected to impact autophagy, including PI3K/Akt/mTOR and JNK. Moreover, vGPCR is expressed during lytic replication when the antiviral autophagic response may threaten virion production. Here, vGPCR activity has been definitively shown to trigger autophagy in endothelial cells using immunoblot analysis, fluorescent reporter proteins, and transmission electron microscopy. Furthermore, preliminary data suggest that this stimulatory effect is evoked through JNK activation. Taken together, these findings indicate that vGPCR likely elicits autophagic responses during KSHV lytic replication.

Recently, autophagy has been recognized as a molecular barrier to tumourigenesis, influencing cell survival, cell death, or a form of cell cycle arrest called oncogene-induced senescence (OIS). Remarkably, like many host oncogenes, ectopic expression of vGPCR triggers OIS in endothelial cells. This response is dependent on vGPCR signalling activity, as an inactive form of vGPCR (R143A) fails to trigger OIS. Furthermore, vGPCR OIS is atypical in that it does not involve DNA damage responses (DDRs). Together, these autophagic and senescence responses to ectopic vGPCR expression illustrate the potency of its oncogenic potential.

The significance of vGPCR-induced autophagy and senescence during KSHV replication and KS development is presently unclear. I speculate that autophagy represents a hurdle that the virus must overcome *in vivo*. In my working model, potent vGPCR oncogenic signalling activity sets off the alarm, eliciting autophagic responses. It seems likely that additional lytic viral gene products may serve to undermine these autophagic responses and permit viral replication and dissemination *in vivo*.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ACD	autophagic cell death
AIDS	acquired immunodeficiency syndrome
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ATCC	American Type Culture Collection
Atg	autophagy-related gene
BCBL	body-cavity-based lymphoma
Bcl-2	B cell lymphoma 2
bFGF	basic fibroblast growth factor
BME	β-mercaptoethanol
bp	basepairs
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CDK6	cyclin-dependent kinase 6
CMA	chaperone-mediated autophagy
Cox-2	cyclooxygenase-2
CST	Cell Signaling Technology, Inc.
d	days
DDR	DNA damage response
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DRAM	damage-regulated autophagy modulator
DSB	double-strand break
dsDNA	double-stranded deoxyribonucleic acid
EBM	endothelial basal medium
EBSS	Earle's balanced salt solution
EBV	Epstein-Barr virus
EGM	endothelial growth medium
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
EV	empty-vector
FACS	fluorescence-activated cell sorting
FADD	Fas-associated death domain
FBS	fetal bovine serum
FLICE	FADD interleukin-1B-converting enzyme
γ-HV68	gamma-herpesvirus 68
g	gravity
GFP	green fluorescent protein
Gro-α	growth-related oncogene-α
GPCR	G-protein-coupled receptor
Gy	Gray
h	hours
h.p.i.	hours post-infection

HAART	highly active antiretroviral therapy
HBSS	Hank's balanced salt solution
HCMV	human cytomegalovirus
hEGF	human epidermal growth factor
hFGF	human fibroblast growth factor
HDMEC	human dermal microvascular endothelial cell
HI	heat-inactivated
HSV-1	herpes simplex virus type 1
HFF	human foreskin fibroblast
HIF-1α	hypoxia inducible factor-1α
HHV	human herpesvirus
HIV	human immunodeficiency virus
HMGA2	high-mobility group A protein-2
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell
HVS	Herpesvirus saimiri
IF	immunofluorescence
IFN-γ	interferon-γ
IGF	insulin-like growth factor
IL	interleukin
IR	ionizing radiation
JIP-1	JNK-interacting protein-1
JNK	c-Jun N-terminal kinase
kapB	kaposin B
Kb	kilobase pair
kDa	kilodalton
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
kV	kilovolts
l	litre
LB	Luria broth
LC3	microtubule-associated protein1 light chain 3B
m	milli
M	molar
MAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
mg	milligram
mm	millimetre
μ	micro
μg	microgram
μJ	microjoule
MAF	musculoaponeurotic fibrosarcoma
MAPK	mitogen-activated protein kinase
MCD	multicentric Castleman's disease
MEF	mouse embryo fibroblast
MEM	minimum essential medium

MHC	major histocompatibility complex
min	minute
MK2	MAPK-associated protein kinase 2
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MW	molecular weight
n	nano
neo	neomycin
NF-κB	nuclear factor-κB
OIS	oncogene-induced senescence
ORF	open reading frame
p-	phosphorylated
PAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PEI	polyethylenimine
PEL	primary effusion lymphoma
PI3K	phosphatidylinositol 3-kinase
pRb	retinoblastoma protein
PRRs	pattern recognition receptors
PSQ	penicillin/streptomycin/L-glutamine
puro	puromycin
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
Rta	replication transactivation activator
SABG	senescence-associated β-galactosidase
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulphate
sec	second
TEM	transmission electron microscopy
TIME	telomerase-immortalized microvascular endothelial
TBS	Tris-buffered saline
TBST	Tris-buffered saline + 0.05% tween 20
TLR	Toll-like receptor
TNF-α	tumour necrosis factor-α
UTR	untranslated region
UV	ultraviolet
vCyclin	viral cyclin
VEGF	vascular endothelial growth factor
vFLIP	viral FLICE inhibitory protein

vGPCR	viral G-protein-coupled receptor
vIL-6	viral interleukin-6
VSV	vesicular stomatitis virus
w.t.	wild type
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

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CHAPTER 1 – INTRODUCTION

1.1 Overview

Viruses are opportunistic pathogens that are incapable of autonomous replication. As obligate intracellular parasites, viruses must enter host cells and assimilate cellular biosynthetic machinery to orchestrate viral gene expression, assemble progeny virions, and maintain infection. By coevolving with host cells, viruses have developed elegant strategies to undermine antiviral mechanisms. Additionally, many DNA viruses deregulate the cell cycle to provide an optimal environment for genome replication, inadvertently promoting tumourigenesis and cancer development.

Autophagy is an essential cellular mechanism that serves in a myriad of critical biological processes, and dysfunctional autophagy has been associated with a number of diseases including cancer. Autophagy also aids cellular defences against invading pathogens such as viruses; not surprisingly, many viruses have developed mechanisms to subvert autophagy and promote their persistence in the host. Interestingly, no oncogenic pathogen to date has been found to hijack autophagic activity to facilitate tumour development. Accumulating evidence suggests that the Kaposi's sarcoma-associated herpesvirus (KSHV) likely deregulates autophagy in host cells, either to allow viral replication, evade immune surveillance, or (perhaps inadvertently) to promote tumourigenesis. The details remain to be elucidated.

The following introduction provides an overview of the mechanics and regulation of autophagy. The biological importance of autophagy is described with an emphasis on its roles in governing cell fate and facilitating immunity against viruses. Furthermore, KSHV-associated pathologies, virion structure, replication, and gene expression profiles are reviewed, highlighting target viral oncogenes. Finally, the potential links between autophagy and KSHV pathogenesis are illustrated.

1.2 Autophagy

Autophagy (derived literally from the Greek words for 'self' and 'eat') is an intracellular catabolic process that controls the quantity and quality of intracellular biomass (Klionsky and Emr, 2000). Autophagy is evolutionarily conserved across all

eukaryotic organisms, and although it was first described about 50 years ago, the complex molecular underpinnings and physiological importance of autophagy have only recently begun to be appreciated (history of autophagy reviewed in Yang and Klionsky, 2010). Autophagy has essential roles in many important biological processes, including development and differentiation (Mizushima and Levine, 2010), metabolism and homeostasis (Vousden and Ryan, 2009; Rabinowitz and White, 2010), the integrated stress response (Kroemer *et al*., 2010), and immunity (Levine and Deretic, 2007; Levine *et al*., 2011). Not surprisingly, dysfunctional autophagic activity has therefore been implicated in human health and the pathogenesis of many diseases (reviewed in Levine and Kroemer, 2008; Mizushima *et al*., 2008); particularly, deregulated autophagy is instrumental in aging (Madeo *et al*., 2010), neurodegenerative conditions (Nixon, 2006), infectious diseases (Deretic and Levine, 2009; Deretic, 2010), and cancer (Jin and White, 2007; Levine, 2007; Mathew *et al*., 2007; Maiuri *et al*., 2009b; Liang and Jung, 2010). Consequently, the development of therapeutic applications that target autophagic processes has received significant attention (Rubinsztein *et al*., 2007). Nevertheless, advancing our molecular understanding of autophagy and fully elucidating its link to disease remains at the forefront of biomedical research.

1.2.1 The Macroautophagic Process

To date, three different autophagy-related processes have been characterized in eukaryotic cells: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA; Figure 1.1). Microautophagy involves the internalization of cytoplasmic cargo, including large structures, directly at the lysosome by invaginations of the lysosomal membrane; in contrast, CMA selectively targets soluble proteins by chaperone-mediated recognition, and these cytosolic substrates are translocated into lysosomes for degradation (reviewed in Massey *et al*., 2006; Klionsky *et al*., 2007; Mizushima *et al*., 2008; Cuervo, 2010a). Microautophagy and CMA will not be further described here. Macroautophagy (hereafter referred to as autophagy) is the major intracellular catabolic process used for the large-scale degradation and recycling of long-lived proteins, macromolecular aggregates, damaged organelles (such as mitochondria), and other intracellular biomass (Dunn, 1994; Klionsky and Emr, 2000). Selective autophagic

degradation of individual organelles and other cytoplasmic structures, along with the extensive associated nomenclature, is described elsewhere (Klionsky *et al*., 2007; Klionsky *et al*., 2010).

Upon the initiation of autophagy by pro-autophagic stimuli (see section 1.2.2), a flattened membranous sac, known as a phagophore or isolation membrane, begins to sequester the portion of cytosol or organelle destined for degradation (Figure 1.1). The origin of the isolation membrane has remained elusive, although recent evidence has implicated the plasma membrane (Ravikumar *et al*., 2010), mitochondria (Hailey *et al*., 2010), the endoplasmic reticulum (Axe *et al*., 2008), and the trans-Golgi network (Young *et al*., 2006) as disparate autophagosomal membrane sources (Cuervo, 2010b). The phagophore elongates by the addition of new membrane and eventually seals around the cytoplasmic cargos, forming a double-membrane organelle known as an autophagosome, the hallmark structure of autophagy. During maturation, the autophagosome fuses with a late endosome or lysosome to form an autolysosome, which contains lysosomal hydrolytic enzymes. Dissolution of the inner autolysosomal membrane and degradation of its contents follows, and lysosomal membrane permeases mediate export of the resulting macromolecules. Detailed characterizations of the macroautophagic process have been reviewed (Klionsky and Emr, 2000; Yorimitsu and Klionsky, 2005; Mizushima *et al*., 2008).

1.2.2 Molecular Mechanism and Cellular Regulation

Autophagy is a dynamic process that responds to a variety of stresses under different conditions. Not surprisingly, such a process requires a complex interplay between a multitude of mediators, known as the autophagy-related gene (Atg) proteins, which are highly conserved from yeast to higher eukaryotes (Mizushima *et al*., 1998). Importantly, numerous molecular cascades converge on the Atg proteins to regulate the execution of autophagic activity (reviewed in Levine and Klionsky, 2004; Yorimitsu and Klionsky, 2005; Klionsky, 2007; Rubinsztein *et al*., 2007; Maiuri *et al*., 2007; Levine and Kroemer, 2008; Maiuri *et al*., 2009a).

Molecular regulation of autophagy predominantly centers around two key players: the mammalian target of rapamycin (mTOR) and Beclin 1; notably, mTOR is an

important regulator of cell growth and metabolism (Wullschleger *et al*., 2006; Pattingre *et al*., 2008). In the presence of abundant nutrients and growth factors, active signalling through the class I phosphatidylinositol 3-kinase (PI3K) and downstream activation of Akt (also known as protein kinase B) functions to activate mTOR, which is a critical negative regulator of autophagy; however, during starvation conditions the signalling through the PI3K/Akt/mTOR axis is ablated, relieving this mTOR-mediated suppression of autophagy (Figure 1.2; Wullschleger *et al*., 2006; Pattingre *et al*., 2008). Inhibition of mTOR, by nutrient deprivation or with rapamycin, a pharmacological inhibitor of mTOR, permits functional activity of Beclin 1, an essential autophagy protein located downstream of mTOR in a complex with class III PI3K and other regulators (Pattingre *et al*., 2008). Ultimately, active Beclin 1 stimulates a cascade of Atg proteins to execute the autophagic process, and dysfunctional or deregulated Beclin 1 activity abolishes cellular autophagy (Levine and Kroemer, 2008; Mizushima *et al*., 2008).

Importantly, the anti-apoptotic protein Bcl-2 was identified as a negative regulator of Beclin 1 via direct interaction (Pattingre *et al*., 2005; Maiuri *et al*., 2007). This characterization not only revealed a complex interplay between the regulatory mechanisms underlying apoptosis and autophagy (Maiuri *et al*., 2007), but it also led to the identification of c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) as a critical positive regulator of autophagy that acts downstream of mTOR (Wei *et al*., 2008b; 2008a; Sinha and Levine, 2008). During starvation, JNK mediates the phosphorylation of Bcl-2, which results in the dissociation of Bcl-2 from Beclin 1, liberating Beclin 1 to assemble autophagy-promoting class III PI3K complexes.

The last regulatory mechanism of interest is governed by the tumour suppressor protein p53. Once considered the 'guardian of the genome,' p53 is critical in regulating genomic stability, cell proliferation, cell death, and senescence (reviewed in Vousden and Lane, 2007). Although DNA damage activates p53 and triggers a p53-dependent autophagic response (Feng *et al*., 2005), the exact role of p53 in regulating autophagy is complex and controversial (Levine and Abrams, 2008; Maiuri *et al*., 2009b; Green and Kroemer, 2009; Maiuri *et al*., 2009a). In brief, nuclear p53 transactivates a number of p53-target genes, including *DRAM* (damage-regulated autophagy modulator) and *sestrins 1* and *2*, which in turn activate AMP-activated protein kinase (AMPK; Crighton *et al*.,

2006; Budanov and Karin, 2008). AMPK is an energy-sensing kinase that activates autophagy in mTOR-dependent (via TSC2 phosphorylation; Inoki *et al*., 2003) and independent (via ULK1 phosphorylation; Kim *et al*., 2011; Egan *et al*., 2011) mechanisms. In contrast, cytoplasmic p53 inhibits autophagy by an unknown mechanism and must be degraded for autophagic activity to occur (Tasdemir *et al*., 2008). Importantly, deregulation of autophagy at many of its control points results in a dysfunctional process, which has been associated with many human diseases and is emerging as key targets for therapeutic developments (Rubinsztein *et al*., 2007; Levine and Kroemer, 2008; Mizushima *et al*., 2008).

1.2.3 Involvement in Cell Fate

Since the genetic link was established between autophagy and cancer, one of the first diseases associated with dysfunctional autophagy, the molecular underpinnings of this paramount process has been a focal point of biomedical research (Mathew *et al*., 2007; Levine and Kroemer, 2008; Mizushima *et al*., 2008). In fact, the essential autophagy gene *beclin 1* is a haploinsufficient tumour suppressor gene that is mono-allelically deleted in 75% of ovarian, 50% of breast, and 40% of prostate cancers (Aita *et al*., 1999), and further evidence identifying Beclin 1 as a tumour suppressor has been obtained from *in vivo* mouse models (Qu *et al*., 2003; Yue *et al*., 2003). This suggests that autophagic activity functions in tumour suppression. Moreover, many tumour suppressor genes that are frequently mutated or silenced in human malignancies (*p53, PTEN, TSC1/TSC2*) stimulate autophagy, whereas many hyperactive oncogenes (those encoding class I PI3K, Akt, mTOR, Bcl-2) inhibit autophagy (Botti *et al*., 2006). Although autophagy is accepted as a true tumour suppressor pathway, a paradoxical role in oncogenesis has also been described (reviewed in Levine, 2007; Levine and Kroemer, 2008). The underlying explanation for this phenomenon dwells on autophagy as a fundamental player in dictating cell fate; indeed, autophagy is particularly important in cytoprotective processes, including cell survival and a form of irreversible cell cycle arrest known as senescence, but autophagy is also controversially considered an important mechanism for non-apoptotic cell death (Levine and Kroemer, 2008;

Mizushima *et al*., 2008; Young and Narita, 2010). Here, the involvement of autophagy in cell survival, senescence, and cell death is briefly described.

1.2.3.1 Cell Survival

Autophagy is predominantly characterized as a pro-survival mechanism for the cell; indeed, basal levels of autophagy serve to maintain normal homeostasis, but autophagy is also a fine-tuned adaptive stress response that is equipped with cytoprotective processes to deal with a variety of conditions (Levine and Kroemer, 2008; Mizushima *et al*., 2008; Moreau *et al*., 2010). In its most basic form, autophagy functions to allow cells to 'self-cannibalize' during starvation stress – when cells are deprived of nutrients, autophagic degradation of non-essential cellular components provides the cell with the essential nutrients to maintain energy metabolism and survival (reviewed in Mizushima and Klionsky, 2007). Importantly, autophagy is not only required for the survival of single-cell organisms, such as yeast, during nutrient deprivation, but it is also essential for organismal survival during starvation, such as during the early neonatal period; indeed, mice lacking *Atg5* or *Atg7*, essential autophagy genes, are defective for autophagy and die during this neonatal stage of development (Kuma *et al*., 2004). Autophagy also limits the accumulation of cytoplasmic misfolded-protein aggregates, which would otherwise build up in the cell and impede normal cellular functions (Rubinsztein, 2006). Mitophagy, the selective autophagic degradation of mitochondria, is important in regulating the quantity and quality of these homeostatic organelles (Klionsky *et al*., 2007). Mitochondria are central players in energy metabolism; they are key mediators of the intrinsic apoptotic pathway; and damaged mitochondria are significant producers of reactive oxygen species. The autophagic removal of damaged mitochondria limits reactive oxygen species production to effectively mitigate this genotoxic cellular stress (Jin and White, 2007; Mathew *et al*., 2007). Interestingly, autophagic activity can be differentially regulated by p53 (see section 1.2.2); as such, autophagy can be stimulated in cells defective for p53 (i.e. mutated or absent), which would be characteristic of many cancer cells, and this effectively promotes cell survival, possibly by mitophagy (Tasdemir *et al*., 2008; Maiuri *et al*., 2009b; Green and Kroemer, 2009). Lastly, autophagy has been recently characterized as an important cellular anti-

microbial defence (see section 1.2.4). Given the paramount importance of autophagy in protecting cells from deleterious stresses, it should come as no surprise that dysfunctional or deregulated autophagic activity is associated with a number of human diseases, including cancer.

1.2.3.2 Cellular Senescence

Cellular senescence is an irreversible form of cell cycle arrest triggered by persistent DNA damage in response to 'replicative exhaustion' or genotoxic stresses. Notably, senescence can be induced by chronic oncogene expression, termed oncogene-induced senescence (OIS), and has been implicated as a fundamental tumour suppressor mechanism. A detailed characterization of OIS, the associated molecular underpinnings, and its biological importance, are described in section 4.1. Importantly, a landmark study has recently demonstrated that autophagic activity is required for efficient execution of the senescence program (Young *et al*., 2009). Expression of H*ras*^{V12}, a robust oncogene and trigger of OIS, induced a persistent DNA damage response (DDR) that led to p53 transactivation of target genes and mTOR-dependent activation of autophagy; furthermore, inhibition of autophagic activity delayed the onset of OIS (Young *et al*., 2009). Although the exact role of autophagy in driving the OIS transition remains unclear, it may function in cellular remodelling, prolonged metabolic homeostasis, and/or the mitigation of cellular damage (discussed in White and Lowe, 2009). This novel link between autophagy and senescence, two putative tumour suppressor pathways, has set the stage to uncover their complex, cooperative roles in oncogenesis and cancer (Young and Narita, 2010).

1.2.3.3 Cell Death

Although autophagy is primarily considered a stress-response pathway that promotes cell survival, some evidence has implicated a role, albeit controversial, for autophagy as a pro-death mechanism. In response to cellular insults and damage that is beyond repair (i.e. following prolonged hyperproliferation), cells may undergo the canonical programmed cell death known as apoptosis, a common tumour suppressor mechanism (Kerr *et al*., 1972). However, a form of non-apoptotic cell death, known as

type II programmed cell death or 'autophagic cell death' ('ACD'), has recently been described (reviewed in Levine and Yuan, 2005; Maiuri *et al*., 2007; Kroemer and Levine, 2008). Morphologically, cells succumbing to 'ACD' lack chromatin condensation, in contrast to apoptotic cells, and there is large-scale autophagic vacuolization of the cytoplasm (Galluzzi *et al*., 2007). Initially, 'ACD' was considered a mechanism whereby cells were dying by autophagy, meaning that cell death was caused by large-scale autophagic degradation of molecules essential for cell survival. However, the emerging consensus from multiple studies involving knockdown of Atg genes suggests that the mere presence of autophagosomes in dying cells is insufficient to clearly implicate autophagy in cell death (Maiuri *et al*., 2007; Kroemer and Levine, 2008). Furthermore, 'ACD' may act in concert with apoptosis, as there is a complex interplay and cross-regulation between both of these processes (Maiuri *et al*., 2007). Overall, there is overwhelming evidence characterizing autophagy as a principal decision-maker in cell fate and associating dysfunctional autophagy with oncogenesis and cancer.

1.2.4 Involvement in Immunity

The autophagic pathway has recently emerged as a central player in the cellular defence against intracellular pathogens. A multipronged attack is elicited by the autophagic machinery that includes the direct elimination of pathogens by their selective sequestration into autophagosomes (a processed known as xenophagy, 'to eat what is foreign'), and the delivery of microbial nucleic acids and peptide antigens to autophagic structures for activation of innate and adaptive immune responses (Figure 1.3). Our understanding of the autophagic arsenal of immune functions is expanding at an incredible rate, and our appreciation of these processes has been extensively reviewed (Kirkegaard *et al*., 2004; Schmid and Münz, 2007; Levine and Deretic, 2007; Orvedahl and Levine, 2008; Münz, 2009; Deretic and Levine, 2009; Levine *et al*., 2011). Here, xenophagy and the involvement of autophagy in activating innate and adaptive immune responses to invading pathogens, as well as viral adaptation to these cellular defences, is briefly described.

1.2.4.1 Xenophagy

Xenophagy is the cell-autonomous elimination of invading pathogens by lysosomal destruction via canonical autophagic activity (Levine, 2005; Mizushima *et al*., 2008; Lin *et al*., 2010b). Xenophagy has been well demonstrated to sequester invading bacteria and parasites following entry and deliver them to lysosomes for degradation, as well as newly synthesized virions during egress from the nucleus into the cytoplasm; however, it is unknown whether xenophagy also targets viruses during cell entry, but this is considered plausible (Levine and Deretic, 2007). Although the exact mechanism(s) underlying how the autophagic machinery specifically targets free, cytosolic pathogens are largely unknown, 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). 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 has been well characterized to limit bacterial infections (Nakagawa *et al*., 2004; Gutierrez *et al*., 2004), evidence regarding the elimination of viruses is lacking; nevertheless, xenophagy is considered critical in capturing newly assembled viruses in host cells for subsequent lysosomal destruction (Kirkegaard *et al*., 2004; Levine, 2005; Wileman, 2007).

1.2.4.2 Innate Immunity

Recently, autophagy has emerged as a novel regulatory process in the immune surveillance of infected host cells. Innate immune activation depends, in part, on PRR-sensing of microbial PAMPs; notably, a subset of TLRs that sense viral nucleic acids have 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 unknown. Interestingly, a landmark study demonstrated that autophagy mediates the recognition of viral ssRNAs by TLR7 in endosomal compartments during vesicular stomatitis virus (VSV) and Sendai virus infections, and this TLR7 activation subsequently triggers a robust type I IFN

antiviral response (Lee *et al*., 2007). Furthermore, autophagic activity can be stimulated upon detection of PAMPS by their cognate PRRs, effectively eliciting a positive feedback loop (Sanjuan *et al*., 2007; Delgado *et al*., 2008). This complex interplay underlies the importance of autophagy in regulating host antiviral innate immune responses.

1.2.4.3 Adaptive Immunity

The role of autophagy in immunity is not restricted to cell-autonomous intracellular pathogen destruction or triggering a robust type I IFN response. Autophagy can promote cytosolic antigen (of viral, self, and tumour origin) presentation on class II major histocompatibility complex (MHC). Following autophagic sequestration and degradation of endogenously synthesized viral antigens, autophagic machinery can deliver the foreign peptides to class II MHC loading compartments (late endosomes) and facilitate antigen presentation in host cells, subsequently activating $CD4^+$ T lymphocytes and an adaptive immune response (reviewed in Schmid and Münz, 2007). This process has been demonstrated with a number of viral pathogens, including influenza (Schmid *et al*., 2007) and EBV (Paludan *et al*., 2005), effectively mediated viral antigen presentation to T cells. Interestingly, canonical class II MHC presentation involves the presentation of exogenous antigens that were acquired by endocytosis and lysosomal degradation; however, autophagic processing permits endogenous antigen presentation to $CD4^+$ T cells (Schmid *et al*., 2007). Together, autophagy's instrumental participation 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.

1.2.4.4 Viral Adaptations to Autophagy

It should come as no surprise that many pathogens have developed elegant strategies to impede autophagic cellular defences, resulting from selective pressure and coevolution with the host to evade immunity and promote persistent infection of the host. The considerable number of anti-autophagic adaptations employed across many different viruses and intracellular bacteria speaks to the paramount importance of this host defence

mechanism; detailed descriptions of these microbial operations have been reviewed elsewhere (Kirkegaard *et al*., 2004; Orvedahl and Levine, 2009; Deretic and Levine, 2009; Dreux and Chisari, 2010; Lin *et al*., 2010b). Although many different autophagic regulators are often targeted, one of the primary targets of viral autophagic subversion is 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; E *et al*., 2009; Leib *et al*., 2009). 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, 2006; Lin *et al*., 2010b). 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; Ke and Chen, 2011), 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-bound vesicles remain as functional components of the autophagic pathway is unknown (Kirkegaard *et al*., 2004; Lin *et al*., 2010b).

1.3 The Herpesviruses

The *Herpesviridae* are a family of large, enveloped viruses with linear, double-stranded DNA (dsDNA) genomes. The herpesviruses can elicit both lytic and latent infections, a hallmark of all herpesvirus replication cycles. Herpesviruses exhibit a

spectrum of cell and tissue tropisms from a wide range of species, including humans, and they are associated with an array of mild to severe diseases. The herpesviruses are categorized into three subfamilies, the alpha-, beta-, and gamma-herpesvirinae, based on commonalities in their size, structure, and genomic homology. The α-herpesviruses are characterized by their relatively short replication cycles and their broad cell tropism; specifically, they can establish latency in neurons. Important human α-herpesviruses include herpes simplex virus type 1 (HSV-1)/human herpesvirus (HHV)-1, HSV-2/HHV-2, and varicella zoster virus (VSV)/HHV-3, the causative agent of chickenpox and shingles. The β-herpesviruses, which include human cytomegalovirus (HCMV)/HHV-5, HHV-6, and HHV-7, largely target leukocytes and typically have long replication cycles. The γ-herpesviruses are known for their restricted host-cell specificity, which primarily include B and T lymphocytes, and latency is preferentially entered following infection. The most relevant members of this subfamily are Epstein-Barr virus (EBV)/HHV-4 and Kaposi's sarcoma-associated herpesvirus (KSHV)/HHV-8. EBV is the causative agent of infectious mononucleosis and, as an oncogenic virus, is also associated with nasopharyngeal carcinoma, Burkitt's lymphoma, and Hodgkin's lymphoma. In addition to the lymphotropism directed by KSHV, this virus can also infect and establish latency in endothelial cells and has been associated with a number of tumour entities. Herpesvirus biology has been well described (Roizman, 1996).

1.4 Kaposi's Sarcoma-Associated Herpesvirus

KSHV is the most recently discovered human herpesvirus and has been identified as the etiological agent for a number of human cancers. The exact mechanisms underlying KSHV pathogenesis remain unknown, but the virus encodes an arsenal of candidate oncogenes that likely work together during lytic replication and latency to orchestrate tumourigenesis. Here, KSHV clinical pathologies, virus structure, replication, and target gene products are reviewed.

1.4.1 Disease Associations

1.4.1.1 Kaposi's Sarcoma

Moritz Kaposi, a Hungarian dermatologist, published a case study in 1872 that described five patients with "idiopathic multiple pigmented sarcomas of the skin," one of whom later developed visceral lesions in the lungs and gastrointestinal tract (Kaposi, 1872; Antman and Chang, 2000). Several years later, Heinrich Koebner coined the term Kaposi's sarcoma (KS) to identify this systemic disease (Mesri *et al*., 2010). There are four distinct epidemiological variants of KS, each representing different clinical manifestations of the same pathological process (reviewed in Beral, 1991; Antman and Chang, 2000; Hengge *et al*., 2002; Boshoff and Weiss, 2002; Szajerka and Jablecki, 2007; Schwartz *et al*., 2008). Classic KS is relatively mild with lesions mainly localized to the skin of the lower extremities, and it predominantly affects elderly men of Mediterranean or Ashkenazi Jewish decent. Endemic KS ranges from benign cutaneous lesions to an aggressive disease invading soft tissue, bone, and viscera; moreover, it is isolated to certain geographical regions of sub-Saharan Africa and patients mainly range from young children to young adults. Iatrogenic, or post-transplant, KS develops in solid-organ transplant recipients receiving chronic immunosuppressive therapies; however, although this type progresses quickly, patients frequently undergo spontaneous remission upon discontinuing immunosuppressive therapies. Epidemic KS, or AIDS-associated KS (AIDS-KS), is the most prevalent disease variant and remains the most common AIDS-associated malignancy worldwide, significantly contributing to the morbidity and mortality of AIDS patients (Beral *et al*., 1990). AIDS-KS is regarded as the most severe form of KS, typically presenting cutaneously on the head, neck, trunk, hard palate, and gums, and later advancing to the lymph nodes, gastrointestinal tract, and lungs. Interestingly, the incidence of AIDS-KS has been greatly reduced since the advent of highly active antiretroviral therapy (HAART), underscoring its dependence on HIV infection and immunosuppression; however, KS continues to be a common AIDS-related comorbidity in developing countries that lack adequate access to HAART (Krown *et al*., 2008; Dittmer, 2010).

Regardless of the clinical manifestation, KS is an unusual malignant neoplasm of the endothelial tissue that commonly presents cutaneously as multifocal dark purple

lesions (Figures 1.4A and B); morphologically, lesions typically progress from patches to plaques to raised nodules, and aggressive forms can lead to systemic illness targeting the viscera (Verma and Robertson, 2003; Mesri *et al*., 2010). The tumour microenvironment is highly vascularized and consists of a prominent inflammatory component; indeed, KS lesions are infiltrated with inflammatory cells (i.e. macrophages), immune cells (i.e. dendritic cells and lymphocytes), as well as vascular and lymphatic endothelial cells (Boshoff and Weiss, 2002). The fundamental driving force of KS is the unique 'spindle cell,' an abnormally elongated endothelial cell whose proliferation directs lesion progression (Figure 1.4C; Browning *et al*., 1994; Boshoff *et al*., 1995; 1997; Boshoff, 1998). Interestingly, the spindle cells comprising each individual KS lesion are clonal, or, in patients who have multiple KS lesions, oligoclonal, signifying metastatic dissemination of spindle cells (Rabkin *et al*., 1995; 1997; Gill *et al*., 1998). The origin of spindle cells has been enigmatic, as these cells simultaneously express markers specific to many cell types, but accumulating evidence suggests they belong to a lymphatic, rather than vascular, endothelial lineage; interestingly, spindle cells have undergone extensive reprogramming of gene expression (Weninger *et al*., 1999; Hong *et al*., 2004). Furthermore, spindle cells are significant contributors to the pro-inflammatory and angioproliferative secretory profile of the tumour microenvironment, expressing high levels of vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), basic fibroblast growth factor (bFGF), tumour necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), key constituents of KS lesions (Salahuddin *et al*., 1988; Ensoli *et al*., 1989; Miles *et al*., 1990; Ensoli *et al*., 1992). Importantly, in 1994 a seminal study isolated and identified herpesvirus-like DNA sequences from KS lesions (Chang *et al*., 1994). This observation led to the discovery of Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8), the etiological agent of KS; indeed, KSHV is the only known risk factor to be essential for KS development (Moore and Chang, 1995; Moore *et al*., 1996). KSHV was quickly associated with a number of lymphoproliferative disorders as well (Cesarman *et al*., 1995), underscoring the paramount clinical importance of this novel oncogenic herpesvirus.

1.4.1.2 Primary Effusion Lymphoma

Primary effusion lymphoma (PEL) is a subtype of non-Hodgkin lymphoma that presents as a lymphomatous effusion in the pleural, pericardial, and peritoneal spaces (Knowles *et al*., 1989; Arvanitakis *et al*., 1996); occasionally, some patients with PEL develop solid tissue masses or lymphadenopathy (Chadburn *et al*., 2004). These 'body cavity-based lymphomas' (BCBLs) occur primarily, albeit not exclusively, in HIV-positive patients suffering from advanced stages of immunosuppression. The effective PEL tumour cells, B lymphocytes, are universally characterized by KSHV infection and are frequently co-infected with EBV (Cesarman *et al*., 1995; Mesri *et al*., 1996; Nador *et al*., 1996; Cesarman *et al*., 1996). Interestingly, KSHV-positive, EBV-negative B-cell lines derived from HIV-negative PEL patients have been established (Arvanitakis *et al*., 1996; Renne *et al*., 1996b). These cell culture systems are used to generate KSHV *in vitro* and have facilitated the detailed study of KSHV molecular biology and pathogenesis.

1.4.1.3 Multicentric Castleman's Disease

Multicentric Castleman's disease (MCD) is the third disorder causally linked with KSHV infection (Soulier *et al*., 1995; Oksenhendler *et al*., 1998). MCD is a rare, B-cell lymphoproliferative disorder that is characterized by recurrent fevers, lymphadenopathy, hepatosplenomegaly, and frequent progression to non-Hodgkin lymphoma (Oksenhendler *et al*., 1996; Oksenhendler, 2002). KSHV has been identified in 100% of HIV-seropositive cases of MCD and nearly 50% of MCD cases in HIV-negative patients (Gessain *et al*., 1996; Dupin *et al*., 1999; Boivin *et al*., 2002). Interestingly, KSHV is specifically associated with a particularly aggressive subtype of MCD, plasmablastic variant MCD, which is characterized by the hyperplasia of KSHV-infected large plasmablastic B cells (Dupin *et al*., 2000). The severity and course of MCD is closely correlated with KSHV viral load, and the disease is thought to be mediated by viral IL-6 (vIL-6); however, further investigation is necessary for successful intervention of MCD (Parravicini *et al*., 1997; Oksenhendler *et al*., 2000; Ahmed *et al*., 2007).

1.4.2 The Virus

1.4.2.1 Virion Structure

KSHV particles (Figure 1.4D) are intermediate in size compared to other herpesviruses; indeed, BCBL-1 particles are approximately 120 to 150 nm in diameter, consisting of a lipid envelope surrounding a 110-nm icosahedral capsid (Said *et al*., 1997; Wu *et al*., 2000). The viral envelope is rich in glycoproteins and, consistent with other herpesvirus virions, a proteinaceous tegument is located between the envelope and the capsid (Said *et al*., 1997). Interestingly, lytic replication in cultured PEL cells triggers the assembly of three distinct types of capsids similar to those found with α- and β-herpesviruses (Nealon *et al*., 2001). Only C type capsids contain the viral genomic DNA, in contrast to the A and B type capsids that are empty and therefore non-infectious (Nealon *et al*., 2001). Furthermore, mature KSHV virions contain the full uniform genome of approximately 165 to 170 kb (Renne *et al*., 1996a; Lagunoff and Ganem, 1997). Although the KSHV genome size is consistent with those of other γ-herpesviruses, it is slightly larger than that of HSV-1, a prototypical α-herpesvirus, and considerably smaller than HCMV, a prototypical β-herpesvirus (Wu *et al*., 2000).

1.4.2.2 Genome Organization

The linear dsDNA genome of KSHV is synthesized and packaged into progeny virions during lytic replication (Renne *et al*., 1996a). Consistent with other γ-herpesviruses, however, the KSHV genome is maintained as a multicopy episome (circular dsDNA) during latency. Genomic circularization occurs through a variable number of tandem 801-bp direct terminal repeats that have a high G + C content (85%); moreover, these terminal repeats are non-coding and they flank the remainder of the KSHV genome, approximately 145 kb in length (Figure 1.5; Russo *et al*., 1996; Neipel *et al*., 1997; Lagunoff and Ganem, 1997). Within this long unique region are at least 87 ORFs, genetically arranged in a strikingly similar manner to Herpesvirus saimiri (HVS), a close γ-herpesvirus relative. KSHV shares 68 conserved genes with HVS that have been numbered consecutively from the 5' to 3' ends of the genome and marked by the prefix 'ORF' (Russo *et al*., 1996); more recently, this conserved genetic organization and gene functionality has been expanded to murine γ-herpesvirus 68 and some primate

rhadinoviruses, other non-human γ-herpesvirus members (Virgin *et al*., 1997; Searles *et al*., 1999; Alexander *et al*., 2000). Viral genes that are unique to KSHV have been designated K1 to K15 (Wen and Damania, 2010). Importantly, KSHV encodes an impressive number of ORFs that share homology to cellular genes, many of which have likely been pirated as a result of viral coevolution within host cells. These viral gene products often have distinct functions compared to their cellular homologs, and they have been strongly implicated in viral pathogenesis (see sections 1.4.4 and 1.4.5).

1.4.3 Replication Cycle: a Brief Overview

A fundamental characteristic of all herpesviruses is the ability to establish both lytic and latent programs; indeed, KSHV is no exception. Consistent with other γ-herpesviruses, latency is predominantly established in clinical KS lesions, KSHV-infected endothelial cell cultures, and cultured PEL specimens, comprising 85 to 99% of infected cells (Zhong *et al*., 1996; Miller *et al*., 1997; Dittmer *et al*., 1998; Sun *et al*., 1999). During latency, replication of the viral genome depends on host-cell DNA-replication machinery and the KSHV episome is propagated during cell division; importantly, no viral progeny are produced in latently infected cells (Renne *et al*., 1996a; 1996b). Furthermore, only a minimal subset of viral genes are expressed during latency (Zhong *et al*., 1996; Sarid *et al*., 1998; Sun *et al*., 1999; Paulose-Murphy *et al*., 2001; Jenner *et al*., 2001; Fakhari and Dittmer, 2002), all of which are putative oncogenes considered instrumental to KS development and tumourigenesis (Liang *et al*., 2008b; Mesri *et al*., 2010). Although it is not clear what stimulates a latently infected cell to undergo lytic reactivation *in vivo*, this transition can be chemically induced *in vitro* using phorbol esters or histone deacetylase (HDAC) inhibitors (Renne *et al*., 1996b). Remarkably, a single viral gene product, ORF50, also known as the replication transactivation activator (Rta), is required to trigger lytic reactivation and initiate the associated gene expression program (Sun *et al*., 1998; Lukac *et al*., 1998; Sun *et al*., 1999; Lukac *et al*., 1999; Jenner *et al*., 2001). This immense lytic expression profile consists of all viral genes that are not expressed during latency, eliciting a productive infection with viral progeny being assembled and released by cell lysis (Sun *et al*., 1999; Paulose-Murphy *et al*., 2001; Fakhari and Dittmer, 2002). Although lytically infected

cells are critical for viral replication and spread, their contribution to KS tumourigenesis is poorly understood as these cells are often short-lived and destined to die.

1.4.4 Latent Gene Expression

The major latency-associated transcripts are all expressed from a small genomic cassette (Figure 1.5; Dittmer *et al*., 1998; Boshoff, 1998). They include the latency-associated nuclear antigen (LANA), viral cyclin (vCyclin), viral FLIP (vFLIP), the kaposins, and the viral-encoded microRNAs (miRNAs). These latent gene products are considered the fundamental viral effectors underlying KS tumourigenesis, promoting cell growth and proliferation, inflammation and neo-angiogenesis, evasion of apoptosis, and profound replication potential; however, together these gene products are unable to induce cell transformation in culture and, consequently, the mechanisms driving KS remain enigmatic (Speck and Ganem, 2010; Mesri *et al*., 2010).

1.4.4.1 LANA

LANA (ORF73) is a large (222-232 kDa) multifunctional nuclear protein that is highly expressed in all KSHV-associated malignancies, and is therefore ubiquitously used as a serological marker for latent KSHV infection (Kedes *et al*., 1996; Gao *et al*., 1996; Simpson *et al*., 1996; Kedes *et al*., 1997). LANA plays a critical role in episomal maintenance during cell division by physically tethering the viral episome to host cellular chromatin (Ballestas *et al*., 1999; Cotter and Robertson, 1999; Ballestas and Kaye, 2001); specifically, the LANA C terminus directly binds to the KSHV genomic terminal repeats, and the N terminus binds to the chromosomal linker protein histone H1 (Cotter *et al*., 2001; Shinohara *et al*., 2002). This functional role is similar to that of EBV nuclear antigen 1 (EBNA1) that maintains EBV genome propagation in viral-infected cells (Cotter and Robertson, 1999). LANA has also been implicated in deregulating cellular gene expression; importantly, some evidence suggests that LANA inhibits the activities of the p53 and pRb tumour suppressor pathways, implicating a pivotal role for LANA as an oncogenic player in KS development (Friborg *et al*., 1999; Radkov *et al*., 2000).

1.4.4.2 vCyclin

A viral homolog of human type D cyclins (vCyclin; ORF72) is encoded by the KSHV latency program (Chang *et al*., 1996). Unlike cellular type D cyclins, vCyclin constitutively activates cyclin-dependent kinase 6 (CDK6) that effectively deregulates cell cycle checkpoints and promotes hyperproliferation (Godden-Kent *et al*., 1997). Specifically, the vCyclin-CDK6 complex inactivates the $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ cell cycle inhibitors (Swanton *et al*., 1997; Mann *et al*., 1999; Ellis *et al*., 1999; Sarek *et al*., 2006). In the absence of functional p53, the true oncogenic potential of vCyclin is elicited despite cytokinesis defects and polyploidy (Verschuren *et al*., 2002). Normally, however, chronic vCyclin expression activates p53 that results in apoptosis or oncogene-induced senescence (OIS), cellular failsafe programs in response to oncogenic stresses (Verschuren *et al*., 2002; Verschuren *et al*., 2004a; Koopal *et al*., 2007). Although the functional consequences of vCyclin during KSHV infection are not clear, Leidal and colleagues have elucidated a pro-autophagic response to vCyclin in endothelial cells, and have found that coexpression with vFLIP elicits bypass of cellular senescence (Leidal *et al*., unpublished work). This coordinated latency profile may effectively subvert these cellular defences to facilitate the proliferation of KSHV-infected cells.

1.4.4.3 vFLIP

KSHV encodes a viral Fas-associated death domain (FADD) interleukin-1B-converting enzyme (FLICE) inhibitory protein (vFLIP). vFLIP (K13; ORF72) was first identified as an inhibitor of caspase 8, effectively protecting cells from Fas-mediated apoptosis *in vitro* (Irmler *et al*., 1997; Djerbi *et al*., 1999); however, following a lack of support for this function *in vivo* this function was later dismissed (Chugh *et al*., 2005). vFLIP is now considered to potently activate host-cell nuclear factor (NF)-κB by directly binding and inactivating the IκB kinase complex (Chaudhary *et al*., 1999; Liu *et al*., 2002; Sun *et al*., 2003). This constitutive NF-κB activation triggers widespread cytokine and chemokine secretion, likely contributing to the pro-inflammatory microenvironment of KS lesions, as well as anti-apoptotic processes by inducing Bcl-2 and Bcl-X_L (Guasparri *et al*., 2004; Sun *et al*., 2006; Sakakibara *et al*., 2009). The morphological spindling of KSHV-infected endothelial cells has been attributed to NF-κB activation by

vFLIP (Grossmann *et al*., 2006; Matta *et al*., 2007), and the NF-κB-dependent transforming potential of vFLIP has also been demonstrated in rodent fibroblasts (Sun *et al*., 2003). Interestingly, vFLIP has recently been characterized to potently suppress autophagic activity by blocking Atg3 binding and processing of LC3 (Lee *et al*., 2009a). This important pro-oncogenic measure was the first identified latent KSHV modulator of autophagy.

1.4.4.4 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 (described in Appendix B.2.1); the principal protein species are kaposins A, B, and C (Sadler *et al*., 1999; Muralidhar *et al*., 2000). Kaposin A (K12) is a small transmembrane protein that has transforming potential in rat fibroblasts, and injecting these cells into athymic nude mice induces the formation of 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 (DR2 and DR1) and shares no homology with kaposin A (Sadler *et al*., 1999). Kaposin B is paramount to establishing the secretory profile of the KS microenvironment, operating in post-transcriptional control of cytokine, growth factor, and proto-oncogene production. 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 (ARE)-containing mRNAs (McCormick and Ganem, 2005; 2006). Upregulating the stability of these transcripts ensures their translation, ultimately increasing cytokine release, such as IL-6 and VEGF, to drive KS development and tumourigenesis. The function(s) of kaposin C have yet to be elucidated.

1.4.4.5 miRNAs

miRNAs are short (~19 to 23 nucleotides) non-coding RNAs that post-transcriptionally regulate gene expression through RNA interference, either targeting mRNAs for degradation or suppressing their translation (Cullen, 2006). Interestingly, many herpesviruses have adapted this strategy to optimize the host cell for successful

infection and pathogenesis, as well as to establish latency and evade immune recognition (reviewed in Cullen, 2006); indeed, KSHV encodes 12 pre-miRNAs (miR-K1 to miR-K12), which engender 18 mature miRNAs that emanate from the kaposin transcript in latently infected cells (Samols *et al*., 2005; Pfeffer *et al*., 2005; Cai *et al*., 2005; Cai and Cullen, 2006; Umbach and Cullen, 2010). Although the functions and cellular targets of most of these viral miRNAs are unknown, some have been identified. miR-K1 targets the NF-κ-B inhibitor IκBα, and, because NF-κB activity is important to suppress lytic gene expression, miR-K1 is implicated in maintaining viral latency (Brown *et al*., 2003; Lei *et al*., 2010). A number of other miRNAs have been found to target thrombospondin 1, an important tumour suppressor and anti-angiogenic factor (Samols *et al*., 2007). Moreover, four other KSHV-encoded miRNAs target and silence the cellular oncogene musculoaponeurotic fibrosarcoma (MAF), an important transcription factor, to induce the reprogramming of lymphatic endothelial cells (Hansen *et al*., 2010). These viral miRNAs may influence spindle-cell differentiation and maintain KSHV latency, contributing to KS development and tumourigenesis.

1.4.5 Lytic Gene Expression

Only a very small proportion (1 - 5%) of KSHV infected cells exhibit lytic gene expression in KS lesions (Chiou *et al*., 2002). The distinct, progressive lytic-gene expression program comprises the majority of the viral genome and generates over 80 protein products (Jenner *et al*., 2001). Although most of these cells are undergoing the lytic viral replication cycle wherein KSHV progeny are being assembled, some cells may have suffered an abortive lytic program due to deregulated lytic gene expression (Sodhi *et al*., 2004a). Regardless, in addition to the sets of structural proteins and those necessary for KSHV genome replication, many lytic viral proteins have putative tumourigenic activities that may contribute to the angioproliferative and inflammatory properties of the KS lesion (reviewed in Mesri *et al*., 2010). Here, two of these lytic candidate oncogenes, vBcl-2 and vGPCR, are described.

1.4.5.1 vBcl-2

Consistent with the other γ-herpesviruses, KSHV encodes a viral homolog of cellular Bcl-2, known as vBcl-2 or ORF16 (Sarid *et al*., 1997). Similar to cellular Bcl-2 and Bcl-X_L, vBcl-2 effectively blocks intrinsic apoptotic cell death in a number of diverse cellular systems (Sarid *et al*., 1997). In contrast to the cellular Bcl-2 family members, however, vBcl-2 is resistant to caspase-mediated cleavage, thus escaping this negative regulation and retaining its anti-apoptotic activities (Bellows *et al*., 2000). Cellular Bcl-2 directly interacts with and inhibits the essential autophagy regulator Beclin 1, effectively blocking autophagic activity and promoting tumourigenesis (Liang *et al*., 1999); importantly, vBcl-2 binds to Beclin 1 with a higher affinity than its host cell counterpart, eliciting a robust and persistent inhibition of autophagy even during environmental stresses (Pattingre *et al*., 2005; Liang *et al*., 2008a). In response to pro-autophagic stimuli (i.e. starvation), JNK-mediated phosphorylation of Bcl-2 alleviates its inhibitory effect on Beclin 1 and thus enables autophagic activation (Wei *et al*., 2008a); however, vBcl-2 lacks the equivalent phosphorylation residues and consequently is resistant to this inactivation (Sinha *et al*., 2008). Furthermore, viral Bcl-2 proteins encoded by other γ-herpesviruses, including murine γ-herpesvirus 68 (Sinha *et al*., 2008; Ku *et al*., 2008) and EBV (Huang *et al*., 2003), have similar functions, suggesting an evolutionary importance of these homologs in herpesvirus replication and/or pathogenesis. Viral acquisition of Bcl-2 homologs may have coevolved to antagonize autophagic antiviral defences, such as clearance by xenophagy and immune recognition, ultimately establishing/maintaining persistent infection; however, this remains to be completely understood.

1.4.5.2 vGPCR

Based on its putative roles in neo-angiogenesis, inflammation, and cell transformation, the KSHV-encoded viral G-protein-coupled receptor (vGPCR; ORF74) has been subject to more rigorous investigation than any other KSHV candidate oncogene (reviewed in Sodhi *et al*., 2004a; 2004b; Yarchoan, 2006; Cannon, 2007; Martin and Gutkind, 2008; Mesri *et al*., 2010). Interestingly, mutant and dysfunctional GPCRs have recently been identified as emerging players in tumourigenesis and metastasis, and a

number of human herpesviruses have pirated one or more viral GPCRs to harness their proliferative and pro-inflammatory potential (Sodhi *et al*., 2004b; Dorsam and Gutkind, 2007). Consisting of seven transmembrane domains (Rosenkilde *et al*., 2008), vGPCR is a constitutively active signalling molecule that shares homology with human CXCR1 and CXCR2, receptors for IL-8/CXCL-8 and growth-related oncogene-α (Gro-α)/CXCL-1, respectively (Arvanitakis *et al*., 1997). Moreover, although vGPCR signalling is ligand-independent, it can recognize ligands more promiscuously than its cellular counterparts, binding both CXC and CC chemokines to further enhance its constitutive activity (Gershengorn *et al*., 1998). Importantly, vGPCR expression has demonstrated transforming potential in a number of cultured cell lines, including fibroblasts and endothelial cells (Arvanitakis *et al*., 1997; Bais *et al*., 1998; 2003; Grisotto *et al*., 2006). Furthermore, a number of seminal studies demonstrated that vGPCR expression, as a transgene or in retrovirally transduced endothelial cells, in multiple murine models leads to the progressive systemic development of angiogenic tumours that are strikingly similar to KS lesions (Yang *et al*., 2000; Guo *et al*., 2003; Montaner *et al*., 2003; Jensen *et al*., 2005). Despite its profound ability to evoke KS-like lesions *in vivo*, vGPCR is expressed in lytically infected cells that are destined to die; consequently, many consider vGPCR to contribute to KS via non-cell autonomous mechanisms (Ganem, 2010; Mesri *et al*., 2010). Nevertheless, the mechanisms underlying vGPCR effects on inflammation and immune evasion, as well as direct (autocrine) and indirect (paracrine) cell transformation, are quickly being unravelled (Figure 1.6).

Constitutive activation of complex signalling networks is the hallmark of vGPCR activity, and this is instrumental to all of the functional phenotypes evoked by vGPCR expression (Sodhi *et al*., 2004b; Martin and Gutkind, 2008). Indeed, point mutations (such as that causing the R143A substitution) that abrogate the constitutive signalling by vGPCR completely abolish its oncogenic potential, confirming that vGPCR hyperactivation of signalling routes is critical to its role in KS tumourigenesis (Holst *et al*., 2001; Sodhi *et al*., 2004c). One of the fundamental effects of vGPCR activity is potent stimulation of the class I PI3K/Akt pathway in endothelial cells (Montaner *et al*., 2001), a critical intracellular route that regulates many processes important in cancer, including cell proliferation, survival, growth, and responses to nutrient availability (Luo

et al., 2003). Furthermore, vGPCR-mediated PI-3K/Akt signalling targets the phosphorylation and activation of mTOR at least in part by phosphorylation and inhibition of tuberin sclerosis complex-2 (TSC2), and this results in the phosphorylation of both p70 S6K and 4EBP1, downstream effectors of mTOR signalling (Sodhi *et al*., 2006). Subversion of the PI3K/Akt/mTOR signalling axis renders vGPCR-expressing endothelial cells resistant to apoptosis and is critical for cell transformation and vGPCR sarcomagenesis *in vivo* (Montaner *et al*., 2001; Bais *et al*., 2003; Sodhi *et al*., 2006). Interestingly, vGPCR-mediated upregulation of secreted factors, such as VEGF, IL-8, and Gro-α (discussed below), engages both autocrine and paracrine mechanisms to further stimulate PI3K/Akt/mTOR activity in vGPCR-expressing and neighbouring cells, respectively (Sodhi *et al*., 2004c). Furthermore, pharmacological inhibition of mTOR activity with rapamycin effectively blocks vGPCR-induced sarcomagenesis *in vivo* (Sodhi *et al*., 2006). The neoplastic potential of vGPCR may result from the autocrine and paracrine conversion on the PI-3K/Akt/mTOR axis, linking its roles in direct and indirect endothelial cell transformation to KSHV tumourigenesis and KS development.

In a KS tumour there are only few cells that are lytically expressing vGPCR (Montaner *et al*., 2003); as such, many consider non-cell autonomous mechanisms to underlie the role of vGPCR in KSHV pathogenesis (Ganem, 2010; Mesri *et al*., 2010). Perhaps the most striking biological activity of vGPCR is its ability to promote paracrine neoplasia, with the vGPCR-associated secretory profile effectively contributing to the cell transformation, inflammation, and angiogenesis in KS lesions (reviewed in Martin and Gutkind, 2008; Mesri *et al*., 2010). These paracrine effects elicited by vGPCR center around stimulation of the mitogen-activated protein kinases (MAPKs), including p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) / stress-activated protein kinase (SAPK; Bais *et al*., 1998). Activation of p38 and ERK increases the phosphorylation of the transcription factor hypoxia-inducible factor (HIF)-1α, which in turn upregulates the expression of VEGF (Sodhi *et al*., 2000), a fundamental angiogenic factor in KS development (Nakamura *et al*., 1997). Importantly, vGPCR-mediated activation of the MAPKs depends, at least in part, on potent activation of Rac1, a member of the Rho family of small guanosine triphosphatases (GTPases; Dadke *et al*., 2003; Montaner *et al*., 2004). Increased Rac1 activity can stimulate the AP-1 and NF-κB

transcription factors, partly by JNK activation, to promote the expression of pro-inflammatory cytokines such as IL-6, IL-8, TNF-α, and bFGF, all of which are key secreted mediators that comprise the KS tumour microenvironment (Bais *et al*., 1998; Montaner *et al*., 2001; Pati *et al*., 2001; Schwarz and Murphy, 2001; Couty *et al*., 2001; Martin *et al*., 2008). Pharmacological ablation of Rac1 activity effectively blocks vGPCR autocrine and paracrine signalling *in vitro*, and causes rapid tumour regression *in vivo* (Montaner *et al*., 2004). The autocrine and paracrine effects evoked by the comprehensive secretory profile of vGPCR, along with direct cell transformation, are paramount to the inflammatory and angioproliferative components of KS tumours (Figure 1.6), thus highlighting its role as a potent viral oncogene.

1.5 Rationale and Objectives

At the time this study was initiated, modulation of autophagy by KSHV was largely unexamined. Given the fundamental importance of autophagy as both a tumour suppressor mechanism and a cellular antiviral defence, others have speculated that tumour-causing viruses, particularly γ-herpesviruses such as KSHV, may orchestrate subversion of autophagy to promote to viral oncogenesis (Deretic and Levine, 2009; Levine *et al*., 2011). Further evidence linking KSHV to autophagy emanates from potent inhibition of the PI3K/Akt/mTOR signalling axis in KSHV-infected endothelial cells, yet no direct association with autophagy has been asserted (Wang and Damania, 2008). The real 'smoking gun' implicating an instrumental role for autophagic subversion in KSHV infection and viral tumourigenesis comes from reports that KS patients treated with rapamycin, a pharmacological activator of autophagy via mTOR inhibition, showed complete tumour regression (Campistol *et al*., 2004; Izzedine *et al*., 2005). Although two KSHV proteins, the lytic-associated vBcl-2 (Pattingre *et al*., 2005) and the latency-associated vFLIP (Lee *et al*., 2009a), have been characterized as robust inhibitors of autophagy, the effects of *de novo* KSHV infection on autophagy, as well as effects by any of the remaining 80+ viral ORFs, remain unexplored. The McCormick lab has been interested in addressing these unanswered questions by searching for novel viral regulators of autophagy.

The KSHV vGPCR protein was hypothesized to be an ideal candidate for deregulating cellular autophagy. vGPCR triggers robust signalling through a number of key regulatory pathways that govern autophagic activity, including the PI3K/Akt/mTOR axis (a negative autophagic regulatory pathway) and JNK/SAPK (a positive autophagic regulatory pathway); vGPCR is expressed during lytic replication when virion progeny are assembled and thus the antiviral effects of autophagy need to be escaped; and the oncogenic potential of vGPCR has been well-documented (Martin and Gutkind, 2008). Here, an array of techniques to examine modulation of autophagy by vGPCR was employed, and vGPCR was identified as a profound activator of autophagy in endothelial cells. This foundation will serve useful for determining the underlying molecular mechanism.

Interestingly, during the course of these studies it was serendipitously discovered that vGPCR is also a robust trigger of OIS. vGPCR is the second putative KSHV oncogene demonstrated to elicit this cellular response; indeed, vCyclin expression induces a prominent autophagic response that necessitates the transition to senescence (Leidal *et al*., unpublished data). In contrast to OIS triggered by most oncogenes, however, vGPCR does not evoke a persistent DDR. The significance of vGPCR-triggered OIS is presently unclear, but acquisition of the senescence phenotype speaks to the extreme oncogenic potential of vGPCR.

The potential of kaposin B, a latency-associated KSHV protein, to modulate autophagic activity was also examined. Kaposin B is a demonstrated activator of MK2, and MK2 activity has been linked to the regulation of autophagy. Although no significant effect on starvation-induced autophagy by kaposin B was observed, we are still actively investigating the potential of the other kaposin proteins to subvert autophagy.

The findings presented in this thesis identify vGPCR as a novel trigger of autophagy and OIS, and should serve as a foundation for further investigation of autophagic subversion by KSHV and the importance of these adaptations in KS development.

1.6 Figures and Tables

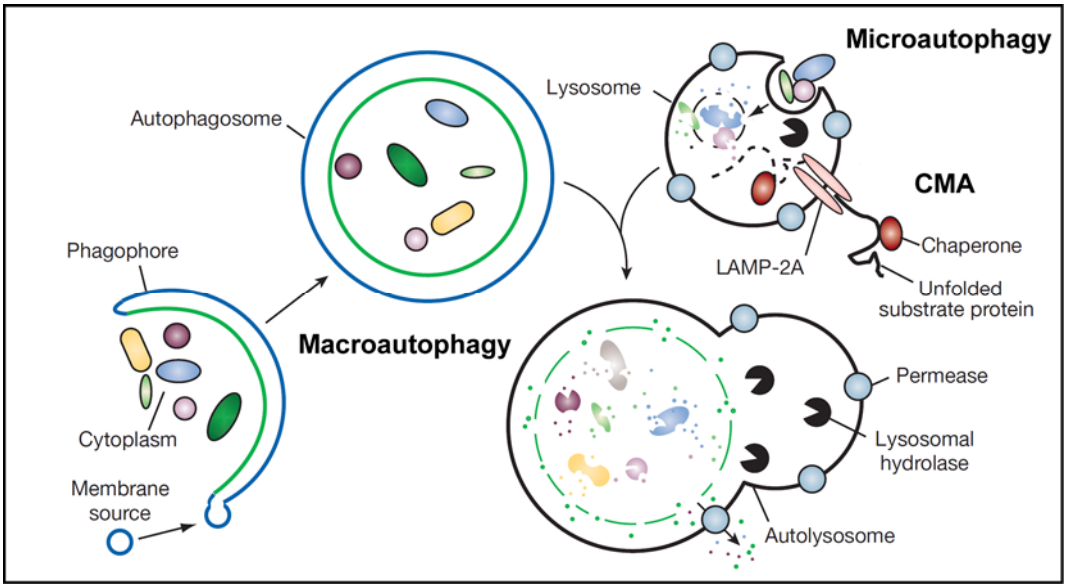

Figure 1.1 Schematic diagram of the different types of autophagy. The processes of macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) are depicted. During macroautophagy, a phagophore or isolation membrane sequesters portions of cytosol to be degraded. Fusion of the phagophore around this material forms a double-membrane compartment called an autophagosome. Following maturation, the autophagosome fuses with a late endosome or lysosome to form an autolysosome, wherein the cargos are degraded to their constituents by lysosomal hydrolytic enzymes, and these macromolecules can then be recycled for use by the cell. Refer to text for additional details. Figure adapted from Mizushima *et al*. (2008).

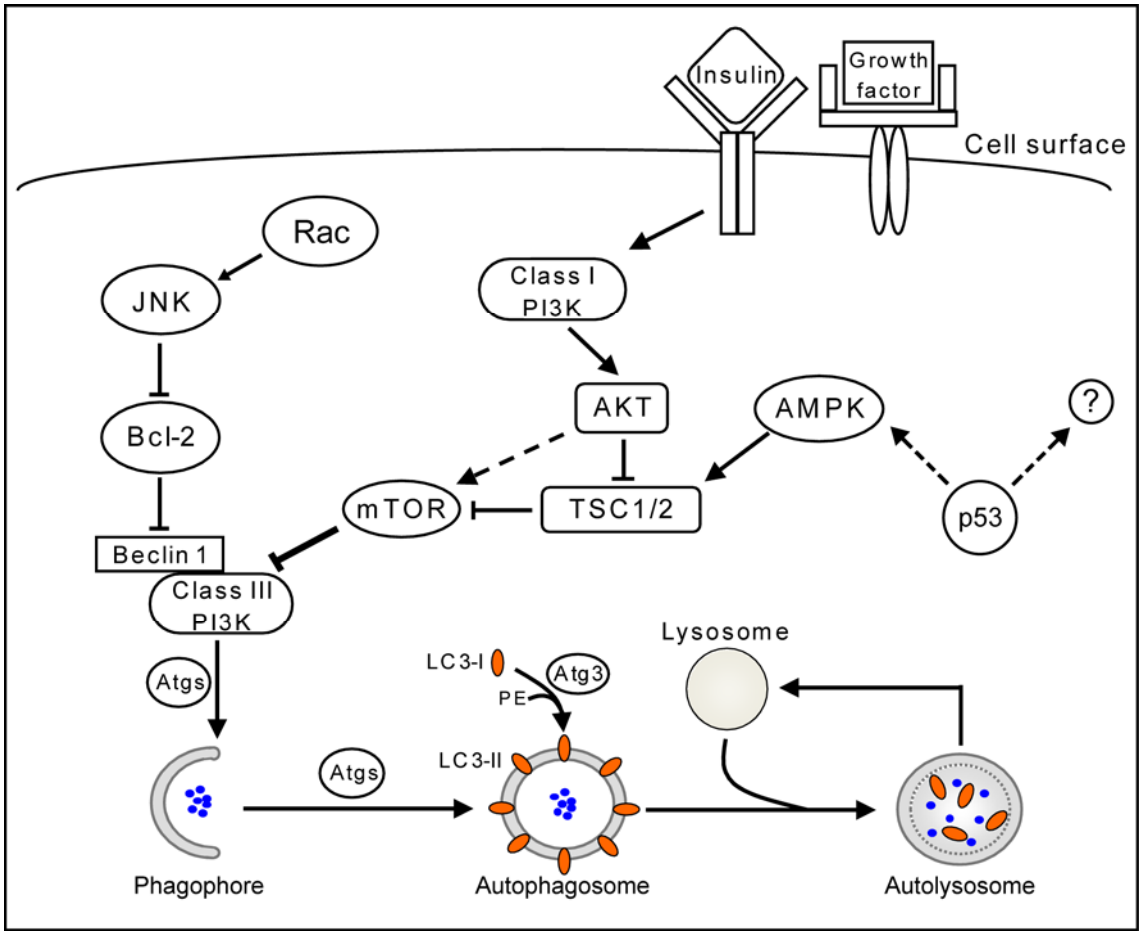

Figure 1.2 Overview of autophagic regulatory pathways. A simplified depiction of the regulation of autophagy by the PI3K/Akt/mTOR signalling axis; JNK-mediated repression of Bcl-2, a negative regulator of Beclin 1; and the complex modulatory roles of p53. The lipidation of LC3, a well-characterized essential autophagosome constituent, and its incorporation into the autophagosomal membrane is noted. Refer to text for additional details.

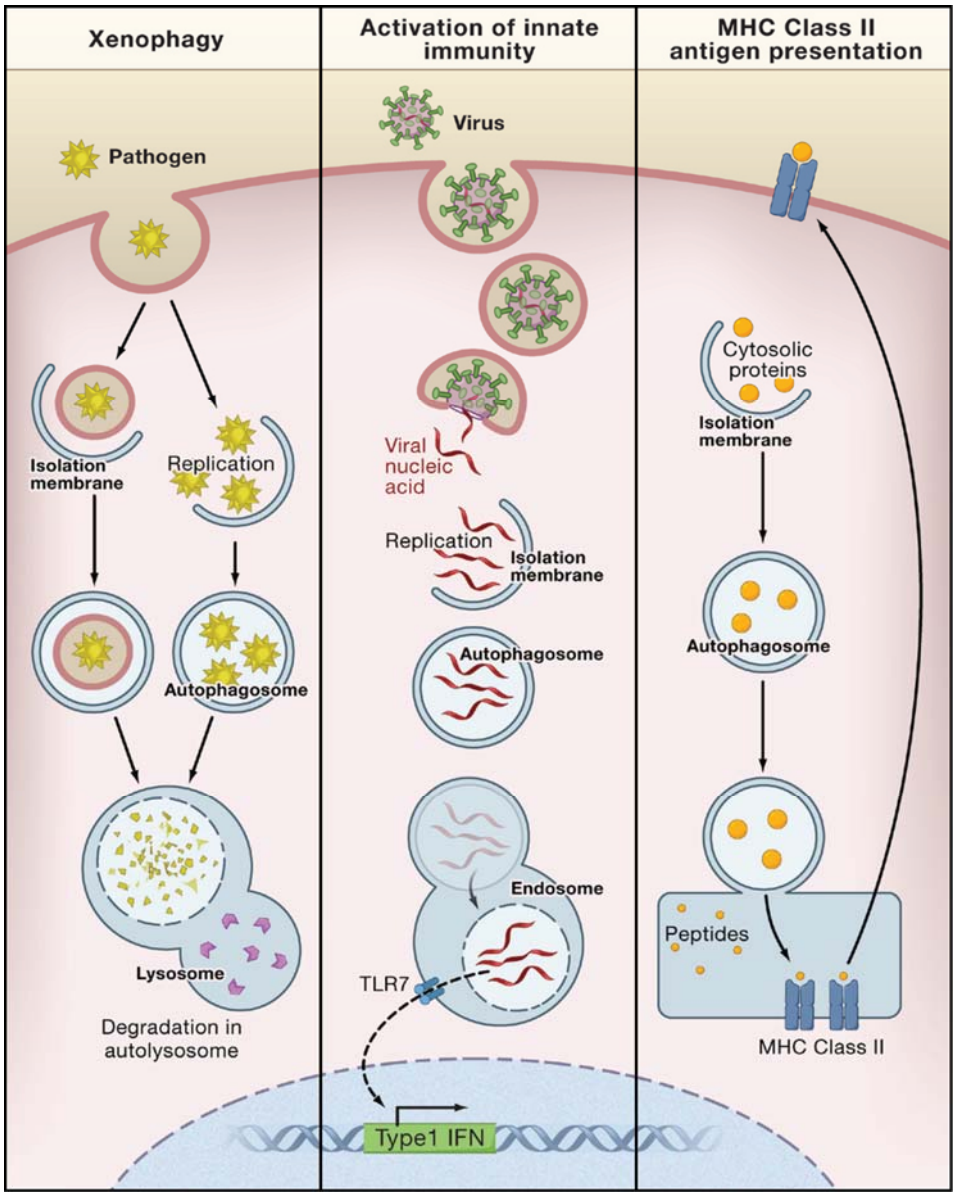

Figure 1.3 Autophagy as a cellular antiviral defence. Autophagy plays an important role in the direct elimination of intracellular pathogens, known as xenophagy, by sequestering pathogens in autophagosomes and delivering them to autolysosomes (left). Autophagy facilitates the activation innate immunity by delivering viral nucleic acids to endosomal Toll-like receptor 7 (TLR7) that triggers type I interferon (IFN) production (middle). Autophagy also serves to load microbial antigens onto class II major histocompatibility complex (MHC) and promote adaptive immunity (right). Figure was reproduced from Levine and Kroemer (2008).

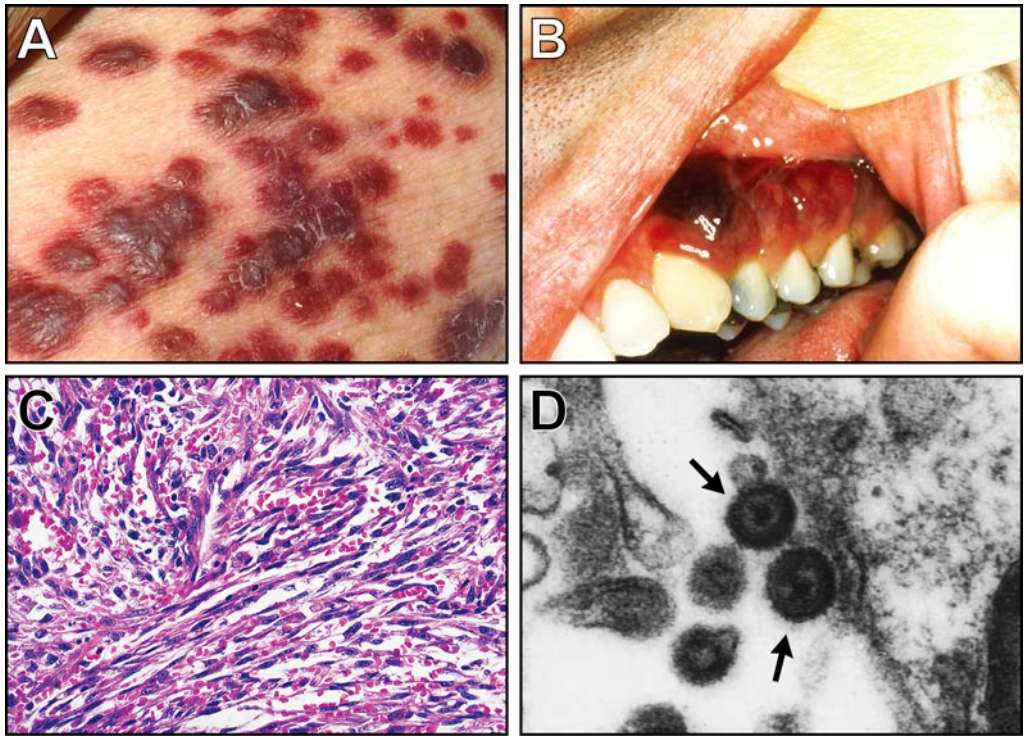

Figure 1.4 Kaposi's sarcoma. Kaposi's sarcoma (KS) often presents as nodular lesions, dark purple or red in colour, primarily localized to the **(A)** skin, but can also be found in the **(B)** oral cavity and, in advanced cases of KS, may disseminate to the lungs and gastrointestinal tract (not shown). **(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).

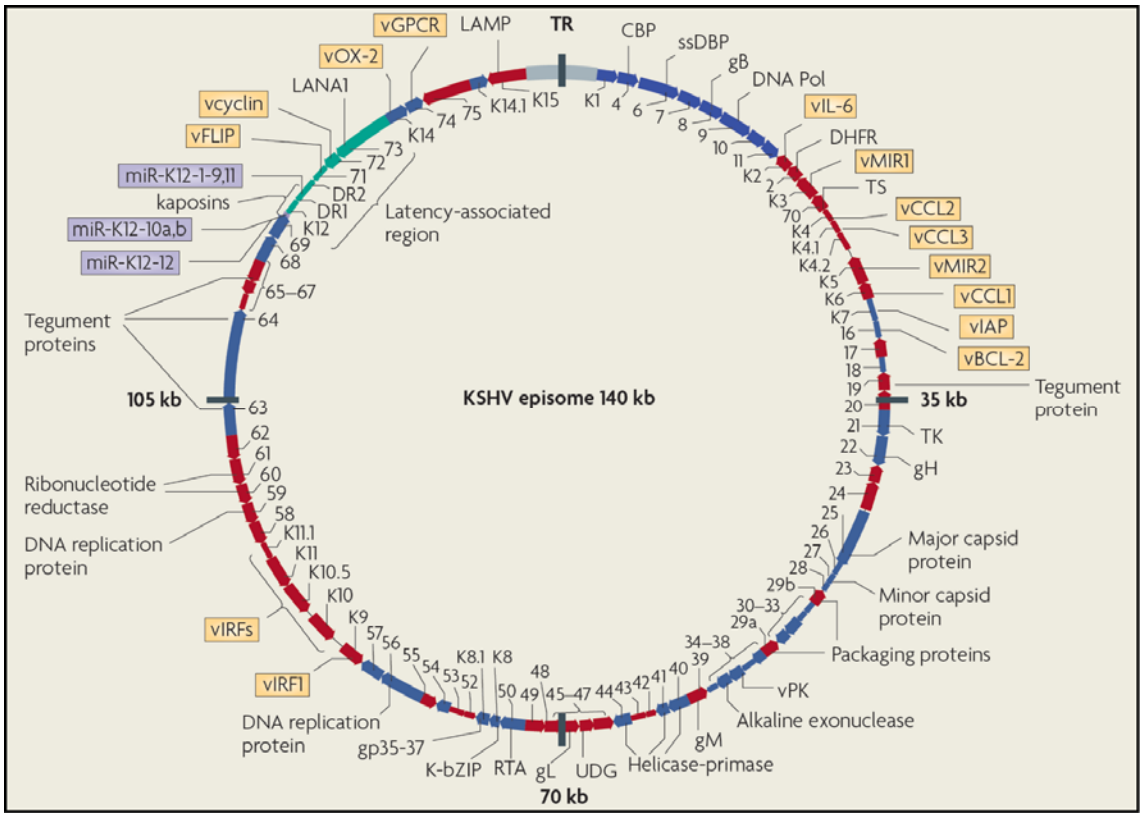

Figure 1.5 The KSHV episome. During latency, the KSHV genome is circularized by way of the terminal repeat (TR) region and maintained as an episome. Like many viral genomes, the KSHV genes are predominantly clustered based on their expression profiles; notably, all of the latent genes are expressed from 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).

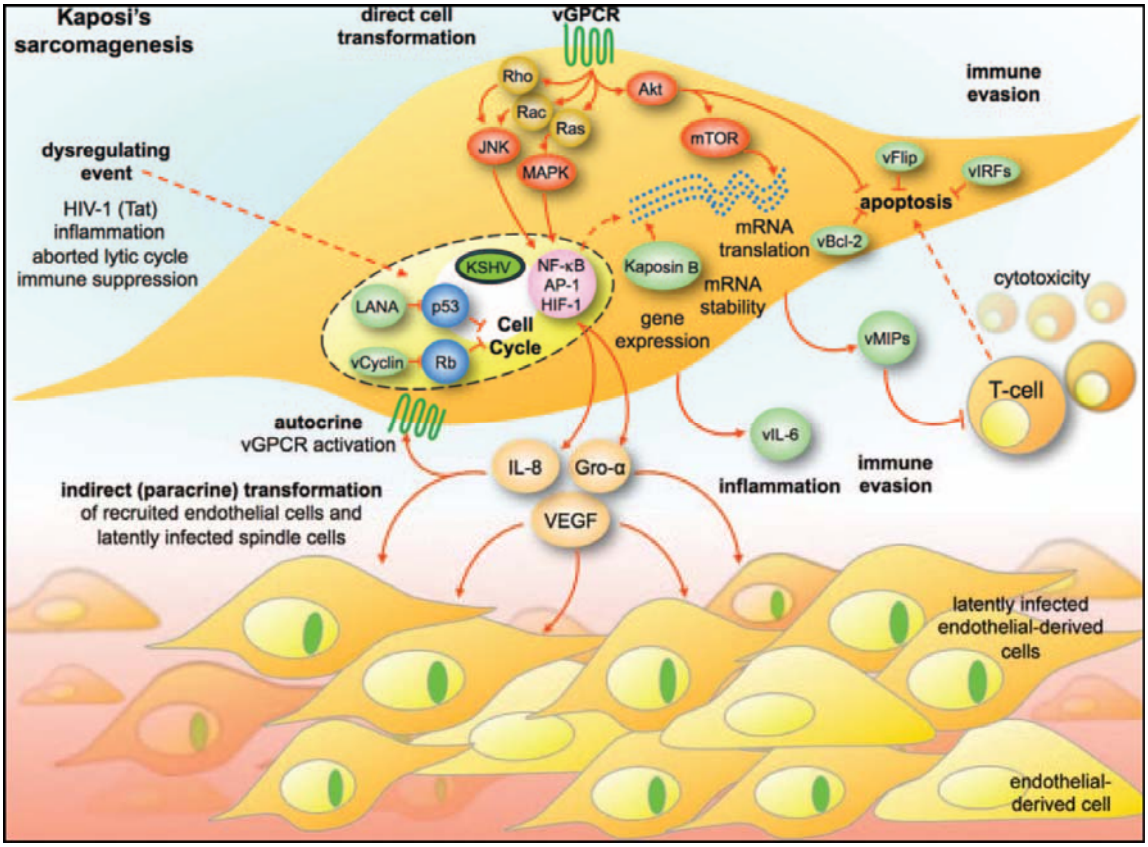

Figure 1.6 Complex interplay between lytic- and latent-associated genes orchestrates KSHV tumourigenesis. Coordinated functions of the latent LANA, vFLIP, vCyclin, and kaposin B proteins promote KSHV pathogenesis in the majority of KSHV-infected cells. Importantly, vGPCR is expressed in cells undergoing lytic replication, and may contribute to KS by direct (autocrine) and indirect (paracrine) cell transformation, pro-inflammatory properties, and facilitating immune evasion. Refer to text for details. Figure was reproduced from Martin and Gutkind (2008).

CHAPTER 2 – MATERIALS AND METHODS

2.1 Plasmid Construction

A pcDNA3 mammalian expression vector encoding the vGPCR open reading frame (ORF) and the 5'- and 3'-untranslated regions (UTRs) was obtained from D. Ganem (University of California San Francisco, San Francisco, CA, USA). The 5'- and 3'-UTRs were removed by PCR amplification of the vGPCR ORF using forward (5'-GAA TTC CAC CAT GGC GGC CGA GGA TTT CCT AAC C) and reverse (5'-CTT TCA TGT CCG GCG CCA CCA CGT AGC TCG AGC) primers that contained flanking *Eco*RI and *Xho*I restriction enzyme sites, respectively. The resulting amplicon was TA-cloned into pCR4-TOPO according to the manufacturer's instructions (TOPO TA Cloning Kit, Invitrogen), and following digestion with *Eco*RI and *Xho*I, the vGPCR ORF was ligated into pCR3.1 to generate pCR3.1-vGPCR. QuikChange Site-Directed Mutagenesis of pCR3.1-vGPCR was performed according to the manufacturer's instructions (Stratagene) to cause an AG to GC (R143A) transition using mutagenic forward (5'-GTG CGT CAG TCT AGT GGC GTA CCT CCT GGT GGC) and reverse (5'-GCC ACC AGG AGG TAC GCC ACT AGA CTG ACG CAC) primers, and template DNA was digested with *Dpn*I prior to transformation of competent *E. coli* cells generating pCR3.1-vGPCR(R143A). Successful cloning and mutagenesis were confirmed by DNA sequencing (McLab).

We used the pBMN retroviral vector system to establish uniform, stable gene expression in cell culture, wherein successfully transduced cells can be selected with antibiotic resistance to puromycin (puro) or neomycin (neo; Swift *et al*., 1999). pBMN-IRES-puro (pBMN-IP) and pBMN-IRES-neo (pBMN-IN) were created by L. Coscoy (D. Ganem lab, UCSF). pBMN-IP-vGPCR and pBMN-IP-vGPCR(R143A) were constructed by excision of the vGPCR ORF or the corresponding vGPCR mutant insert from pCR3.1 with *Eco*RI and *Xho*I, followed by subsequent ligation into pBMN-IP. Creation of pCR3.1-kapB, which bears the kaposin B ORF derived from a pulmonary KS lung isolate, was described previously (McCormick and Ganem, 2005). Clones of pBMN-IP-kapB were generated by excising kapB from pCR3.1 with *Bam*HI and *Eco*RI followed by ligating the resulting fragments into the *Bam*HI and *Eco*RI restriction sites of pBMN-IP.

DNA sequencing analysis was conducted on all kapB plasmid clones (McLab). pBMN-IN-GFP-LC3, which harbours human LC3B bearing a N-terminal GFP fusion, was constructed from pBMN-IN and pEGFP-LC3 (obtained from K. Kirkegaard, Stanford University, Stanford, CA, USA; Jackson *et al*., 2005); this plasmid was generated and provided by A. Leidal (Dalhousie University, Halifax, NS, Canada; Leidal *et al*., unpublished work). All restriction enzymes were obtained from New England Biolabs, and all primers were obtained from Invitrogen. All sequencing analysis was examined using DNAStrider software (freeware; C. March, Service de Biochemie et de Genetique Molculaire in France).

2.2 Cell Culture

All cells were grown at 37°C in a humidified 5% CO_2 atmosphere. HeLa (human cervical carcinoma) cells were a kind gift from C. Richardson (Dalhousie University, Halifax, NS, Canada). Phoenix-amphotropic retrovirus packaging cells were generously provided by G. Nolan (Stanford U; Swift *et al*., 1999). Human foreskin fibroblast (HFF) cells were generously provided by D. Ganem (UCSF). HeLa, Phoenix, and HFF cells were cultured in Dulbecco's modified Eagle medium (DMEM; Hyclone) supplemented with 10% heat-inactivated (HI) fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (1% PSQ; Wisent, Inc.). WI-38 human lung fibroblasts were obtained from ATCC (Manassas, Virginia, USA), and were grown in minimum essential medium (MEM; Gibco) supplemented with 10% HI FBS and 1% PSQ. All of the above cell lines were subcultured two to three times per week to maintain a cell density of 20-95% confluency in standard tissue culture flasks.

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Lonza Walkersville, Inc. (Walkersville, MD, USA) and were cultured according to the supplier's instructions. In brief, HUVECs were grown in endothelial growth medium (EGM)-2 (Lonza), composed of endothelial basal medium (EBM)-2 supplemented with EGM-2 Singlequots (2% FBS, hydrocortisone, hEGF, VEGF, hFGF-B, R^3-IGF-1, ascorbic acid, heparin, and gentamicin/amphotericin-B; Lonza), and were seeded onto tissue culture plates that had been pre-coated with 0.1% gelatin. HUVECs were maintained between approximately 20-85% confluency and were not allowed to over

grow; as such, HUVECs were split 1:4 to 1:6 every 2-4 days (d) and their growth media replaced every 2 d as needed. HUVECs were grown in 6-well or 10-cm plates and were not cultured beyond passage 8. Telomerase-immortalized microvascular endothelial (TIME) cells, a dermal microvascular endothelial cell line immortalized with hTERT (Venetsanakos *et al*., 2002), were kindly provided by D. Ganem. TIME cells were cultured as described above for HUVECs with the following exceptions: TIME cells were growth in EGM-2 developed for microvascular endothelial cells (EGM-2-MV), composed of EBM-2 supplemented with EGM-2-MV Singlequots (5% FBS, hydrocortisone, hEGF, VEGF, hFGF-B, R^3-IGF-1, ascorbic acid, and gentamicin/amphotericin-B; Lonza), and were cultured beyond passage 8.

Body-cavity-based lymphoma (BCBL)-1 cells, a BCBL cell line that is KSHV-positive and EBV-negative, were provided by D. Ganem (Renne *et al*., 1996b). BCBL-1 cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Wisent) supplemented with 10% HI FBS, 1% PSQ, and 55 μM β-mercaptoethanol (BME; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). BCBL-1 cells were maintained in suspension at a cell density of 2 x 10^5 to 1 x 10^6 cells per ml in 175-cm^2 tissue culture flasks.

2.3 Viruses

2.3.1 KSHV Preparation and Infections

Preparation of KSHV inoculum and infections were performed as described (Renne *et al*., 1998; Lagunoff *et al*., 2002) with modification. In brief, BCBL-1 cells were grown to a cell density of 1.0 x 10^6 cells/ml in 175-cm^2 tissue culture flasks. To induce KSHV production, valproic acid (Sigma), a deacetylase inhibitor, was added to each flask to a concentration of 0.3 mM and the cells were incubated at 37°C and 5% CO_2 as described (Shaw *et al*., 2000). At 7 d post-induction, cells were pelleted by centrifugation at 2,000 rpm for 10 min in a JS-5.3 rotor and the virus-containing supernatant was passed through a 0.45-μm filter (VWR). KSHV virions were then pelleted by centrifugation at 15,000 rpm for 1-2 h in a JLA-16.250 rotor at 4°C, viral pellets were resuspended in 1/100 of the original volume using EGM-2, and the KSHV inoculum was aliquoted and stored at -80°C. To determine the infectious titre of the

KSHV stock, serial dilutions of the viral inoculum were used to infect the target cells (as described below), 48 hours post-infection (h.p.i.) the infected cell monolayers were processed for immunofluorescence (IF) microscopy (section 2.7.1), and the highest dilution to yield > 90% LANA-positive was identified.

For KSHV infection of target cells, HUVECs (1.0×10^5) or TIME cells (2.0×10^4) were seeded per well of a 6-well plate 1 d prior to infection. The 30 to 50% confluent monolayers were overlaid with 3 ml of the diluted viral inoculum supplemented with 8 μg/ml polybrene, or were mock-infected with medium and polybrene alone, and the culture plates were centrifuged at 2,000 rpm for 2 h in a JS-5.3 rotor at 30°C; indeed, both polybrene (Lagunoff *et al*., 2002) and centrifugation (Yoo *et al*., 2008) have been demonstrated to enhance KSHV infection. Following centrifugation, cells were washed twice with phosphate buffered saline (PBS; Wisent) and overlaid with fresh growth medium.

2.3.2 Recombinant Retrovirus Production and Transductions

Preparation of recombinant retroviruses was performed as described (Swift *et al*., 1999) with modification. In brief, Phoenix cells (1.0×10^7) were seeded into 150-mm culture dishes 24 h prior to polyethylenimine (PEI; Sigma) transfection of 15.7 μg of retroviral plasmid DNA (section 2.1) with 47 μg/ml of PEI in serum- and antibiotic-free DMEM. Transfection medium was replaced 6 h post-transfection with fresh growth medium. At 48 h post-transfection the virus-containing supernatants were passed through a 0.45-μm filter, supplemented with 5 μg/ml sequabrene (Sigma), aliquoted and stored at -80°C.

For retroviral transductions, target cells were seeded into 6-well plates and grown to 70% confluency. Culture medium was replaced with virus-containing supernatants and the culture plates were centrifuged at 2,000 rpm for 2 h in a JS-5.3 rotor at 30°C. Following centrifugation, the cells were washed twice with PBS, overlaid with fresh growth medium and allowed to recover for 24 h. Transduced cells were subsequently selected in 1 μg/ml puromycin (Sigma) or 800 μg/ml G418 (Sigma) as required; importantly, a mock-transduced well was always used as a kill control for complete selection. Cells were washed twice with PBS following selection, cultured in growth

medium for 24-48 h, and re-seeded into tissue culture dishes as necessary for individual assays. For coincident transductions with differentially selectable retroviral vectors, cells were transduced and selected sequentially as described above. A GFP-bearing retroviral vector was used to assess transfection efficiency of Phoenix cells and transduction efficiency of target cells. Morphological changes of transduced cells were examined using an Olympus CKX41 culture microscope equipped with a QImaging monochromatic digital camera, and phase contrast images were captured with QCapture imaging software (QImaging, Surrey, BC, Canada).

2.4 Cell Treatments

To examine starvation-induced autophagy in HUVECs or TIME cells, culture medium was replenished and 4 h later the cell monolayers were washed twice with PBS and overlaid with complete culture medium (EGM-2 or EGM-2-MV, respectively), EBM-2, Earle's balanced salt solution (EBSS; Sigma), or Hank's balanced salt solution (HBSS; Sigma) for the indicated times prior to lysis. To investigate effects on autophagic flux, culture medium was replenished and 4 h later the cells were treated with Bafilomycin A1 (Sigma) at 100 nM for 4 h prior to lysis.

To insult cellular genomic DNA and elicit a DNA damage response (DDR), TIME cells were exposed to 10 Gy γ-irradiation (ionizing radiation; IR) and overlaid with fresh culture medium 24 h prior to lysis or fixation. Irradiated cells also served as positive controls when examining DNA damage and the DDR-associated signalling pathways. Alternatively, TIME cells were treated with 62.5 μM etoposide (Sigma) for 6 h, washed twice with PBS, and overlaid with fresh culture medium. To examine the effects on cellular senescence, DNA-damaged cells were subcultured for 5 d post-damage and subjected to senescence-associated β-galactosidase (SA β-gal) staining (section 2.9).

The positive control coincidently used when examining Akt/mTOR signalling-pathway activation included overlaying cells with culture medium supplemented with 20% FBS for 4 h prior to lysis. The positive control used to activate signalling through SAPK/JNK included exposing cells in PBS to 2500 $\mu J/cm^2$ ultraviolet (UV) radiation with a HL-2000 HybriLinker (UVP Laboratory Products)and refreshing the culture medium 30 min prior to lysis.

2.5 Antibodies

Primary antibodies used for immunoblot analysis are listed in Table 2.1. Primary antibodies used for indirect immunofluorescence (IF) microscopy included rabbit anti-53BP1 PAb at 1:200 (Santa Cruz Biotechnology, Inc.; sc-22760), rabbit anti-γH2AX PAb at 1:50 (Cell Signaling Technology [CST]; #2577), and rabbit anti-LANA PAb at 1:500 generously provided by D. Ganem (UCSF; Polson *et al*., 2001).

Secondary antibodies used for immunoblot detection or primary antibodies included Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (CST; #7076) and HRP-conjugated goat anti-rabbit IgG (CST; #7074). All secondary antibodies for immunoblotting were used at 1:2000 to 1:10,000. Secondary antibodies used for indirect detection of primary antibodies included Alexa Fluor® 488-conjugated goat anti-rabbit IgG at 1:500 (Molecular Probes; A-11008) and Alexa Fluor® 555-conjugated goat anti-rabbit IgG at 1:500 (Molecular Probes; A-21428).

2.6 Immunoblotting

Twenty-four h prior to harvesting cell lysates, cells were seeded into 10-cm culture plates at approximately 60% confluency. Unless otherwise indicated, culture medium was refreshed 24 h post-seeding and cells were lysed 4 h later. To prepare whole-cell extracts, cells were washed twice with ice-cold PBS, scraped, collected into microcentrifuge tubes and pelleted at 700 x g for 10 min; importantly, all preceding steps were performed on ice or at 4°C. Cells were then lysed in equal volumes of 2x Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS), sonicated, boiled for 3 min, and vortexed briefly. Ten μl of sample lysates were aliquoted to quantify protein concentrations using the Bio-Rad DC Protein Assay Kit according to the manufacturer's instructions (Bio-Rad Laboratories Ltd.). The remaining lysates were supplemented with 5% BME and 0.01% bromophenol blue (Bio-Rad), aliquoted to minimize freeze-thaw cycles, and stored at -80°C. Upon thawing, sample lysates were boiled again for 3 min and 10 – 25 µg of total protein were resolved by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western transferred to methanol-activated Immun-Blot PVDF Membrane (Bio-Rad) by standard wet-transfer techniques

(Gallagher, 2006). Membranes were blocked for 1 h at room temperature (RT) in milk blocking buffer (Tris-buffered saline [TBS; 20 mM Tris pH 7.5, 150 mM NaCl] containing 0.05% Tween 20 [TBST] and 5% w/v non-fat dry milk powder), washed thoroughly in TBST and incubated with the indicated primary antibodies at the specified dilutions (section 2.5) in TBST containing 5% w/v bovine serum albumin (BSA; Fisher) and 0.2 μM sodium azide (Sigma) overnight at 4°C. Membranes were then washed thoroughly in TBST, incubated with the indicated HRP-conjugated secondary antibodies at the specified dilutions (section 2.5) in milk blocking buffer for 1 h at RT. Importantly, 5% w/v BSA in TBST was used as a blocking buffer and for secondary-antibody dilution when immunoblotting for phosphorylated target proteins, and 1% w/v casein (Sigma) in TBST was used as a blocking buffer and for primary- and secondary-antibody dilutions when immunoblotting for LC3. Membranes were washed thoroughly in TBST, developed using the enhanced chemiluminescence assay according to the manufacturer's instructions (Amersham ECL Plus Western Blotting Detection System; GE Healthcare), and visualized with a Kodak Image Station 4000MM PRO (Carestream Health, Inc.). Image capture and densiometric quantification of protein signals were performed using Carestream Molecular Imaging Software (Carestream Health, Inc.). Protein signals were measured as an integrated volume with correction for a defined background, followed by normalization to β-actin signals. Images were processed and annotated using Adobe Photoshop CS2 (Adobe) and Adobe Illustrator CS2 (Adobe). Prestained molecular-weight markers (New England Biolabs) were resolved by SDS-PAGE and transferred to PVDF membrane coincident with sample protein lysates.

2.7 Fluorescence Microscopy

GFP-LC3 TIME cells were seeded onto glass coverslips and cultured for 1-2 d until the monolayer reached approximately 70% confluency. Culture medium was refreshed and 4 h later cells were washed twice in PBS, fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 10 min at RT, and washed thoroughly in PBS. Coverslips were then mounted onto microscope slides with Vectashield Mounting Medium for Fluorescence with DAPI H-1200 (Vector Laboratories, Inc.). Fluorescence was examined using a Zeiss Axiovert 200M equipped with a Hamamatsu Orca R2

Camera and images were digitally captured using Axiovision software (Carl Zeiss, Inc.). To examine the autophagic status of GFP-LC3 TIME cells, 50 cells were evaluated for the accretion of more than 20 GFP-LC3 puncta within the cytoplasms and were classified as positive or negative for autophagy, respectively.

2.7.1 Indirect Immunofluorescence Microscopy

Cells to be processed for indirect immunofluorescence (IF) microscopy were seeded, cultured, and fixed as described above (section 2.7). Following fixation, cells were permeabilized in ice-cold 90% methanol for 10 min, washed thoroughly in PBS, blocked in filtered 3% FBS in PBS for 1 h at RT, and incubated with primary antibodies (section 2.5) in blocking buffer overnight at 4°C. Subsequently, cells were washed 3 times in PBS, incubated with secondary antibodies (section 2.5) in blocking buffer for 1 h at RT, and washed thoroughly in PBS. Coverslips were then mounted onto microscope slides and examined as described above (section 2.7).

2.8 BrdU Incorporation Assay

To examine effects on cell proliferation, bromodeoxyuridine (BrdU) incorporation and immunolabelling were performed according to the manufacturer's instructions (BD Pharmingen). In brief, cells were cultured on glass coverslips and pulsed with 100 μM BrdU for 12 h in a humidified 5% CO_2 atmosphere at 37°C. Cells were subsequently washed in PBS, fixed in pre-warmed 3.7% formaldehyde for 20 min, washed twice, permeabilized in ice-cold 90% methanol for 10 min at RT, washed twice, and blocked in 3% FBS in PBS for 1 h at RT. To expose the BrdU, the cell's DNA was then denatured with 300 μg/ml DNase I (Qiagen) in a humidified chamber for 1 h at 37°C. The cells were subsequently washed twice with PBS and incubated with a 1/10 dilution of Alexa Fluor 488-conjugated mouse anti-BrdU MAb (BD Pharmingen) at RT overnight. Cells were washed and counterstained with DAPI, mounted onto microscope slides and visualized (section 2.7). Approximately 1000 cells for each condition were evaluated for BrdU incorporation and enumerated.

2.9 Senescence-Associated β-Galactosidase Staining

Senescent cells were identified using the Senescence β-Galactosidase Staining Kit according to the manufacturer's instructions (CST); importantly, this protocol specifically detects senescence-associated β-galactosidase (SA β-gal) activity at pH 6, a unique characteristic of senescent cells (Dimri *et al*., 1995). Following fixation, cells were stained for 24 h at 37°C in a non-humidified incubator, washed and overlaid with PBS for storage at 4°C. SA β-gal staining was examined using a Nikon Inverted Diaphot-TMD Microscope equipped with a Nikon D300s camera and images were digitally captured with Camera Control Pro 2 (Nikon Corporation). Positive SA β-gal activity is indicated by blue staining. The number of senescent cells were determined by evaluating SA β-gal staining of approximately 1000 cells at 100 x total magnification for each condition.

2.10 Transmission Electron Microscopy

To prepare samples for transmission electron microscopy (TEM), cells were washed twice with PBS and trypsinized from their culture plates, resuspended in ice-cold growth medium, and pelleted at 3,500 rpm for 5 min in a JS-5.3 rotor at 4°C. The cells were subsequently fixed with 0.1 M Sorensen's phosphate buffer containing 3.5% glutaraldehyde and 0.25 M sucrose for 2 h at RT with gentle agitation overnight at 4°C. Samples were post-fixed with1% osmium tetroxide in 0.1 M cacodylate buffer at 4°C, treated with 0.25% uranyl acetate overnight, and subsequently dehydrated with acetone. Samples were then infiltrated with an acetone and epon-araldite mixture, embedded in 100% epon-araldite resin, and subsequently cured at 45°C in a vacuum oven for 24 h followed by 24 h at 60°C in a standard oven. Ultrathin sections were cut with a LKB ultramicrotome using a diamond knife and post-stained for 10 min with 2% w/v aqueous uranyl acetate followed with modified Sato's lead citrate for 4 min (Hanaichi *et al*., 1986). Thin-section samples were observed in a JEOL 1230 TEM operating at 80 kV equipped with a Hamamatsu 2K X 2K ORCA-HR high-resolution (2,000 x 2,000 pixels) digital camera. The number of autophagic vacuoles per cell section was enumerated from recorded images of 30 cell sections per condition to calculate the mean number of autophagic vacuoles per cell section. Autophagic vacuoles were defined as double-membrane vacuolar structures containing recognizable cytoplasmic contents as

determined using 50,000 x magnification, as discussed previously (Yla-Anttila *et al*., 2009; Mizushima *et al*., 2010).

2.11 Figures and Tables

Table 2.1 Primary antibodies used for immunoblot analysis.

Target (clone)	Species	Company	Catalogue #	Dilution
p-4E-BP1 T37/46	Rabbit, PAb	CST	#9459	1:1000
4E-BP1	Rabbit, PAb	CST	#9452	1:1000
p-Akt T308	Rabbit, PAb	CST	#9275	1:1000
p-Akt S473 (D9E)	Rabbit, MAb	CST	#4060	1:1000
Akt	Rabbit, PAb	CST	#9272	1:1000
p-AMPKα T172 (40H9)	Rabbit, MAb	CST	#2535	1:1000
AMPKα (23A3)	Rabbit, MAb	CST	#2603	1:1000
p-ATF-2 T71	Rabbit, PAb	CST	#9221	1:1000
β-actin (JLA20)	Mouse, MAb	Calbiochem	CP01	1:10,000
p-c-Jun S63	Rabbit, PAb	CST	#9261	1:1000
Cox-2 (29)	Mouse, MAb	Santa Cruz	sc-19999	1:1000
HMGA2 (FL-109)	Rabbit, PAb	Santa Cruz	sc-30223	1:200
p-JNK/SAPK T183/Y185 (81E11)	Rabbit, MAb	CST	#4668	1:1000
JNK/SAPK (56G8)	Rabbit, MAb	CST	#9258	1:1000
Kaposin B	Rabbit, PAb	Kind gift from D. Ganem*		1:5000
LC3 (2G6)	Mouse, MAb	Nanotools	#0260-100	1:200
p-mTOR S2448	Rabbit, PAb	CST	#2971	1:1000
mTOR	Rabbit, PAb	CST	#2972	1:1000
p21 (F-5)	Mouse, MAb	Santa Cruz	sc-6246	1:1000
p-p53 S15	Rabbit, PAb	CST	#9284	1:1000
p53 (DO-1)	Mouse, MAb	Santa Cruz	sc-126	1:1000
p-p70 S6K S389	Rabbit, PAb	CST	#9208	1:1000
vGPCR	Rabbit, PAb	Kind gift from G. Hayward*		1:2000

* Rabbit anti-vGPCR was kindly provided by G.S. Hayward (Johns Hopkins School of Medicine, Baltimore, MD, USA; Chiou *et al*., 2002). Rabbit anti-kaposin B was kindly provided by D. Ganem (UCSF).

CHAPTER 3: vGPCR INDUCES AUTOPHAGIC ACTIVITY IN ENDOTHELIAL CELLS

3.1 Introduction

Autophagy is a highly conserved catabolic mechanism that controls the quality and quantity of intracellular biomass in eukaryotic cells; as such, this process is fundamental in maintaining homeostasis with particular effects on cell survival. Importantly, dysfunctional regulation of autophagy underlies a physiological role for this process in health and disease (reviewed in Levine and Kroemer, 2008; Mizushima *et al*., 2008). Notably, autophagic activity is an important antiviral defence, and many viruses have consequently evolved elegant strategies to exploit or evade this antiviral defence mechanism, effectively promoting their replication and persistence in the host (reviewed in Levine and Deretic, 2007; Deretic and Levine, 2009; Levine *et al*., 2011).

The McCormick lab is inherently dedicated to furthering the understanding of KSHV pathogenesis and its contribution to disease, which are yet to be fully elucidated; interestingly, modulation of autophagy by KSHV has been largely unexamined. Only one lytic-associated KSHV protein has been identified to modulate autophagy; indeed, vBcl-2 is a potent inhibitor of autophagic activity (Pattingre *et al*., 2005). However, the effects of autophagy on KSHV replication during the lytic cycle, viral adaptations to autophagy during *de novo* infection, and potential autophagic deregulation by any of the remaining 80+ lytically expressed ORFs have not yet been explored.

Others have speculated that oncogenic γ-herpesviruses, KSHV in particular, may subvert cellular autophagy to effectively ablate this tumour suppressor mechanism and promote viral oncogenesis (Deretic and Levine, 2009; Levine *et al*., 2011). Furthermore, treatment with rapamycin, a well-known activator of autophagy, has yielded complete tumour regression in transplant KS patients (Campistol *et al*., 2004; Izzedine *et al*., 2005), suggesting that autophagy may play an important role in KSHV tumourigenesis. The latent protein vFLIP has been found to suppress autophagy by preventing autophagosome formation (Lee *et al*., 2009a), but the effects of other viral candidate oncogenes on autophagy, as well as the importance of autophagy in KSHV-mediated tumourigenesis, have yet to be elucidated.

For these reasons, I hypothesized that KSHV replication triggers host autophagic responses that must be actively suppressed by these anti-autophagic proteins, which is further essential for KS development. The McCormick lab is interested in identifying additional KSHV deregulators of autophagy, to begin to reveal the impact of autophagic processes on KSHV infection.

Characteristics of the KSHV G-protein-coupled receptor (vGPCR) identified it as a candidate protein to subvert cellular autophagy. Firstly, this potent signalling activator impinges on a multitude of signal-transduction pathways that tightly regulate autophagic function, including the PI3K/Akt/mTOR (inhibitory effect on autophagy) and JNK/SAPK (stimulatory effect on autophagy) pathways (reviewed in sections 1.2.2 and 1.4.5.2). Secondly, vGPCR is expressed during the lytic phase of the KSHV replication cycle and may support the production of viral progeny by modulating the autophagic antiviral defences (reviewed in section 1.2.4). Lastly, the oncogenic potential of vGPCR has been well-documented (Martin and Gutkind, 2008), and we can therefore surmise that vGPCR may also hijack the autophagic machinery to further contribute to KS tumourigenesis. Here, a set of diverse techniques were employed to investigate the modulation of autophagy by vGPCR, and vGPCR was identified as a strong inducer of autophagic activity in endothelial cells.

3.2 Results

3.2.1 Establishing a vGPCR Retroviral Transduction System

To accurately characterize effects on autophagic activity by vGPCR, a model system to reliably express a gene of interest had to be established. Transient transfection of expression vectors using a variety of transfection reagents induces a robust autophagic response (Mizushima *et al*., 2010); furthermore, endothelial cells do not generally transfect with high efficiency (data not shown). Consequently, recombinant retroviral transductions were employed based on the Phoenix Retroviral Expression System developed by G. Nolan (Stanford U; Swift *et al*., 1999). Retroviral vectors encoding the *vGPCR* open reading frame (ORF), a *vGPCR-R143A* mutant (hereafter referred to as R143A), or an empty-vector (EV) control plasmid, were constructed. The R143A mutation constitutes an arginine-to-alanine substitution at residue 143 that renders

vGPCR incapable of proper signalling activity, which may result from conformational changes in the receptor or impaired interactions with G-proteins (Ho *et al*., 2001). Following transduction and antibiotic selection of primary human umbilical vein endothelial cells (HUVECs), vGPCR-expressing HUVECs, but not the EV control cells, presented a 'spindle-like' morphological change (Figure 3.1A); notably, R143A-transduced cells also did not exhibit a morphological change (data not shown). This altered cellular morphology is a well-characterized phenotype of vGPCR expression that results from increased expression of various cell adhesion molecules (Bais *et al*., 2003). Furthermore, vGPCR-transduced HUVECs demonstrated increased levels of cyclooxygenase-2 (Figure 3.1B), an inflammatory regulator that has been identified as a downstream target of active vGPCR signalling (Shelby *et al*., 2007). Moreover, expression of vGPCR was confirmed by immunoblot analysis (Figure 3.1B). vGPCR protein products were detected as ~90-kDa smears (Figure 3.1B), suggesting that vGPCR may be post-translationally modified by glycosylations or phosphorylations (Chiou *et al*., 2002).Together, the retroviral transduction system was validated by establishing stable vGPCR-expression in primary endothelial cells with a functional output.

3.2.2 vGPCR Enhances Starvation-Induced Autophagy in HUVECs

Given the known signalling activities of vGPCR, it could modulate starvation-induced autophagy, a cellular stress response that is tightly regulated by many pathways that overlap with those activated by vGPCR (see sections 1.2.2 and 1.4.5.2). To address this, immunoblot analysis of microtubule-associated protein 1 light chain 3 (LC3) was performed; LC3 has been well-characterized as an essential, reliable marker used to monitor the autophagic status of a cell (Kabeya *et al*., 2000). Endogenous LC3 is present in two forms in the cell: LC3-I is cytosolic, whereas LC3-II is conjugated to phosphatidylethanolamine (PE) and is incorporated into isolation membranes and autophagosomes (Figure 1.2; Kabeya *et al*., 2000; Kabeya *et al*., 2004). The amount of LC3-II is tightly correlated to the number of autophagosomes in the cell, and therefore serves as a good indicator of autophagosome formation (Kabeya *et al*., 2000). Although the molecular weight of LC3-II is greater than that of LC3-I due to lipidation, the resulting extreme hydrophobicity yields a downward mobility shift following SDS-PAGE

that can be observed by immunoblot detection (Kabeya *et al*., 2004). During a short period of starvation, LC3-I is rapidly converted into LC3-II as autophagy is induced; however, if cells are subjected to longer periods of starvation the levels of LC3-II decrease. This is because LC3-II is present on both the inner and outer autophagosomal membranes, where the former is degraded in the autolysosome and the latter becomes deconjugated from PE, recycled into LC3-I, and returned to the cytosol (Tanida *et al*., 2005). Indeed, methods for accurate interpretation of LC3 immunoblotting have been extensively reviewed (Mizushima and Yoshimori, 2007; Klionsky *et al*., 2008; Mizushima *et al*., 2010).

To confirm that changes in cellular autophagic activity could be readily detected during nutrient deprivation in primary endothelial cells, HUVECs were overlaid with fresh culture medium 4 h prior to starvation to decrease the basal level of cellular autophagy, as basal autophagy had been determined to be fairly prevalent following 1-2 d of growth in culture (data not shown). Subsequently, the medium was replaced with various forms of starvation, including endothelial basal medium (EBM-2; Figure 3.2A) or Earle's balanced salt solution (EBSS; Figure 3.2B) for the indicated times. HUVECs starved in either EBM-2 or EBSS demonstrated a robust increase in LC3-II by 30 min after nutrient deprivation was initiated, but these heightened levels of LC3-II quickly declined and by 2 h they had returned to baseline levels (Figures 3.2A and B). At the time, this was the first documented observation of starvation-induced autophagy in primary HUVECs by LC3 immunoblotting, which exhibited the expected progression of LC3 turnover as described above.

To investigate the possible modulation of starvation-induced autophagy by vGPCR, HUVECs were transduced with retroviruses bearing EV or vGPCR and starved in EBM-2 as described above. Immunoblot analysis revealed that, despite both cell populations responded as expected to prolonged starvation, vGPCR-expressing HUVECs demonstrated enhanced autophagic activity immediately following nutrient deprivation (Figure 3.3A), and this augmented response was maintained throughout starvation (Figure 3.3B). Furthermore, increased levels of LC3-II were observed in vGPCR-expressing cells even before starvation was initiated (Figure 3.3A). Given the robust autophagic deregulation by vGPCR during both starvation and nutrient-rich conditions, it

was hypothesized that vGPCR may be modulating the basal activity of cellular autophagy. Therefore, examination of autophagic deregulation by vGPCR was continued only at the basal level under nutrient-rich conditions.

3.2.3 vGPCR Induces Autophagic Activity in Endothelial Cells

To determine the effect of vGPCR expression on autophagy during nutrient-rich conditions in endothelial cells, HUVECs or telomerase-immortalized microvascular endothelial (TIME) cells were transduced with EV, vGPCR, or R143A retroviruses. The culture medium was refreshed and 4 h later the cell monolayers were harvested and subjected to immunoblot analysis of LC3. In both HUVECs (Figure 3.4A) and TIME cells (Figure 3.4B), vGPCR-expressing cells exhibited a 1.8-fold or 2.7-fold increase in LC3-II over EV-transduced cells, respectively. This pronounced increase in LC3-II was not observed in cells expressing the R143A mutant vGPCR that is incapable of signalling activation. Interestingly, vGPCR-expressing cells also demonstrated increased levels of LC3-I compared to EV- and R143A-transduced cells (Figures 3.3A, B, and 3.4B), indicating that vGPCR may upregulate the synthesis of total LC3. Although LC3-II is predominantly incorporated into autophagic membranes, a proportion of LC3-II can be ectopically generated in an autophagy-independent manner, which has been described (Mizushima *et al*., 2010); as such, additional approaches are necessary to accurately assess autophagic activity. These results suggest that vGPCR expression promotes autophagosome accumulation in endothelial cells and that this phenomenon is dependent on its robust signalling potential.

It is a common misconception that increased levels of LC3-II must indicate an induction of autophagic activity. Autophagy is a very dynamic process and autophagic structures are in constant flux; indeed, autophagosomes are generated and then fuse with autolysosomes, and thus the number of these intermediate structures is a function of this balance (Tanida *et al*., 2005; Mizushima *et al*., 2010). Therefore, an accumulation of autophagosomes (and thus LC3-II) may represent either an induction of autophagy, or a suppression of events that are downstream of autophagosome formation (Mizushima and Yoshimori, 2007; Mizushima *et al*., 2010). For example, matrix protein 2 of influenza A virus was found to inhibit autophagy by blocking autophagosomal fusion with the

autolysosome (Gannagé *et al*., 2009). To differentiate between an induction of autophagic flux and a block in a late autophagic event, cells can be treated with a lysosomal inhibitor such as bafilomycin A1. Bafilomycin is a specific chemical inhibitor of vacuolar H^+ ATPase that effectively blocks lysosomal acidification and impairs autophagic degradation and LC3 turnover (Yoshimori *et al*., 1991; Yamamoto *et al*., 1998; Mizushima and Yoshimori, 2007). If vGPCR is inducing autophagic flux, then bafilomycin treatment would promote further accumulation of LC3-II; however, if vGPCR is blocking a late stage of autophagy, then bafilomycin treatment would have no effect on the level of LC3-II. Treatment of EV-, vGPCR-, and R143A-expressing TIME cells with bafilomycin yielded a marked increase in LC3-II compared to untreated cells (Figure 3.5). Together, these data indicate that vGPCR induces autophagic flux in endothelial cells.

3.2.4 Accretion of Autophagic Puncta in vGPCR-Expressing GFP-LC3 TIME Cells

A number of other techniques commonly used to examine cellular autophagic activity directly or indirectly monitor LC3 localization to autophagic structures. One such method employs LC3 with EGFP fused at its N terminus (GFP-LC3), and this marker can be visualized by fluorescence microscopy (Kabeya *et al*., 2000; Mizushima *et al*., 2004). GFP-LC3 is localized into a diffuse cytosolic pool under normal, nutrient-rich conditions; however, upon the induction of autophagy, lipidated GFP-LC3 assembles into discrete punctate structures throughout the cytoplasm. Although this fluorescent autophagosomal marker is a useful tool to survey effects on autophagy, cautions must be taken when conducting these studies and interpreting the results, and these caveats have been well described (Klionsky *et al*., 2008; Mizushima *et al*., 2010). For example, overexpression of GFP-LC3, or coexpression with other aggregation-prone proteins, can lead to aggregation of GFP-LC3 and these aggregates are often indistinguishable from true autophagosomes by fluorescence microscopy (Kuma *et al*., 2007). To rule out these experimental artifacts, it has been recommended to establish stable cell lines with low levels of GFP-LC3 expression and to avoid transient transfections (Mizushima *et al*., 2010). Therefore, a retroviral vector encoding GFP-LC3 was constructed and TIME cell line that stably expresses this fluorescent autophagy marker was generated (Leidal *et al*.,

unpublished work). These GFP-LC3 TIME cells constitutively expressed low levels of GFP-LC3 that diffusely localized throughout the cell under normal conditions, but became rapidly localized into discrete cytoplasmic puncta when cells were treated with bafilomycin, confirming that these reporter cells accurately display changes in cellular autophagic activity (Figure 3.6).

To investigate the subversion of autophagy by vGPCR using this fluorescent marker, TIME cells were sequentially transduced with retroviruses bearing GFP-LC3 followed with EV, vGPCR, or R143A. Fluorescence microscopic examination revealed a marked accretion of cytoplasmic GFP-LC3 puncta in vGPCR-expressing TIME cells, but not in EV- or R143A-transduced cells (Figure 3.7A). There are a number of techniques commonly used to quantify the autophagic status of GFP-LC3 cells (Mizushima *et al*., 2010). A threshold level of 20 GFP-LC3 puncta per cell was selected to effectively distinguish "autophagy active" and "autophagy inactive" states, and then the percentage of cells containing greater than 20 GFP-LC3 puncta represents the number of autophagic cells. As such, 77.8% of vGPCR-expressing cells were exhibiting autophagic activity, compared to 23.0% and 31.9% of EV- and R143A-transduced cells, respectively (Figure 3.7B). Expression of vGPCR was confirmed by immunoblot analysis (Figure 3.7C). These results further support a robust autophagic response to vGPCR expression in endothelial cells.

3.2.5 Autophagic Structures Accumulate in vGPCR-Expressing Cells

Ultrastructural identification of autophagosomes and autolysosomes, the hallmark subcellular structures of autophagy, remains the most direct measure of cellular autophagic activity and often accompanies the biochemical assays described above (Klionsky *et al*., 2008). This method, using transmission electron microscopy (TEM), relies on accurately classifying the autophagic structures based on specific characteristics (Yla-Anttila *et al*., 2009; Mizushima *et al*., 2010). Autophagosomes are vacuoles that possess at least a portion of double membrane, or occasionally multiple membranes, and enclose undigested cytoplasmic contents but no lysosomal proteins. In contrast, autolysosomes may not possess more than a single membrane and their contents are often partially or completely degraded by lysosomal hydrolytic enzymes; importantly,

lysosomal contents are very electron dense, and are transferred to autolysosomes following autophagosome-lysosome fusion (Yla-Anttila *et al*., 2009; Mizushima *et al*., 2010). Caveats regarding ultrastructural identification of autophagic structures by TEM have been well described (Eskelinen, 2008).

Ultrastructural analysis of vGPCR-expressing TIME cells was implemented to confirm that the enhanced autophagic activity inferred using biochemical assays corresponds to increased numbers of autophagic vacuoles. Although EV- and R143A-transduced cells were largely void of autophagosomes and autolysosomes, the cytoplasms of vGPCR-expressing cells were replete with both of these autophagic structures (Figure 3.8A). On average, there were 11.1 autophagosomes and 5.5 autolysosomes (total, 16.6 ± 1.8) per thin section of vGPCR-expressing cells; in contrast, thin sections of EV- and R143A-transduced cells contained 3.7 autophagosomes and 1.9 autolysosomes (total, 5.6 ± 0.7) and 4.4 autophagosomes and 3.1 autolysosomes (total, 7.5 ± 1.0), respectively (Figure 3.8B). The increased numbers of both types of autophagic structures further indicates an induction of autophagic activity by vGPCR and not a late-stage suppression of autophagy; indeed, a late-stage block would have resulted in an accumulation of autophagosomes only (Mizushima *et al*., 2010). Collectively, these data have definitively demonstrated that vGPCR expression in endothelial cells triggers a robust autophagic response.

3.2.6 Immunoblot Screen for Mechanism(s) Driving vGPCR-Induced Autophagy

To investigate the molecular mechanism(s) that are responsible for the cellular autophagic response to vGPCR expression, extensive immunoblot analysis of candidate signal-transduction pathways and their associated effector proteins was performed. The PI3K/Akt/mTOR signalling axis plays a critical role in autophagic regulation, and vGPCR has been well documented to potently activate this pathway; however, the activation of Akt/mTOR by vGPCR would be expected to have a suppressive effect on cellular autophagy (Kamada *et al*., 2000; Sodhi *et al*., 2004c). Immunoblot analysis of vGPCR-expressing TIME cells did not reveal increased levels of Akt T308, Akt S473, or mTOR S2448 phosphorylation over that found in EV- and R143A-transduced cells, nor were there increased levels of 4E-BP1 T37/46 or p70 S6K T389 phosphorylation,

downstream effects of mTOR activation (Figure 3.9). Notably, the lack of activation of these effectors by 20% FBS (positive control) suggests that the approach or experimental system for examining these factors may be confounded by other variables (discussed in section 3.3.6).

Similar to many other G-protein-coupled receptors, vGPCR has been characterized to constitutively activate a number of stress-response pathways including the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and the p38/mitogen-activated protein kinase (MAPK) pathways, as well as their downstream effector proteins (Arvanitakis *et al*., 1997; Bais *et al*., 1998). Importantly, JNK activation has been shown to stimulate autophagy by phosphorylating the anti-apoptotic Bcl-2 protein, effectively disrupting negative regulation by Bcl-2 of the essential pro-autophagic protein Beclin 1 (Wei *et al*., 2008a). Given that Bcl-2-mediated repression of Beclin 1 acts downstream of mTOR signalling, activation of JNK by vGPCR may bypass the anti-autophagic effects of vGPCR-mediated stimulation of PI3K/Akt/mTOR signalling, ultimately inducing a pro-autophagic cellular response (Figure 1.2). Preliminary immunoblot analysis of vGPCR-expressing TIME cells revealed increased phosphorylation at T183/Y185 of both the 46-kDa and 54-kDa JNK isoforms compared to that seen for EV- and R143A-transduced cells (Figure 3.10); of note, functional differences between the 46-kDa and 54-kDa JNK isoforms have not been reported (Ip and Davis, 1998). Surprisingly, phosphorylation of two downstream nuclear targets of JNK signalling, c-Jun S63 and ATF-2 T71, was not upregulated in response to the vGPCR-mediated JNK activation (Figure 3.10). Interestingly, JNK has both cytoplasmic and nuclear functions, and thus the lack of c-Jun and ATF-2 phosphorylations may suggest that JNK is retained in the cytoplasm in response to vGPCR expression, unable to activate its downstream nuclear effectors (see section 3.3.4). Further careful examination of JNK activation by vGPCR is necessary to accurately draw conclusions regarding its involvement in vGPCR-induced autophagy.

Canonical DNA damage responses (DDRs) and activation of the tumour suppressor p53 have been linked to cellular autophagy, but the precise role of p53 in regulating autophagy is complex and enigmatic (Maiuri *et al*., 2009b; Green and Kroemer, 2009; Maiuri *et al*., 2009a). In brief, nuclear p53 acts as an autophagy-inducing

transcription factor, stimulating gene expression of pro-autophagic effectors including damage-regulated autophagy modulator (DRAM), sestrin 1, and sestrin 2 (Crighton *et al*., 2006; Crighton *et al*., 2007; Budanov and Karin, 2008; Green and Kroemer, 2009). Particularly, the sestrins modulate mTOR signalling via adenosine monophosphate-activated protein kinase (AMPK) activity that subsequently phosphorylates the tuberin sclerosis complex 2 (TSC2) subunit of the TSC1/2 complex, a negative regulator of mTOR activity (Budanov and Karin, 2008). In contrast, cytoplasmic p53 exerts an autophagy-inhibitory function, but the molecular mechanism behind this inhibition is unclear; indeed, the degradation of cytoplasmic p53 is required for successful induction of autophagy (Tasdemir *et al*., 2008). Preliminary immunoblot analysis revealed an absence of p53 S15 phosphorylation in vGPCR-expressing TIME cells, suggesting that p53 is not effectively transactivating pro-autophagic target genes (Figure 3.11). Furthermore, this lack of p53 activation is reflected in low levels of AMPK T172 phosphorylation, implying low levels of sestrin 1 and 2, and also in low levels of $p21^{WAF1/Cip1}$, a negative regulator of cell cycle progression (Harper *et al*., 1993; el-Deiry *et al*., 1993; Budanov and Karin, 2008). Interestingly, total p53 levels are diminished in cells expressing vGPCR compared to EV- and R143A-transduced cells (Figure 3.11). This decrease may correspond to enhanced degradation of cytoplasmic p53, a proposed requirement for efficient autophagic induction (Tasdemir *et al*., 2008). Further study is necessary to understand the importance of p53 in vGPCR-induced autophagic activity.

3.3 Discussion

3.3.1 Summary

Autophagy is a conserved eukaryotic process that has been implicated in many biological processes and diseases; importantly, autophagy has been characterized as a tumour suppressor mechanism, and it also functions to limit viral infections. Through coevolution with their hosts, viruses have developed elegant strategies to hijack this cellular antiviral defence to establish infection and support their persistence in the host; however, no oncogenic virus has been identified that subverts host cell autophagy to directly promote tumourigenesis. Accordingly, the autophagic status during KSHV infection is presently unknown. Although two KSHV proteins, the lytic vBcl-2 and the

latent vFLIP, have been characterized as powerful suppressors of autophagic activity, the modulation of autophagy by *de novo* KSHV infection, the susceptibility of KSHV to autophagic activity, and the effects of other viral gene products on autophagy have not been explored.

In this study the KSHV vGPCR has been characterized as a novel deregulator of cellular autophagy. This lytically expressed, constitutively active pleiotropic signalling molecule is considered a critical component of KSHV pathogenesis and establishment of KS tumours. Using a set of different experimental assays to monitor autophagy, vGPCR was definitively identified as a robust trigger of autophagic activity in endothelial cells. Moreover, preliminary data suggest that the underlying molecular mechanism functions through JNK activation, which may result in JNK-mediated suppression of Bcl-2-inhibited Beclin 1. To my knowledge, this is the first report of a G-protein-coupled receptor, either cellular or virally acquired, which activates a cellular autophagic response.

3.3.2 Why Would vGPCR, a Putative Oncogene Expressed During the Lytic Cycle, Trigger Autophagy?

Upon examination of the documented functions of vGPCR, one would be quick to hypothesize that it should inhibit cellular autophagy. Indeed, vGPCR is a potent activator of the PI3K/Akt/mTOR axis (Sodhi *et al*., 2004c; 2006), a critical signalling cascade that suppresses autophagic activity (Wullschleger *et al*., 2006; Pattingre *et al*., 2008); vGPCR is expressed during the lytic cycle, during which antiviral autophagic functions could threaten active virus replication and particle assembly; and vGPCR is believed to be important in KS initiation and development, and therefore the tumour suppressive nature of autophagy would need to be ablated. However, vGPCR did not regulate autophagy as expected; surprisingly, it triggered a robust autophagic phenotype. How vGPCR is able to stimulate this cellular response may be derived from its pleiotropic signalling activities, which are discussed below. But why vGPCR expression would upregulate autophagy, given its aforementioned features, is another question.

Although most viruses have mechanisms to deregulate cellular autophagy, not all viruses suppress this process in infected host cells. In fact, many viruses stimulate

autophagy and exploit this process to facilitate viral replication (reviewed in Deretic and Levine, 2009; Lin *et al*., 2010b). However, most documented cases of virally upregulated autophagy occur with positive-sense ssRNA viruses that use autophagosomes for scaffolding (Lin *et al*., 2010b). Perhaps KSHV requires autophagic activity for efficient replication; indeed, the exact mechanism(s) underlying KSHV lytic replication have yet to be fully elucidated, but it may be possible that autophagy facilitates virion egress from the host cell. Interestingly, human parvovirus B19 upregulates autophagy to promote the survival of infected cells and facilitate a longer replication time (Nakashima *et al*., 2006). It is possible that vGPCR may activate autophagy in KSHV-infected cells to extend the lifespan of lytically reactivated cells to enhance viral production.

Aside from directly impacting KSHV replication, autophagic subversion by vGPCR may serve alternative functions that benefit KSHV infection. Recently, Wen and colleagues demonstrated that autophagy is upregulated by the replication transactivation activator (Rta) during the switch from latent to lytic replication (Wen *et al*., 2010). Their data suggested that this enhanced autophagic activity was necessary to remodel and reorganize the protein profile, both cellular and viral, of KSHV-infected cells, and that this was required to generate a cellular environment that is conducive to lytic reactivation (Wen *et al*., 2010). Moreover, Rta directs expression of vGPCR during the lytic cycle (Chiou *et al*., 2002); as such, perhaps coordinate gene expression of Rta and vGPCR serves to remodel the cytosolic environment to mediate lytic reactivation.

Alternatively, vGPCR may control autophagy to mediate its own expression in infected host cells. The latent membrane protein 1 (LMP1) encoded by EBV evokes distinct functions that depend on its quantity in the cell (Lee and Sugden, 2008a); at low levels, LMP1 promotes cell proliferation and tumourigenesis, but at high levels it triggers antiproliferative responses that result in cell cycle arrest and inhibited protein synthesis (Lee and Sugden, 2008b). Interestingly, LMP1 is able to induce varying degrees of autophagic activity, which effectively control the quantity of LMP1 in infected cells by mediating LMP1 turnover via autophagic degradation (Lee and Sugden, 2008b). The elegant feedback mechanism assures that EBV-infected cells maintain their tumourigenic potential by regulating low levels of LMP1. It is possible that vGPCR evokes an analogous quality-control mechanism during lytic reactivation, effectively maintaining

low levels of vGPCR during KSHV infection as to not trigger host-cell defences while continuing to execute oncogenic outcomes. Further study is required to understand the autophagic status of lytically infected cells and its importance on KSHV replication.

3.3.3 Could vGPCR-Induced Autophagy be Ablated by Other Lytic Proteins?

The majority of viruses have evolved mechanisms to evade or block cellular autophagy (Deretic and Levine, 2009; Lin *et al*., 2010b). It is frequently advantageous for a virus to suppress autophagic activity during viral replication and virion assembly in order to circumvent the antiviral properties of autophagy, such as xenophagy and the activation of innate and adaptive immune responses (Levine and Deretic, 2007; Deretic and Levine, 2009). Particularly, herpesviruses have developed multipronged modes of autophagic subversion, suggesting that deregulating this fundamental cellular process is of paramount importance to viral infection and/or pathogenesis. For example, the HSV-1-encoded ICP34.5 protein effectively antagonizes autophagic function by directly interacting with Beclin 1 and also through PKR/eIF2α inhibition, and this activity is essential for HSV-1-mediated neurovirulence *in vivo* (Tallóczy *et al*., 2006; Orvedahl *et al*., 2007). Remarkably, ΔICP34.5 HSV-1 mutants are efficiently targeted for xenophagy and also readily processed for antigen presentation to $CD4^+$ T cells *in vivo* (Alexander *et al*., 2007; English *et al*., 2009a; 2009b; Leib *et al*., 2009). The γ-herpesviruses also encode lytic proteins, including the Bcl-2 homologs of KSHV and γMH68, which suppress autophagy by interacting with and antagonizing Beclin 1, similar to ICP34.5 (Pattingre *et al*., 2005; Sinha *et al*., 2008; Ku *et al*., 2008).

Taken together, herpesviruses express inhibitors of autophagy during the lytic cycle, and autophagic activity, at least in the case of HSV-1, is deleterious to viral replication and pathogenesis. As such, one can speculate that most herpesviruses ultimately seek to block the antiviral and tumour suppressive activities of autophagy, and that KSHV is no different. Then why does vGPCR induce a robust autophagic response? Perhaps this increase in cellular autophagy is merely an unfortunate consequence of vGPCR expression, and the cell is simply responding to this molecular trigger of oncogenic, inflammatory, and angiogenic stress. If this is the case, and autophagy is detrimental to KSHV lytic replication, then perhaps the virus evolved a countermeasure

to antagonize this host-cell response. If this autophagic response is mediated by JNK activation (see section 3.3.4), then coordinated viral expression of vBcl-2 during the lytic cycle may be sufficient to block vGPCR-induced cellular autophagy. By working in concert, vGPCR and vBcl-2 may be instrumental in establishing KSHV infection and facilitating viral tumourigenesis. Interestingly, this situation would be the second example (vFLIP and vCyclin) of a KSHV-coordinated gene expression program to subvert an autophagic response that is consequently triggered by another viral gene product (discussed in sections 1.4.4.2 and Appendix A; Leidal *et al*., unpublished work). Further study is necessary to determine what the status of autophagy is in lytically infected cells, if autophagy is detrimental to KSHV lytic replication, and if the coexpression of vGPCR with vBcl-2 is sufficient to mitigate this cellular autophagic response.

3.3.4 How Could JNK Activation Impact Autophagy?

Upon the discovery that vGPCR expression triggers a robust autophagic response, it was clear that this response is unlikely to result from constitutive vGPCR-signalling through the PI3K/Akt/mTOR axis, which would negatively regulate cellular autophagy. Instead, vGPCR-mediated JNK activation, which would positively regulate autophagy, emerged as a candidate underlying mechanism (Figure 3.10). JNK has been identified to play a critical role in phosphorylating Bcl-2 during nutrient deprivation, promoting the dissociation of Bcl-2 from Beclin 1; indeed, the Bcl-2 interaction with Beclin 1 suppresses autophagic activity (Pattingre *et al*., 2005), and the JNK-mediated phosphorylation of Bcl-2 effectively relieves this negative regulatory process so that autophagy can be induced (Wei *et al*., 2008a). Activation of JNK by vGPCR may trigger an autophagic response by this mechanism. Interestingly, the KSHV vBcl-2 exhibits a higher binding affinity for Beclin 1 than its host-cell counterpart, and vBcl-2 does not harbour the JNK-targeted phosphorylation sites (Pattingre *et al*., 2005; Wei *et al*., 2008a); as such, this supports the notion that vBcl-2 may effectively antagonize a cellular autophagic response to vGPCR during KSHV lytic replication.

There are a number of alternative, or additional, potential mechanisms that may mediate vGPCR-induced autophagy. vGPCR may directly upregulate the expression of

the essential autophagy gene Atg5, which could effectively induce autophagic activity. Recent studies have shown that stimulation of the Rac1/MKK7/JNK pathway by oncogenic $Hras^{V12}$ triggers increased transcription of Atg5, which in turn activated cellular autophagy (Byun *et al*., 2009). Although the status of Rac1 or MKK7 activation was not examined, vGPCR has been previously characterized to activate JNK by these regulatory molecules (Montaner *et al*., 2004), and vGPCR may enhance Atg5 expression by activating this signalling pathway. Interestingly, even though the exact mechanism driving JNK-mediated Atg5 expression was not elucidated, vGPCR may induce Atg5 gene expression by upregulating the transcription factor C/EBPβ (Choi and Nicholas, 2010), which has been shown to target the *Atg5* promoter element (Yuk *et al*., 2009).

Furthermore, despite increased JNK phosphorylation the activation of downstream nuclear targets of JNK, c-Jun and ATF-2, were not observed (Figure 3.10). These data suggest that activated JNK may be retained in the cytoplasm, and is therefore unable to translocate to the nucleus and phosphorylate c-Jun and ATF. Although the underlying mechanism is unclear, it is possible that in vGPCR-expressing cells activated MKK7 is working as a cytoplasmic anchoring protein for JNK and is thus restricting nuclear entry (Wang *et al*., 2011). Alternatively, there is an extensive cytoplasmic scaffolding network comprised of JIP-1 (JNK-interacting protein 1) and POSH that may also be retaining JNK in the cytoplasm (Engström and Granerus, 2009). Although these alternative processes are currently speculative, further investigation into fully understanding vGPCR-mediated activation of JNK and the lack of downstream signalling is necessary. Importantly, characterizing the importance of JNK in vGPCR-induced cellular autophagy is critical to elucidating the biological consequence of this process.

3.3.5 Why are Total p53 Levels Decreased in vGPCR-Expressing Cells?

The tumour suppressor p53 has a relatively complex and enigmatic role in regulating cellular autophagy; indeed, the cytoplasmic and nuclear pools of p53 elicit opposing effects on autophagy (reviewed in Maiuri *et al*., 2009b; 2009a; Green and Kroemer, 2009). Nuclear p53, which is activated by various genotoxic and oxidative stresses, can transactivate a number of genes that induce autophagy, such as DRAM and sestrins 1 and 2. On the other hand, cytoplasmic p53 inhibits autophagy by an unknown

mechanism. Interestingly, recent evidence suggests that depletion of the cytoplasmic pool of p53 is required for autophagy to be efficiently induced by other stimuli, such as starvation (Tasdemir *et al*., 2008; Green and Kroemer, 2009). Although increased p53 activation would have supported the robust autophagy observed in vGPCR-expressing cells, the absence of this phosphorylation event was not surprising as this has not been previously reported. However, vGPCR-expressing cells exhibited a pronounced decrease in total p53 levels (Figures 3.11 and 4.4), which, if this corresponds to the cytoplasmic p53 pool, is a necessary contributor to the enhanced autophagic phenotype. Interestingly, this decrease in p53 may result from the well-characterized Akt activation elicited by vGPCR (Montaner *et al*., 2001). Akt has been previously shown to physically interact with MDM2 and phosphorylate it at two distinct residues, ultimately enhancing MDM2-mediated ubiquitination and degradation of p53 (Zhou *et al*., 2001; Ashcroft *et al*., 2002; Gottlieb *et al*., 2002; Ogawara *et al*., 2002). It would be interesting to determine if vGPCR mediates p53 degradation by chronic stimulation of the PI3K/Akt signalling axis, and if this event is required for the vGPCR-induced cellular autophagic response.

3.3.6 Why is the Akt/mTOR Axis Not Upregulated by vGPCR in Our System?

Activation of the PI3K/Akt/mTOR signalling axis by vGPCR has been extensively studied and well characterized (reviewed in Sodhi *et al*., 2004b; Martin and Gutkind, 2008). Needless to say, the absence of hyperactivated Akt, mTOR, and the downstream mTOR targets p70 S6K and 4EBP1 in vGPCR-expressing cells, was surprising (Figure 3.9). There are a few possible explanations to account for these anomalies. Endothelial cell-specific culture medium is extremely rich in nutrients and growth factors (see section 2.2), and the PI3K/Akt/mTOR pathway is sensitively responsive to extracellular factors; as such, the components of the medium may have consequently raised the basal level of PI3K/Akt/mTOR activity in these cells, effectively diminishing any detectible increase in activity evoked by vGPCR signalling. This possibility is signified by a lack of Akt/mTOR activation during high-serum conditions, which serves as a positive control (Figure 3.9). If this is the case, then the basal level of Akt/mTOR activation could be decreased by starving the cells for a short period before harvesting them. Alternatively, the lack of vGPCR-mediated stimulation of Akt/mTOR

may be a consequence of the chronic vGPCR expression that results from the experimental system. Following successful transduction and antibiotic selection, vGPCR had been expressed in these cells for 5 days before the transductants were assayed for autophagy and the status of signal-transduction pathways. It is possible that the cells had responded to the prolonged vGPCR signalling and adapted as necessary. Therefore, it may be significant to examine vGPCR functions and effects on signalling pathways at earlier time's post-initial expression. To address this issue, an inducible TIME cell line has been generated that expresses vGPCR in a doxycycline-dependent manner (data not shown). Using this new system, the temporal deregulation of autophagy and its control elements by vGPCR in endothelial cells will be able to be elegantly characterized.

3.3.7 Does R143A Function as a Valid Negative Control?

Mutational analysis has revealed that charged residues at the intracellular ends of transmembrane helices 2 and 3, particularly the arginine at position 143 in transmembrane helix 2, are critical to the constitutive activity of vGPCR, and an R143A substitution effectively abolishes vGPCR signalling capabilities (Ho *et al*., 2001). Although the R143A mutant has been commonly used as a signalling-defective control in studies examining signal-transduction pathways modulated by vGPCR (Sodhi *et al*., 2004c; Montaner *et al*., 2004; 2006; Sodhi *et al*., 2006), the molecular basis underlying the dysfunctional R143A signalling capabilities is presently unclear; however, Ho and colleagues have speculated that the R143A substitution may evoke changes in receptor conformation or disrupt interactions with the G protein (Ho *et al*., 2001). Here, immunoblot detection of R143A consistently revealed decreased protein levels compared to vGPCR, and the banding pattern corresponding to R143A resolved at a higher molecular weight than that for vGPCR (Figures 3.4 and 3.5). Considering there are no documented R143A immunoblots in the literature, this raises a number of interesting questions regarding the validity of R143A as a negative control for vGPCR-related studies. Although the decreased detection of R143A may result from inefficient antibody binding, decreased R143A protein levels may alternatively suggest that R143A is targeted for proteolytic degradation, and the increased molecular weight may be a product of enhanced polyubiquitination. If the R143A mutation evokes a conformational change

in the receptor, the mutant proteins may form macromolecular aggregates in the cell that could be subject to ubiquitination and proteolysis, as well as selective degradation by autophagy (Pankiv *et al*., 2007; Kirkin *et al*., 2009; Kraft *et al*., 2010). Interestingly, R143A-expressing cells consistently demonstrated increased autophagy compared to EV-transduced cells, albeit to a lesser degree than that elicited by vGPCR (Figures 3.4, 3.5, 3.7 and 3.8). This enhanced autophagic activity may be a result of R143A protein aggregates accumulating in the cell, and the associated cellular stress response may further account for the observed activation of JNK (Figure 3.10). These previously uncharacterized attributes of R143A expression indicate that it may not serve as a valid control to assess effects of vGPCR signalling on cellular autophagy. Alternatively, the signalling activity of vGPCR could be exogenously ablated with pertussis toxin, which effectively prevents the interaction of G-proteins with G-protein-coupled receptors (GPCRs) and has been frequently used to inhibit vGPCR activity (Couty *et al*., 2001; Cannon and Cesarman, 2004). Regardless, further study is necessary to assess the validity of R143A as a negative control for examining vGPCR-mediated effects on cellular autophagy.

3.3.8 Do Other GPCRs Trigger a Cellular Autophagic Response?

To our knowledge, the KSHV-encoded vGPCR is the first example of a GPCR, viral or cellular, that induces autophagy as a result of functional signalling activity. Only one other GPCR has been linked to autophagic activity: when overexpressed, the Parkinson's disease-associated orphan receptor GPR37 forms large protein aggregates that induce an ER-stress response resulting in autophagy (Marazziti *et al*., 2009). GPCRs are commonly subverted or hijacked by viruses to facilitate successful viral replication and pathogenesis. Some viruses directly activate cellular GPCRs during infection (i.e. HIV), whereas other larger viruses encode their own GPCRs (i.e. KSHV, HHV-6, HHV-7, poxviruses, etc.) or their own GPCR-modulating cytokines and chemokines (called 'virokines') that were likely pirated from their cellular hosts over time (reviewed in Sodhi *et al*., 2004b). Considering the breadth of this molecular piracy, it will be interesting to investigate the potential impact of these other viral GPCRs and associated molecules on host autophagic responses. Moreover, deregulated GPCRs have been associated with

tumour progression and metastasis, contributing to cell survival, autonomous proliferation, immune evasion, and neo-angiogenesis (reviewed in Dorsam and Gutkind, 2007). In light of these studies, it will be interesting to investigate whether autophagy represents a universal barrier to oncogenesis mediated by deregulated cellular GPCR activity, which must then be circumvented by additional mutations to allow tumour progression. The importance of GPCR-directed autophagic activity in other viral infections, as well as in cancers that lack an underlying viral etiology, warrants further investigation.

3.4 Figures and Tables

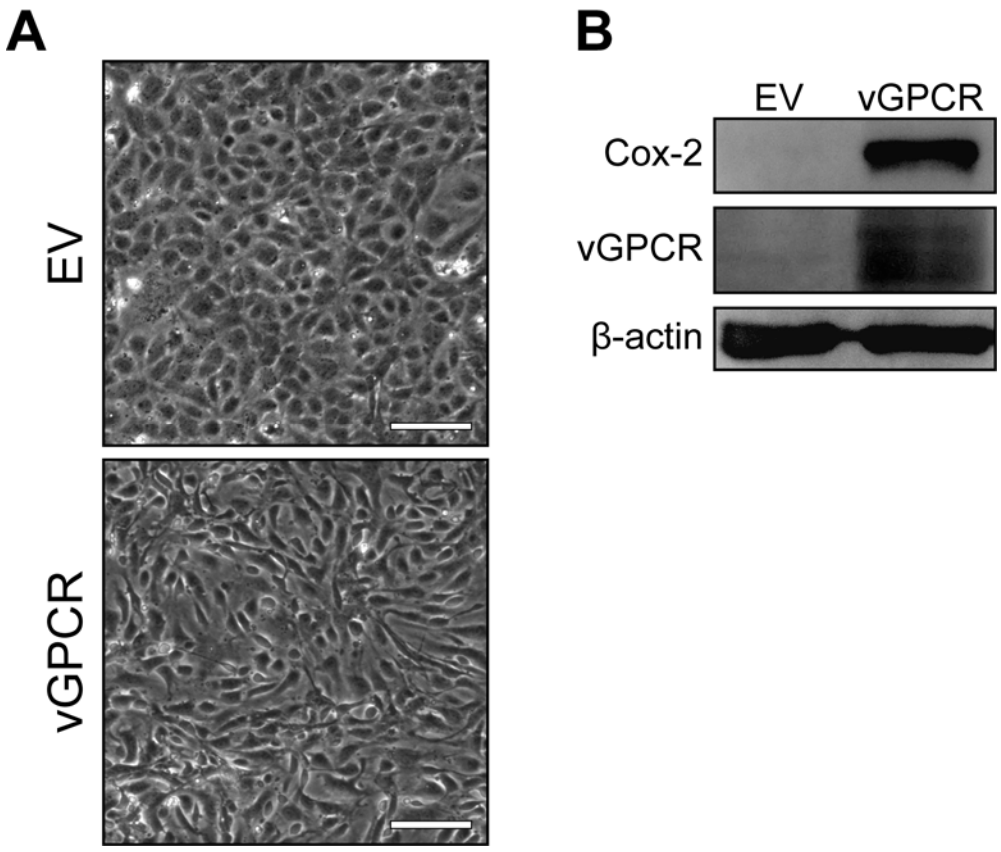

Figure 3.1 Functional confirmation of vGPCR-expression in transduced HUVECs. HUVECs were transduced with EV or a retroviral vector encoding vGPCR and selected in puromycin. At 2 d post-selection cell monolayers were **(A)** examined by light microscopy or **(B)** harvested and portions of the lysates were resolved by SDS-PAGE and subjected to immunoblot analysis of Cox2 and vGPCR. β-actin served as a protein loading control. Scale bars, 100 μm.

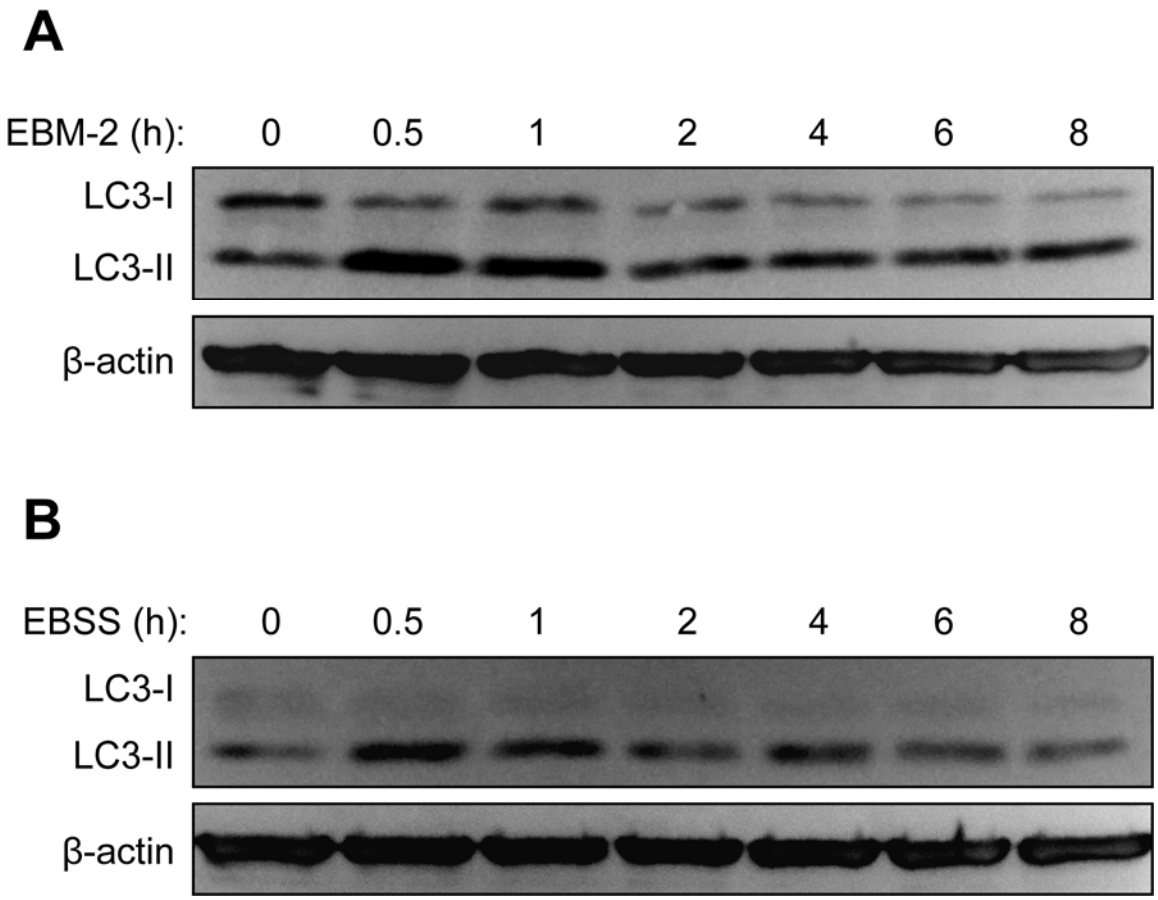

Figure 3.2 Induction of autophagy during nutrient deprivation in primary endothelial cells. HUVECs were grown to subconfluent monolayers. The culture medium was refreshed, 4 h later the cells were washed and overlaid with **(A)** EBM-2 or **(B)** EBSS and the cell monolayers were harvested at the indicated times. Portions of the lysates were resolved by SDS-PAGE and subjected to immunoblot analysis of LC3. β-actin served as a protein loading control.

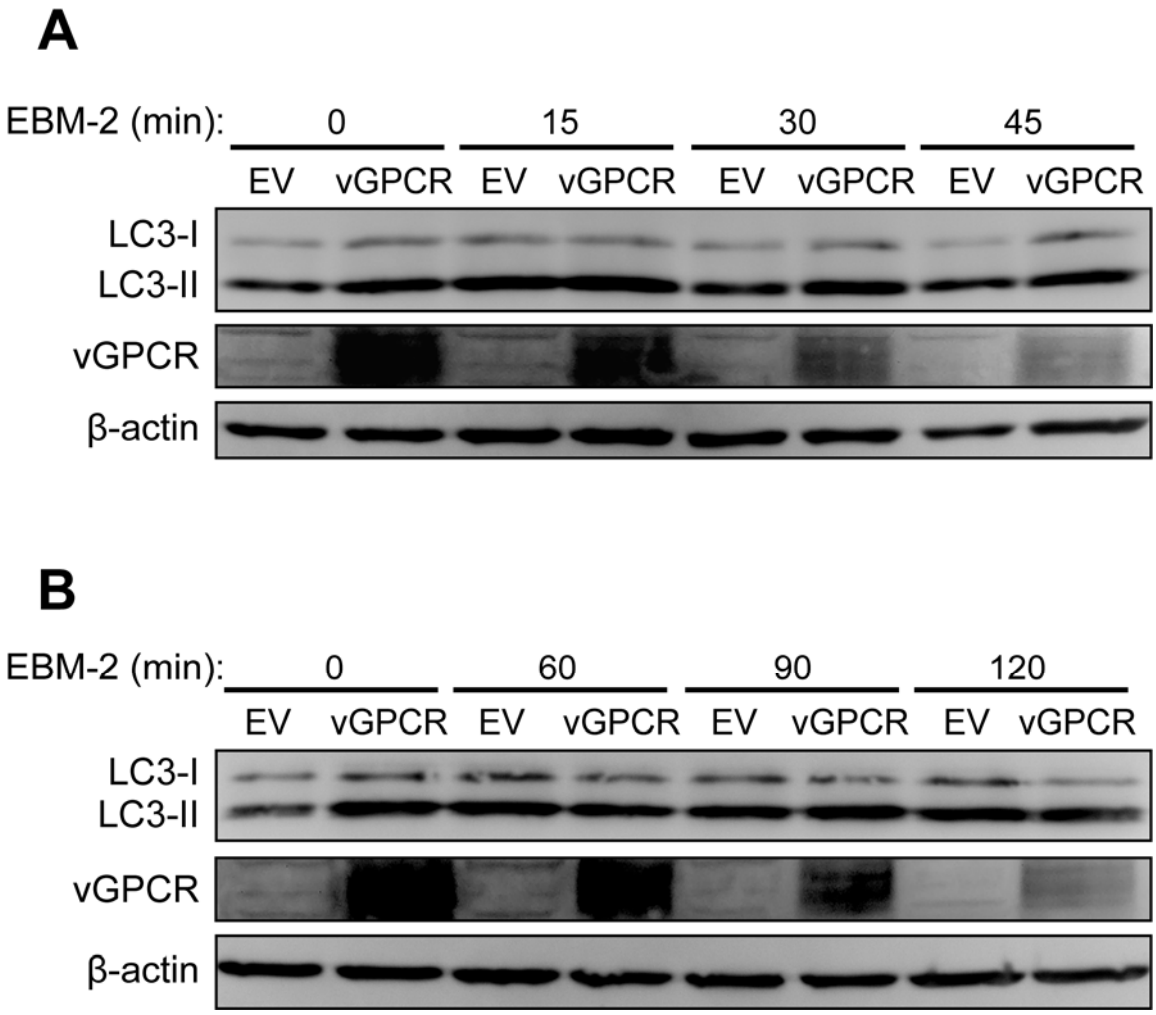

Figure 3.3 vGPCR enhances LC3-II accumulation during nutrient deprivation in HUVECs. HUVECs were transduced with an empty retroviral vector (EV) or one encoding vGPCR and selected in puromycin. At 2 d post-selection the culture medium was refreshed, 4 h later the cells were washed and overlaid with EBM-2, and at the **(A)** early or **(B)** late indicated times the cell monolayers were harvested. Portions of the lysates were resolved by SDS-PAGE and subjected to immunoblot analysis of LC3 and vGPCR. β-actin served as a protein loading control.

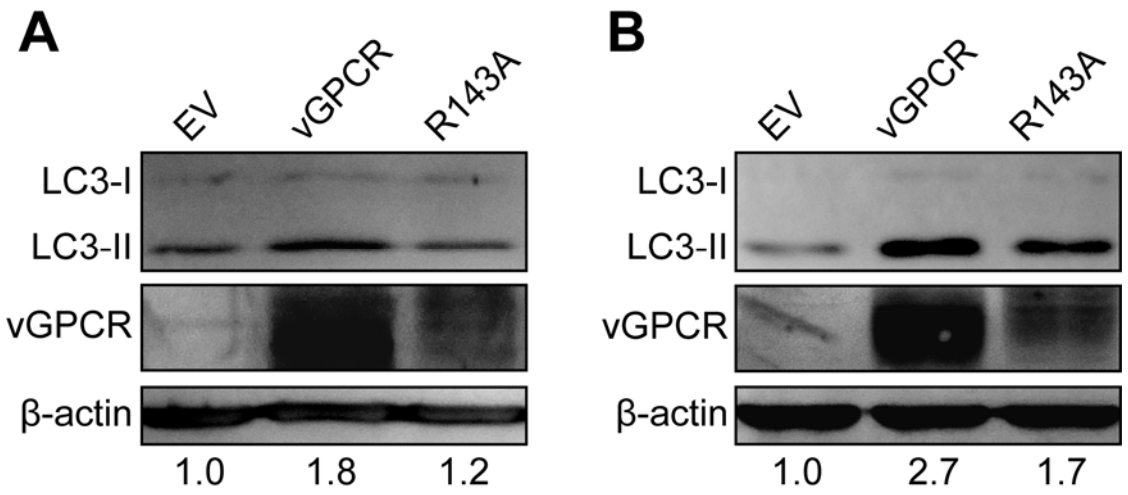

Figure 3.4 vGPCR enhances LC3-II accumulation under nutrient-rich conditions in endothelial cells. (A) HUVECs or **(B)** TIME cells were transduced with an empty retroviral vector (EV) or a retroviral vector encoding vGPCR and selected in puromycin. At 2 d post-selection the culture medium was refreshed and 4 h later the cell monolayers were harvested. Portions of the lysates were resolved by SDS-PAGE and subjected to immunoblot analysis of LC3 and vGPCR. β-actin served as a protein loading control. To quantify relative levels of LC3-II, band intensities were measured and normalized to that of β-actin in each lane and expressed relative to LC3-II levels of EV control.

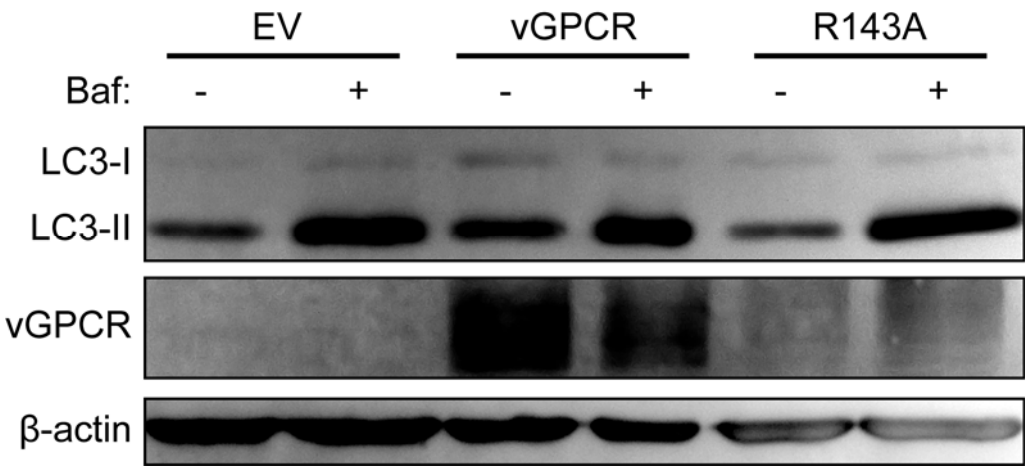

Figure 3.5 vGPCR induces autophagic flux in endothelial cells. TIME cells were transduced with EV or a retroviral vector encoding vGPCR or R143A and selected in puromycin. At 2 d post-selection the culture medium was refreshed, 4 h later the cells were overlaid with EGM-2 ± 160 μM bafilomycin for 4 h, at which time the cell monolayers were harvested. Portions of the lysates were resolved by SDS-PAGE and subjected to immunoblot analysis of LC3 and vGPCR. β-actin served as a protein loading control.

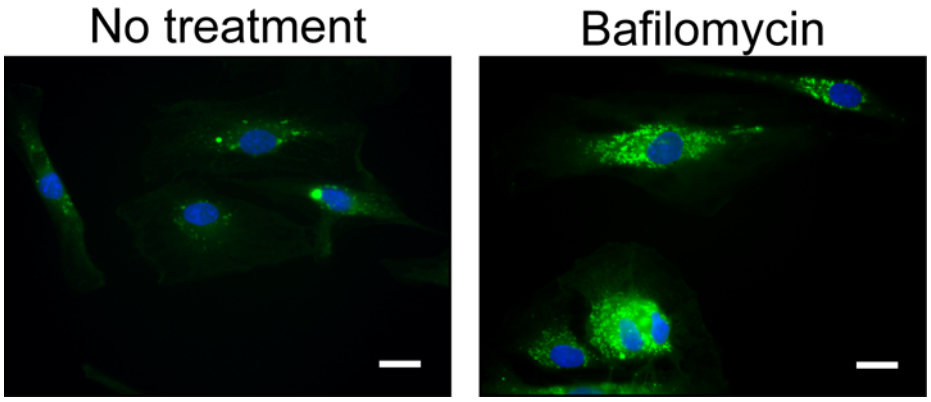

Figure 3.6 Accretion of cytoplasmic GFP-LC3 puncta in TIME cells. TIME cells were transduced with a retroviral vector bearing GFP-LC3 and selected in G418. The resulting cell line was seeded onto glass coverslips, the culture medium was refreshed and 4 h later the cells were overlaid with EGM-2 ± 160 μM bafilomycin for 4 h, at which time the cell monolayers were fixed in 4% paraformaldehyde and counterstained with DAPI. Fluorescence marking GFP-LC3 localization and DAPI were observed by fluorescence microscopy at 488 nm and 350 nm, respectively. Scale bars, 20 μm.

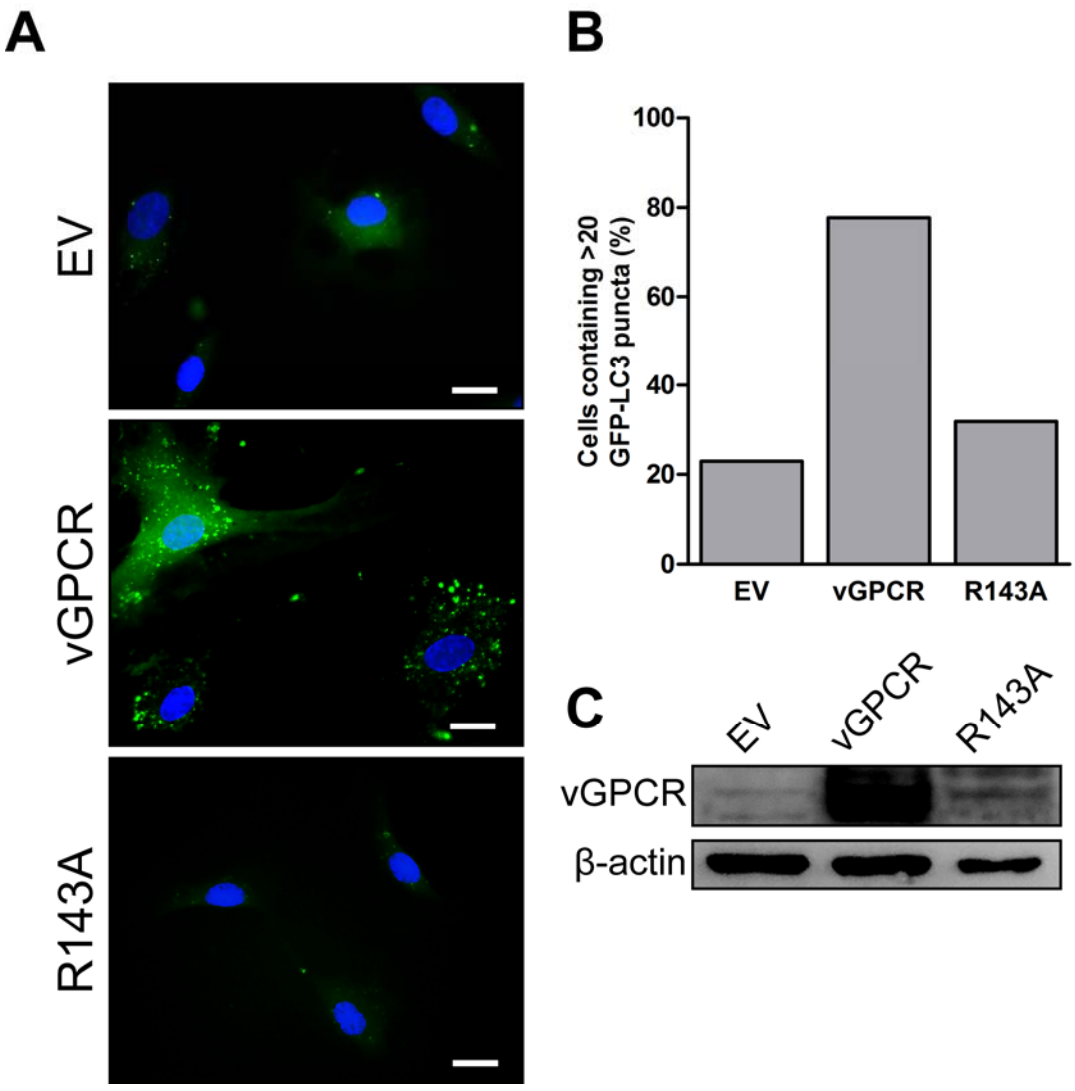

Figure 3.7 vGPCR triggers cytoplasmic accretion of GFP-LC3 in TIME cells. GFP-LC3 TIME cells were transduced with EV of retroviral vectors encoding vGPCR or R143A and selected in puromycin. At 4 d post-selection the culture medium was refreshed and cell monolayers were **(A)** fixed in 4% paraformaldehyde and counterstained with DAPI. Fluorescence marking GFP-LC3 localization and DAPI were observed by microscopy at 488 nm and 350 nm, respectively. Scale bars, 20 μm. **(B)** Quantification of cells in exhibiting >20 GFP-LC3 puncta per 50 cells from one experiment. **(C)** Cell monolayers were also harvested and portions of lysates were resolved by SDS-PAGE and subjected to immunoblot analysis to confirm vGPCR and R143A expression. β-actin served as a protein loading control.

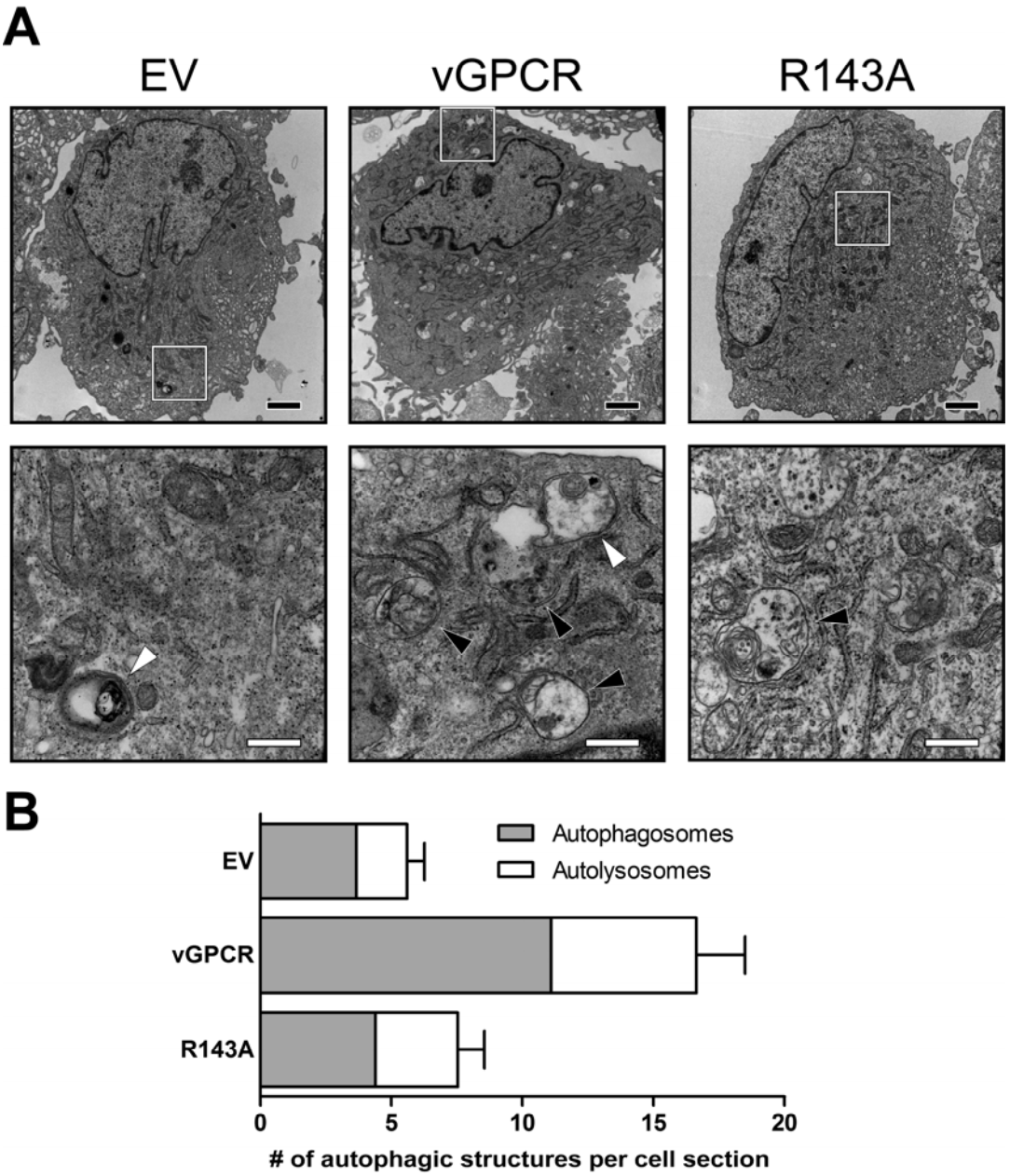

Figure 3.8 vGPCR triggers accumulation of autophagic structures in TIME cells. TIME cells were transduced with EV or a retroviral vector encoding vGPCR or R143A and selected in puromycin. At 3 d post-selection the culture medium was refreshed and 4 h later the cell monolayers were fixed and processed for transmission electron microscopy. **(A)** Representative images depicting autophagic structures in TIME cells. Black arrowheads, autophagosomes; white arrowheads, autolysosomes; black scale bars, 2 μm; white scale bars, 500 nm. **(B)** Quantification of autophagic structures per cell section from 30 cell sections from one experiment. The data are expressed as the means of autophagosomes and autolysosomes ± SEM of total autophagic structures per cell section.

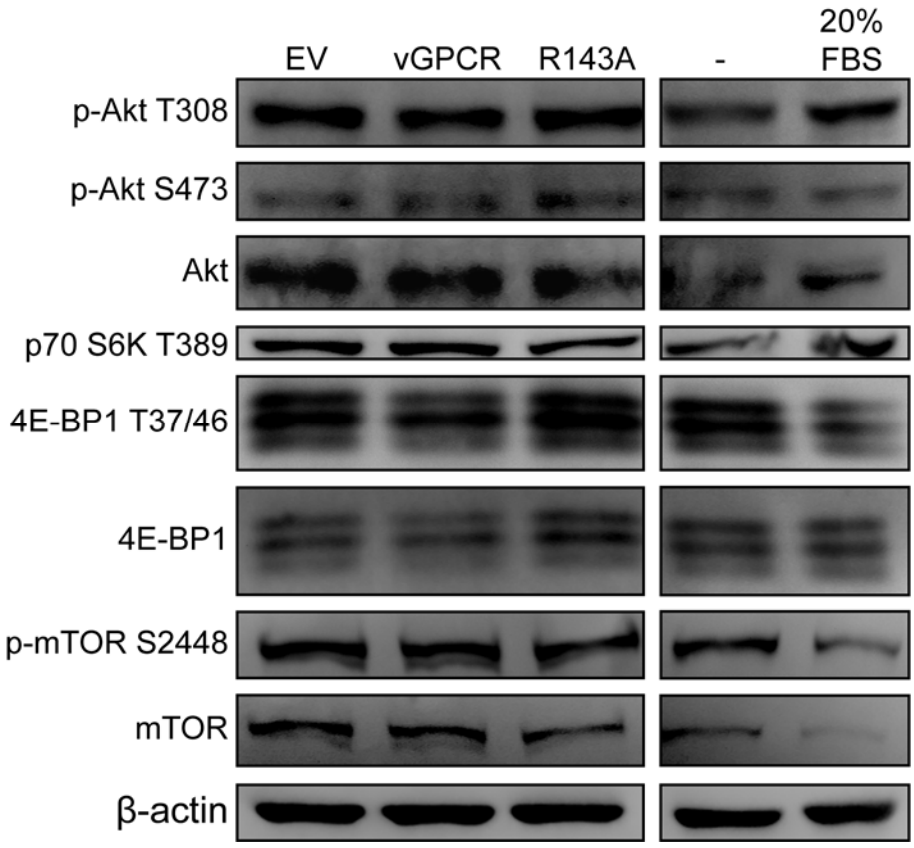

Figure 3.9 Examination of Akt/mTOR activation in vGPCR-TIME cells. TIME cells were transduced with EV or a retroviral vector encoding vGPCR or R143A and selected in puromycin. At 2 d post-selection the culture medium was refreshed and 4 h later the cell monolayers were harvested. TIME cells treated with 20% FBS for 4 h served as a positive control. Portions of the lysates were resolved by SDS-PAGE and subjected to immunoblot analysis of the indicated Akt/mTOR-pathway proteins. β-actin served as a protein loading control.

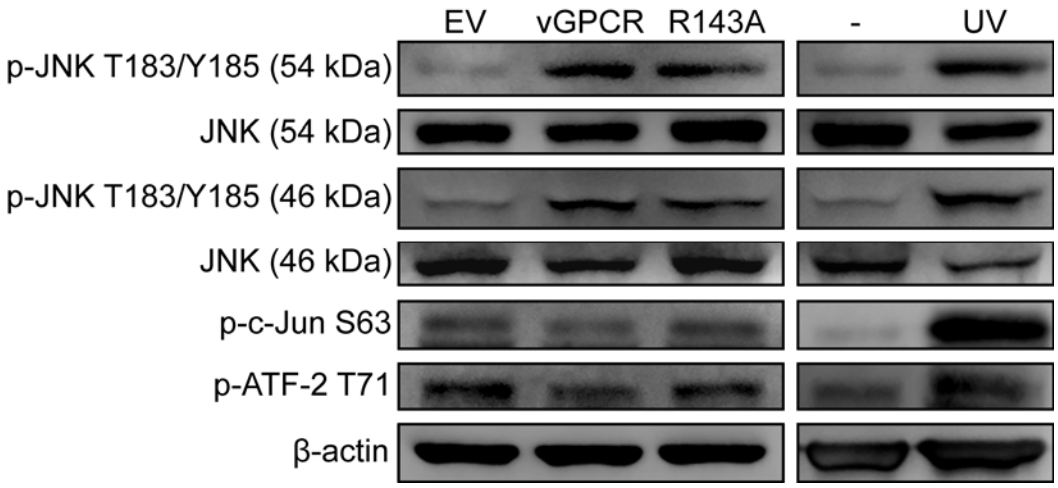

Figure 3.10 Examination of JNK activation in vGPCR-TIME cells. TIME cells were transduced with EV or a retroviral vector encoding vGPCR or R143A and selected in puromycin. At 2 d post-selection the culture medium was refreshed and 4 h later the cell monolayers were harvested. TIME cells exposed to 2500 μJ/cm^2 UV radiation and harvested 30 min later served as a positive control. Portions of the lysates were resolved by SDS-PAGE and subjected to immunoblot analysis of the indicated JNK-pathway proteins. β-actin served as a protein loading control.

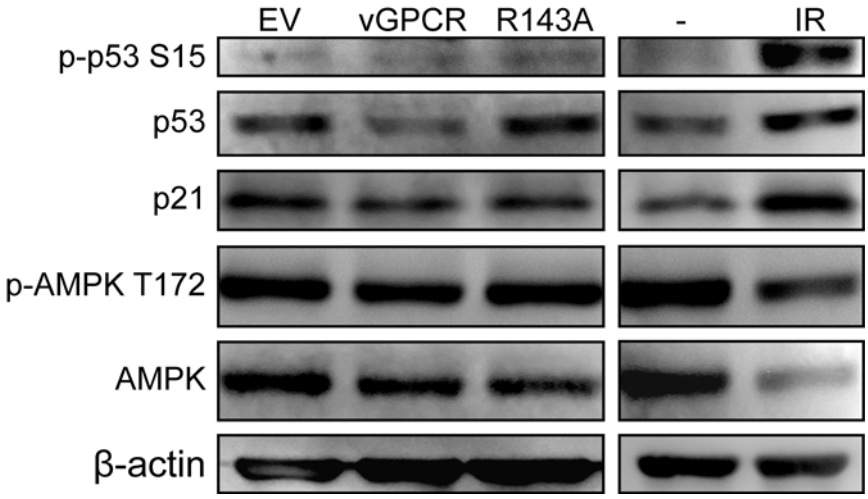

Figure 3.11 Examination of DNA damage response (DDR) pathway activation in vGPCR-TIME cells. TIME cells were transduced with EV or a retroviral vector encoding vGPCR or R143A and selected in puromycin. At 2 d post-selection the culture medium was refreshed and 4 h later the cell monolayers were harvested. TIME cells exposed to 10 Gy γ irradiation and harvested 1 d post-irradiation served as a positive control. Portions of the lysates were resolved by SDS-PAGE and subjected to immunoblot analysis of the indicated DDR-pathway proteins. β-actin served as a protein loading control.

CHAPTER 4: vGPCR TRIGGERS ONCOGENE-INDUCED SENESCENCE

4.1 Introduction

Nearly half a century ago the term 'cellular senescence' was first used to formally describe the limited proliferation of primary cells in culture (Hayflick and Moorhead, 1961; Hayflick, 1965). This particular form of arrest, known as replicative senescence, has been attributed to a critical limit imposed by progressive telomere shortening after each cell division, ultimately provoking a persistent DNA damage response (DDR) and genomic instability (Harley *et al*., 1990; Shay and Wright, 2000). In contrast to quiescence, the senescence growth arrest is essentially irreversible as senescent cells have permanently exited the cell cycle, yet they can remain metabolically viable in this interminably differentiated state for long periods of time (reviewed in Goldstein, 1990). Furthermore, senescence has been identified as a principal cellular response to stress and damage from exogenous and endogenous sources (reviewed in Campisi and d'Adda di Fagagna, 2007; Pazolli and Stewart, 2008; Kuilman *et al*., 2010). Notably, oncogene-induced senescence (OIS) is evoked by chronic oncogene expression or inactivation of tumour suppressor genes in cultured primary cells and *in vivo* (Serrano *et al*., 1997; Braig *et al*., 2005; Chen *et al*., 2005; Collado *et al*., 2005; Michaloglou *et al*., 2005; Courtois-Cox *et al*., 2006; Dankort *et al*., 2007; Sarkisian *et al*., 2007). OIS has therefore been implicated, not surprisingly, as an instrumental molecular barrier to tumourigenesis, as oncogene activation is a hallmark of cell transformation and cancer (reviewed in Campisi, 2005a; 2005b; Collado *et al*., 2007; Collado and Serrano, 2010). Upon oncogene expression, cells typically undergo a hyperproliferation phase that triggers the activation of severe DDRs and the activation of p53 and/or retinoblastoma protein (pRb) tumour suppressor pathways, ultimately propelling the transition to senescence (Di Micco *et al*., 2006; d'Adda di Fagagna, 2008). Furthermore, autophagy has recently been found to be necessary for efficient execution of the cellular senescence program (Narita *et al*., 2009; Young *et al*., 2009; Young and Narita, 2010).

The importance of senescence as a cellular response to infection by oncogenic viruses such as KSHV has yet to be examined. One KSHV gene product, a homolog of

cellular D-type cyclins, vCyclin, has been found to trigger a robust DDR in primary cells and induce OIS (Koopal *et al*., 2007). The McCormick lab is actively investigating the significance of OIS and viral subversion of this host-cell response (Leidal *et al*., unpublished work). Interestingly, the KSHV vGPCR has been demonstrated to evoke cell transformation in culture and induce 'KS-like' endothelial tumours in murine model systems (see section 1.4.5.2); surprisingly, vGPCR expression in TIME cells triggers a profound OIS transition. Here, this acquisition of cellular senescence is further characterized and the molecular mechanism underlying this unexpected phenotype has begun to be elucidated.

4.2 Results

4.2.1 Prolonged vGPCR Expression in TIME Cells Induces a Senescence-Like Morphological Change

Senescent cells display a wide variety of specific characteristics that facilitate their identification both *in vitro* and *in vivo*, many of which may serve as biomarkers for detecting senescent cells in pre-malignant tumours (Collado and Serrano, 2006; 2010). Cellular senescence is commonly associated with profound morphological changes in culture, where senescent cells become very large, flat, and often multinucleated (Bayreuther *et al*., 1988; Serrano *et al*., 1997; Kuilman *et al*., 2010). Light microscopic examination revealed that subconfluent TIME cells stably expressing vGPCR exhibited a number of these senescence-like morphology phenotypes (Figure 4.1). By 14 d post-selection, vGPCR-transduced TIME cells featured enlarged nuclei and extraordinarily vast, flattened cytoplasms, and an increased proportion of cells possessed two nuclei; in contrast, no morphological changes were detected in EV-transduced cells (Figure 4.1). These extreme alterations in the morphology of TIME cells undergoing chronic vGPCR expression were suggestive of a transition to cellular senescence.

4.2.2 Cell Proliferation is Arrested in vGPCR-Expressing TIME Cells

An irreversible exit from the cell cycle, and thus a permanent cell growth arrest, is the only indispensable characteristic for classifying cellular senescence; importantly, however, cell cycle arrest is not unique to senescent cells and must therefore be used in

conjunction with alternative measures to identify senescence (Kuilman *et al*., 2010). To monitor the proliferative profiles of vGPCR-expressing TIME cells, examination of cell growth and bromodeoxyuridine (BrdU)-labelling was utilized. Transductants were seeded at subconfluent densities, and although EV- and R143A-transduced cells grew at a steady rate until confluency was reached, by 5 d post-selection vGPCR-expressing TIME cells had ceased to divide (data not shown). BrdU is a nucleoside analog that can be incorporated into the newly synthesized DNA of actively replicating cells. BrdU can then be detected by immunofluorescence microscopy and the BrdU-positive cells are those that are undergoing DNA synthesis and cell cycle progression (Miltenburger, 1987). The proportion of BrdU incorporation in the control EV- and R143A-transduced cells was relatively high, 31.2 ± 0.2% and 31.4 ± 0.8% respectively; in contrast, only 3.6 ± 0.6% of vGPCR-expressing cells were BrdU-positive (Figures 4.2A and B). These data indicate that vGPCR induces a cell cycle arrest in TIME cells, further supporting the idea that vGPCR triggers a transition into cellular senescence.

4.2.3 vGPCR Triggers Oncogene-Induced Senescence of TIME Cells

The single most accepted and widely used biomarker for the detection of senescent cells is the presence of senescence-associated β-galatosidase (SA β-gal) activity (Dimri *et al*., 1995; Debacq-Chainiaux *et al*., 2009). Although most human cells express a lysosomal β-galactosidase enzyme that is functional at pH 4.0 (Morreau *et al*., 1989), senescent cells exhibit a distinct β-galactosidase activity that is detectable at pH 6.0, referred to as SA β-gal activity (Dimri *et al*., 1995). The increased SA β-gal activity in senescent cells is due to an expansion of the lysosomal compartment, producing a sharp increase in β-gal activity that can be detected at suboptimal pH 6.0 (Kurz *et al*., 2000). SA β-gal activity can be assayed in cultured cells or tissue sections using the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), which can be cleaved specifically by SA β-gal at pH 6.0 to yield an insoluble blue compound. Few robust biomarkers are available to qualify senescence *in vivo*, but SA β-gal staining has proven fruitful in application to reveal senescence both in cultured cells as well as in murine and human tissue biopsies (Collado and Serrano, 2006; Debacq-Chainiaux *et al*., 2009; Collado and Serrano, 2010); however, it should be noted that SA β-gal activity is

not completely specific to cellular senescence and has been identified in cells undergoing a number of stresses (Yang and Hu, 2005). To attribute the vGPCR-induced growth arrest to cellular senescence, TIME cells were transduced with vGPCR and examined for SA β-gal activity. Following prolonged expression, positive staining for SA β-gal activity was observed in 96.5 ± 0.8% of vGPCR-transduced cells, compared to only 6.6 ± 0.8% and 9.0 ± 1.0% positive activity in control EV- and R143A-transduced cells (Figures 4.3A and B). These data suggest that vGPCR, as a candidate viral oncogene, stimulates an OIS transition in TIME cells.

Another biomarker commonly used to identify senescent cells is the presence of a global alteration in chromatin structure (Narita *et al*., 2003; Zhang *et al*., 2005). These dense genomic regions are known as senescence-associated heterochromatic foci (SAHFs). SAHFs function to permanently repress the expression of genes with crucial roles in proliferation, particularly those regulated by the E2F family of transcription factors, largely contributing to the hallmark irreversible growth arrest that characterizes OIS. Although SAHFs can be microscopically visualized as DAPI-stained clusters of condensed chromatin, they cannot be readily detected in TIME cells (data not shown). The senescence-associated high-mobility group A protein-2 (HMGA2) has been found to accumulate on the chromatin of senescent cells and is an essential structural component of SAHFs (Narita *et al*., 2006). Importantly, HMGA2 protein levels sharply increase during cellular senescence and can therefore be used as a biomarker for senescent cells, indirectly identifying SAHFs (Narita *et al*., 2006). Immunoblot analysis revealed elevated levels of HMGA2 in vGPCR-expressing TIME cells, compared to EV- and R143A-transduced cells, at 5 d post-selection (Figure 4.4). Together, these data further suggest that vGPCR triggers a form of OIS, ultimately eliciting a permanent growth arrest that results from chronic vGPCR expression in TIME cells.

4.2.4 A DNA Damage Response is Not Elicited in vGPCR-Expressing TIME Cells

Canonical cellular senescence, oncogene-induced and replicative, is triggered by the engagement of DDR pathways as a result of either DNA hyperreplication or telomere attrition, respectively (d'Adda di Fagagna, 2008). The generation of DNA double-strand breaks (DSBs) is a powerful activator of DDRs, and this relies on the activation of the

ataxia-telangiectasia mutated (ATM) kinase, an instrumental molecular sensor of DSBs and stimulator of DDRs (reviewed in Shiloh, 2003; 2006). ATM phosphorylates S139 of histone variant H2AX molecules that flank the site of DNA damage, and this DDR relies on a number of important mediators including p53-binding protein 1 (53BP1), ultimately facilitating the focal assembly of DNA-repair and checkpoint factors (Shiloh, 2003; 2006). Importantly, nuclear foci of 53BP1 and γH2AX (phosphorylated-H2AX) develop at DSBs as a result of these DDR checkpoints in senescent cells, and can therefore serve as reliable markers for DNA damage (d'Adda di Fagagna *et al*., 2003). To investigate if vGPCR evokes DNA damage in stably transduced TIME cells, we employed immunofluorescence microscopic examination of 53BP1 and γH2AX localization. Interestingly, 53BP1 (Figure 4.5A) and γH2AX (Figure 4.5B) nuclear foci were absent in vGPCR-expressing TIME cells at 5 d post-selection, similar to EV- and R143A-transduced cells. In contrast, γ-irradiated cells displayed a marked increased in 53BP1 and γH2AX foci, indicative of extensive DNA damage (Figures 4.5A and B). Furthermore, the activated of p53 is a critical downstream effector of DDRs and paves the transition to OIS (Di Micco *et al*., 2006; d'Adda di Fagagna, 2008). Not only was phosphorylation of p53 at S15 not observed in senescent vGPCR-expressing TIME cells, but total p53 was also diminished; in contrast, phosphorylated p53 and total p53 were both elevated in γ-irradiated cells (Figure 4.4). These results are consistent with previous observations regarding the p53 status in autophagy-active vGPCR-expressing cells (Figure 3.11). Although this finding is surprising, provocation of DDRs and p53 activation are not universal features of OIS, nor are they required for cellular senescence to be efficiently established (reviewed in Kuilman *et al*., 2010). Together, these data suggest that vGPCR triggers a non-canonical form of OIS in the absence of p53 activation and a robust DDR.

4.3 Discussion

4.3.1 Summary

Cells respond to oncogenic events by triggering OIS, an irreversible form of cell cycle arrest. The senescence program is typically evoked by persistent DDRs that are associated with p53 activation. The KSHV vGPCR is widely considered a potent

oncogene based on its pronounced signalling activity and transforming potential in culture and *in vivo*. Remarkably, however, vGPCR expression in TIME cells triggered an arrest of cell proliferation and a robust OIS phenotype. Moreover, these cells demonstrated no significant DNA damage that would be associated with OIS, nor did they show increased p53 activation. Indeed, these findings have raised more questions then they have answered: Why has this vGPCR-mediated senescence phenotype not been previously documented? Is this senescence transition significant during KSHV infection? How does vGPCR trigger OIS in the absence of DNA damage and p53 activation? These issues are discussed below.

4.3.2 Why has vGPCR-Mediated OIS Not Been Previously Observed?

The oncogenic potential of vGPCR has been extensively studied and well-documented throughout the literature. In addition to its prominent signalling activities and paracrine effects, vGPCR has a tumourigenic nature that is best exemplified by its ability to directly transform cells in culture and induce endothelial tumours in mice (reviewed in Martin and Gutkind, 2008). How, then, can the observation that vGPCR triggers a robust OIS phenotype in TIME cells be reconciled? The initial studies demonstrating vGPCR-mediated cell transformation used COS-1 cells (Arvanitakis *et al*., 1997), NIH3T3 fibroblasts (Bais *et al*., 1998), and HUVECs (Bais *et al*., 2003); interestingly, none of these cell lines are capable of undergoing OIS. COS-1 cells are African green monkey kidney cells that were immortalized with the simian virus 40 (SV40) large-T antigen (Gluzman, 1981), a well-characterized oncogene that inhibits the p53 and pRb tumour suppressor proteins (Ali and DeCaprio, 2001); importantly, these cells do not senesce. NIH3T3 fibroblasts are also defective for the OIS program as they do not senesce in response to $Hras^{V12}$ expression (Lee *et al*., 2009b). Although primary HUVECs undergo replicative senescence in culture, they do not demonstrate $Hras^{V12}$-provoked OIS (A. Leidal, personal communication). Furthermore, the *in vivo* murine model system that demonstrated vGPCR-mediated 'KS-like' lesions used transduced SVEC4-10 cells (SV40-infected mouse endothelial cells) that were implanted into the mouse (O'Connell and Edidin, 1990; Montaner *et al*., 2003); indeed, SVEC4-10 cells will not senescence as they are immortalized with large-T antigen (see above). Together, these

previous studies utilized cell lines that have disabled or defective OIS machinery, which sufficiently explains why they did not observe a vGPCR-induced growth arrest. This study used telomerase-immortalized microvascular endothelial (TIME) cells, which effectively undergo OIS in response to many different oncogenic stresses, including $Hras^{V12}$ expression and DNA-damaging agents such as etoposide (Leidal *et al*., unpublished data). Interestingly, one other study has reported a p53-independent cell cycle arrest triggered by vGPCR in PEL cells; however, they did not test for OIS, nor did they allude that a senescence transition may have occurred (Cannon *et al*., 2006). Thus, the work described here is the first documented evidence, to my knowledge, of confirmed vGPCR-mediated cellular senescence.

To rule out the possibility that vGPCR-mediated OIS is an artifactual event specific to TIME cells, it is necessary to determine if vGPCR evokes OIS in other cell types. Preliminary results from vGPCR-transduced WI38 fibroblasts or human foreskin fibroblasts (HFFs), two cell lines that are commonly used for senescence research, demonstrated a retardation in cell growth but not a complete arrest of cell proliferation (data not shown). This phenotype was similar to that observed in $Hras^{V12}$-transduced cells in this experiment, and therefore it needs to be repeated. It is possible that vGPCR-mediated OIS may be an endothelial cell-specific effect, and therefore it would be appropriate to investigate this phenotype in human dermal microvascular endothelial cells (HDMECs), a primary culture that sustains both $Hras^{V12}$- and vCyclin-mediated OIS (Koopal *et al*., 2007).

4.3.3 What is the Significance of vGPCR-Mediated OIS During KSHV Infection?

vGPCR is expressed in KSHV-infected cells undergoing lytic reactivation. These cells comprise a very small proportion of the infected cells in a KS lesion (1 - 5%) and are ultimately destined to die by lysis (discussed in sections 1.4.3 and 1.4.5). However, given that vGPCR may be instrumental in establishing KS tumours based on its direct and indirect transforming capabilities, the idea that vGPCR may be expressed outside the context of the classic lytic cycle has been suggested (Sodhi *et al*., 2004a). Although a number of candidate mechanisms have been proposed, deregulated expression of a subset of lytic genes during an abortive lytic-cycle program is the most plausible, as abortive

lytic infections have been shown in a number of other herpesviruses (Sodhi *et al*., 2004a). Instead of vGPCR being expressed transiently in 'normal' lytically infected cells, an abortive lytic program may result in prolonged, enhanced vGPCR expression. This hypothesis unfortunately lacks any direct evidence, but it may explain how the angiogenic, inflammatory, and proliferative potential of vGPCR is able to evoke biologically significant effects in the KS tumour microenvironment.

If an aberrant lytic expression program exists, prolonging vGPCR expression during KSHV infection, these cells may undergo vGPCR-mediated OIS over time. Although senescence is considered a tumour suppressor mechanism and may therefore limit the direct cell-transforming potential of vGPCR, the effect of senescence on viral replication has not been characterized. Moreover, senescent cells have a unique secretion profile, producing many cytokines and growth factors that enforce the senescence phenotype and may further contribute to the environment of the KS lesion (Kuilman and Peeper, 2009). The senescence status of KSHV-infected cells in KS lesions is currently unknown. This status can be readily determined by staining histological sections for vGPCR and SA β-gal activity and would directly indicate if senescence contributes to the KS lesion and if vGPCR is able to evoke this stress response *in vivo*. At the very least, the ability of vGPCR to elicit the OIS phenotype in culture simply reinforces the true oncogenic potential of this virally pirated host-cell G-protein-coupled receptor.

4.3.4 vGPCR Triggers OIS by a Non-Canonical Mechanism

OIS is often triggered by chronic oncogene expression or tumour suppressor inactivation, which culminates in irreparable DNA damage and a persistent DDR that is associated with p53 activation (Di Micco *et al*., 2006; d'Adda di Fagagna, 2008). However, there have been numerous cases of OIS that are unaccompanied by a DDR and that does not require p53 activation (Olsen *et al*., 2002; Nicke *et al*., 2005; Kuilman *et al*., 2008; Efeyan *et al*., 2009; Lin *et al*., 2010a). In terms of previously characterized vGPCR functions, the absence of DNA damage and p53 activation in vGPCR-senescent cells was not surprising, as these effects have not been previously reported. These data do signify, though, that vGPCR triggers cellular senescence by a so-called 'non-canonical' mechanism. Recent evidence has suggested that chronic signalling through the

PI3K/Akt/mTOR axis activates a senescence program, albeit weaker than a $Hras^{V12}$-directed response (Kennedy *et al*., 2011). This constitutively active Akt-driven senescence response does not trigger DNA damage and p53 activation, nor does it induce cellular autophagy, which is essential to efficiently establishing the senescence phenotype (Young *et al*., 2009; Kennedy *et al*., 2011). vGPCR, on the other hand, elicits pleiotropic signalling effects that not only target the PI3K/Akt/mTOR pathway but also subvert the MAPKs (see section 1.4.5.2). MAPK activation by vGPCR induces an elaborate cytokine secretion profile that may contribute to the acquisition of OIS via autocrine effects on vGPCR-expressing cells, and it may promote a senescence transition in neighbouring cells by paracrine signalling (Kuilman and Peeper, 2009). Moreover, JNK stimulation by vGPCR may underlie the robust cellular autophagic response to vGPCR expression (see Chapter 3), further enhancing the observed OIS phenotype (Young *et al*., 2009). Further study is needed to investigate the molecular mechanism that drives vGPCR-mediated OIS, and to address whether this is important in KSHV-pathogenesis and KS development or if this is an unfortunate consequence of an oncogenic stress response that must be attenuated by other lytic proteins in KSHV-infected cells. This study may serve as a foundation to better our understanding of how OIS may be triggered in the absence of DNA damage in other settings. Particularly, deregulated and pirated cellular GPCRs are characteristic of many other viral pathologies (reviewed in Sodhi *et al*., 2004b), as well as a number of human cancers (reviewed in Dorsam and Gutkind, 2007), and by enhancing our knowledge of GPCR effects on autophagy and senescence we hope to help guide the development of therapeutics that exploit these cellular failsafe programs in KS and other malignancies.

4.4 Figures and Tables

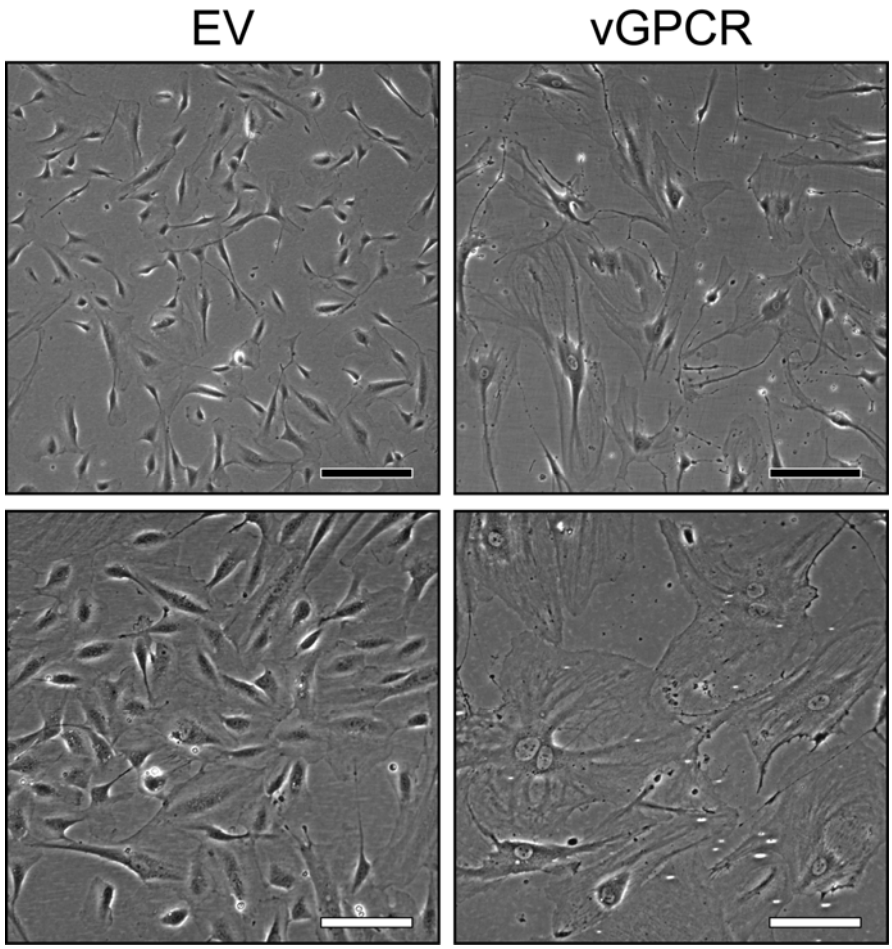

Figure 4.1 Prolonged vGPCR-expression induces morphological changes in TIME cells indicative of senescence. TIME cells were transduced with EV or a retroviral vector encoding vGPCR and selected in puromycin. Following selection, transductants were subcultured for 14 d and examined by light microscopy. Black scale bars, 500 μm; white scale bars, 100 μm.

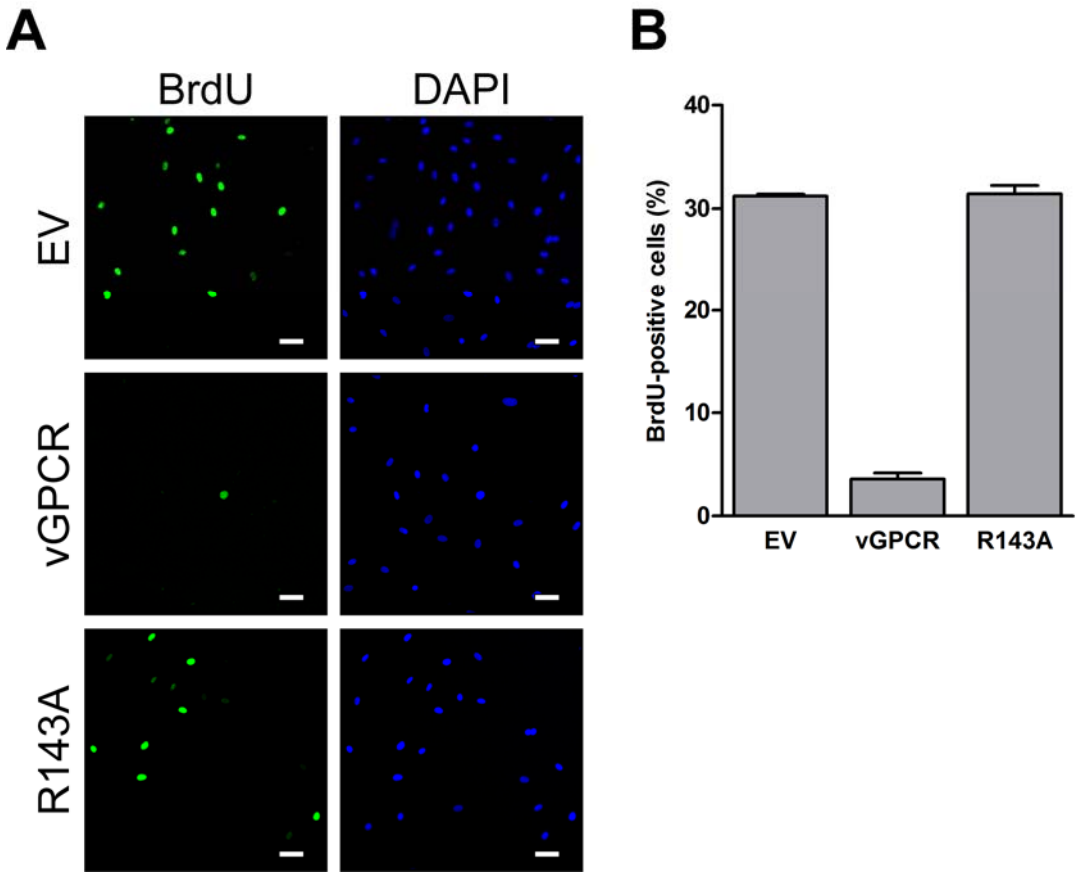

Figure 4.2 vGPCR expression diminishes proliferation of TIME cells. TIME cells were transduced with EV or a retroviral vector encoding vGPCR or R143A and selected in puromycin. At 5 d post-selection, transductants were pulsed with BrdU for 12 h, fixed in 3.7% formaldehyde, and processed for immunofluorescence detection of BrdU; nuclei were counterstained with DAPI. BrdU and DAPI were observed by fluorescence microscopy at 488 nm and 350 nm, respectively. **(A)** Representative images depicting BrdU incorporation. Scale bars, 50 μm. **(B)** Quantification of BrdU-positive cells expressed as the mean ± SEM of three replicates from one experiment.

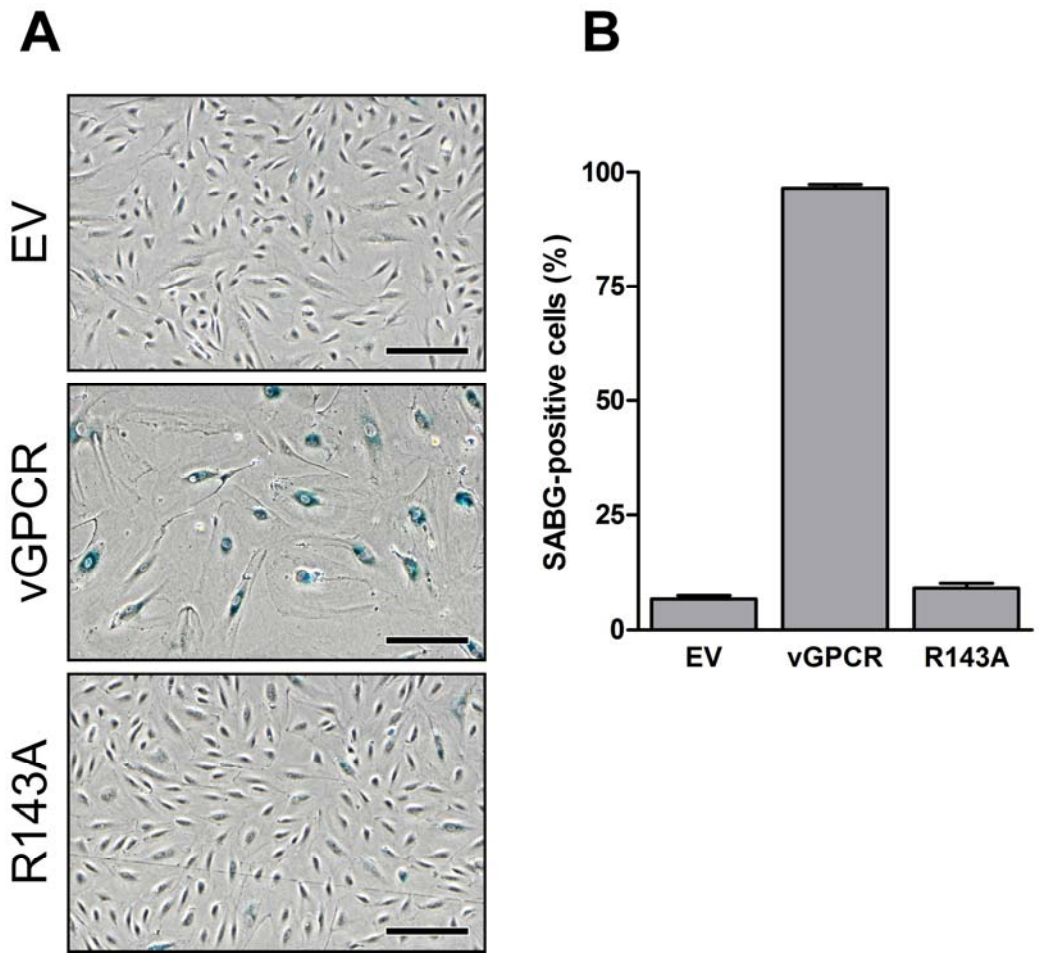

Figure 4.3 vGPCR triggers oncogene-induced senescence in TIME cells. TIME cells were transduced with EV or a retroviral vector encoding vGPCR or R143A and selected in puromycin. At 14 d post-selection, transductants were fixed and stained for senescence-associated β-galactosidase (SA β-gal) activity at pH 6.0. **(A)** Representative images of light microscopy examination depicting SA β-gal staining. Scale bars, 200 μm. **(B)** Quantification of cells positively stained for SA β-gal activity is expressed as the mean per field ± SEM from one experiment.

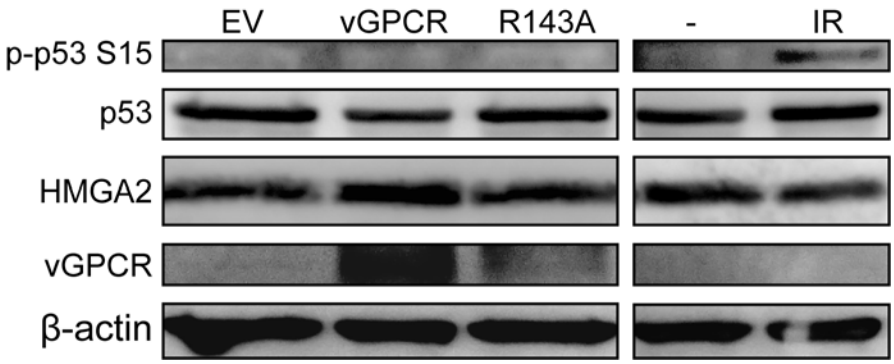

Figure 4.4 vGPCR induces OIS in the absence of p53 activation in TIME cells. TIME cells were transduced with EV or a retroviral vector encoding vGPCR or R143A and selected in puromycin. At 5 d post-selection, transductants were harvested and portions of lysates were resolved by SDS-PAGE and subjected to immunoblot analysis for phosphor-p53 S15, total p53, HMGA2 and vGPCR. TIME cells exposed to 10 Gy γ irradiation (IR) served as a positive control for p53 activation, but not increased HMGA2, and 1 d later the cell monolayer was harvested and processed as described. β-actin served as a protein loading control.

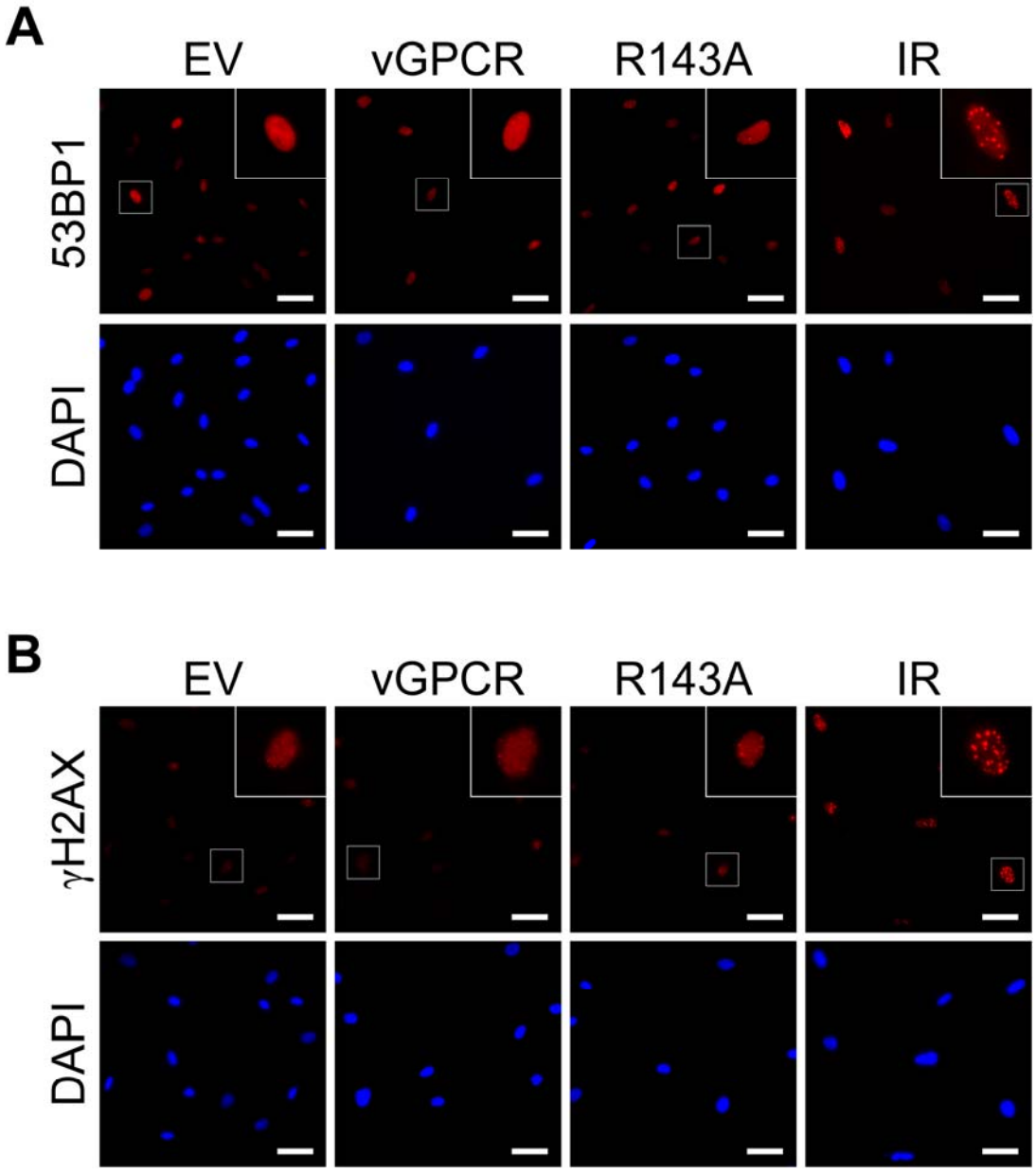

Figure 4.5 vGPCR does not elicit DNA damage in TIME cells. TIME cells were transduced with EV or a retroviral vector encoding vGPCR or R143A and selected in puromycin. At 5 d post-selection, transductants were fixed in 3.7% formaldehyde, processed for immunofluorescence detection of **(A)** 53BP1 or **(B)** γH2AX, and the nuclei were counterstained with DAPI. TIME cells exposed to 10 Gy γ irradiation (IR) served as a positive control, and 1 d later the cell monolayer was fixed and processed as described. DNA damage and DAPI were observed by fluorescence microscopy at 555 nm and 350 nm, respectively. Scale bars, 40 μm.

CHAPTER 5: CONCLUSION

The rapidly growing number of identified viral mediators of autophagy highlights the importance of this process for antiviral defence. We are just beginning to understand the diversity of the multipronged attacks that viruses use to subvert host cell autophagic machinery, which is paramount to successful viral replication and often contributes to pathogenesis. Interestingly, despite the overwhelming evidence linking autophagic dysfunction to cancer, no study has yet clearly demonstrated that an oncogenic virus can usurp autophagy to promote tumourigenesis. Several KSHV gene products have autophagy-deregulating functions, suggesting that autophagy restricts viral replication *in vivo*. However, much work remains to be done to understand how autophagy affects KSHV replication and KS development.

In this study a novel cellular autophagic response to vGPCR, a putative oncogene expressed during the KSHV lytic cycle, was identified. This intensively studied signalling molecule is considered instrumental in establishing KS and developing the tumour microenvironment. Here, a stable cell culture system was developed and a battery of experimental assays were optimized to investigate the potential of vGPCR to modulate autophagic activity. Interestingly, expression of vGPCR in endothelial cells induced a profound activation of cellular autophagy. Preliminary evidence suggested that this increased autophagic flux may be a result of vGPCR-mediated JNK activation; JNK serves as a positive regulator of autophagy by suppressing Bcl-2-mediated inhibition of Beclin 1. Further examination is necessary to characterize the molecular mechanism underlying vGPCR-induced autophagy, and to understand the importance of this process in KSHV-infected host cells.

During the course of these studies, I serendipitously observed that vGPCR activity induced senescence of endothelial cells. Remarkably, vGPCR fails to trigger any DNA damage responses that are typically characteristic of OIS, suggesting that vGPCR may trigger senescence via a non-canonical mechanism. The precise underlying mechanism(s) of vGPCR-mediated OIS, as well as the importance of this process in KSHV infection and pathogenesis, are currently unknown. Nevertheless, these data further support the oncogenic potential of vGPCR.

I am confident that the results presented in this thesis will serve as a solid foundation for further investigation into understanding the importance of autophagy and senescence in KSHV replication and tumourigenesis. To my knowledge, vGPCR is the first G-protein-coupled receptor demonstrated to orchestrate an autophagic response and trigger senescence, potentially revealing an uncharacterized downstream biological consequence of this family of signalling effectors. A better understanding of these mechanisms may unveil novel aspects of the regulatory networks that control autophagy and senescence, and this knowledge may further guide the design of novel therapies that reinforce these antiproliferative responses to block tumour development, both in KS and in cancers that lack an underlying viral etiology.

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APPENDIX A: EXAMINING THE KSHV-MEDIATED IMPAIRED SENESCENCE RESPONSE

A.1 Introduction

Similar to many DNA tumour viruses, KSHV evokes a robust DNA damage response (DDR) upon latent infection of human endothelial cells (Leidal *et al*., unpublished work; Lilley *et al*., 2007). This oncogenic assault is fundamentally attributed to wide-spread deregulation of the cell cycle by viral cyclin (vCyclin), the latent KSHV homolog of cellular D-type cyclins (Verschuren *et al*., 2002; Verschuren *et al*., 2004a; 2004b). Expression of vCyclin in cultured cells stimulates aberrant cell cycle progression and engages the p53 tumour suppressor pathway; consequently, vCyclin expression activates antiproliferative checkpoints and triggers oncogene-induced senescence (Koopal *et al*., 2007). However, KSHV-infected cells fail to efficiently trigger oncogene-induced senescence (OIS) despite this viral attack on host genomic stability, implicating the presence of another viral protein that elicits bypass of this cellular oncogenic defense (Leidal *et al*., unpublished work). Interestingly, autophagy has recently been characterized as a critical effector mechanism for acquisition of the senescence phenotype (Young *et al*., 2009), and we have demonstrated that autophagic activity precedes vCyclin OIS (Leidal *et al*., unpublished work). vFLIP is a KSHV latent gene product that has been found to suppress autophagic activity by inhibiting the essential autophagy protein Atg3 (Lee *et al*., 2009a), and we found that vFLIP efficiently blocks vCyclin-induced autophagy and senescence (Leidal *et al*., unpublished work). Overall, we have revealed a coordinated viral gene-expression program that has evolved to efficiently facilitate host-cell hyperproliferation by impairing autophagy and OIS machinery despite persistent DDRs.

Here, I have begun to address the first of a series of outstanding questions that stem from this work. Given that KSHV-infected cells demonstrate an impaired OIS phenotype despite a robust virally induced DDR, I hypothesized that KSHV infection may confer cellular resistance to senescence induced by exogenous DNA-damaging agents or genotoxic stresses.

A.2 Results

A.2.1 KSHV Infection Impairs DNA Damage-Induced Senescence

To investigate whether KSHV infection can impede the onset of cellular senescence triggered by exogenous genotoxic stresses, TIME cells were infected with KSHV, or mock-infected, and at 48 h post-infection cells were treated with or without etoposide. Etoposide is a DNA-damaging agent that elicits pronounced double-strand breaks (DSBs) by inhibiting topoisomerase II, ultimately triggering persistent DDRs and activating an effective cellular senescence transition (Krizhanovsky *et al*., 2008; Young *et al*., 2009). At 5 d following etoposide treatment, cells were stained for SA β-gal activity (Figure A.1A). Although no considerable differences in SA β-gal activity were observed between untreated mock- and KSHV-infected cells (5.8 ± 0.6% and 6.0 ± 0.4%, respectively), there was a marked difference in SA β-gal activity following etoposide treatment; indeed, 41.4 ± 1.0% of mock-infected cells stained positive for SA β-gal activity, whereas only 17.5 ± 1.9% of KSHV-infected cells expressed SA β-gal activity (Figure A.1B). Furthermore, despite a minor loss in cell number following chronic KSHV infection, immunofluorescence microscopic detection of LANA revealed that nearly the entire infected cell monolayer had established KSHV latency (Figure A.1C). These results suggest that KSHV implements a coordinated latency program that can not only elicit bypass of vCyclin-mediated OIS, but it can also facilitate circumvention of a cellular antiproliferative response to exogenous genotoxic stresses.

A.3 Discussion

A.3.1 Summary

We have previously demonstrated that although latent KSHV infection evokes DNA damage, and this damage can be attributed to the expression of a single KSHV latent-gene product, vCyclin, which can trigger OIS when expressed alone (Leidal *et al*., unpublished work). However, KSHV-infected cells fail to senesce despite these oncogenic insults, and this senescence bypass is mediated, at least in part, by vFLIP (Leidal *et al*., unpublished work). Since KSHV effectively deregulates the senescence program of infected cells, I hypothesized that KSHV-infected cells may be resistant to senescence triggered by exogenous genotoxic stresses. Indeed, fewer KSHV-infected

cells senesced following etoposide treatment compared to the mock-infected control cells.

A.3.2 Are the Effects of Senescence Deleterious to KSHV Pathogenesis?

KSHV predominantly establishes latent infection, similar to all herpesviruses, and successful viral pathogenesis and persistence in the host relies on a coordinated latent-gene expression program. The subset of KSHV genes expressed during latency function in episomal maintenance, immune evasion, inflammation, and the long-term survival and proliferation of infected host cells (see section 1.4.4). Leidal and colleagues (unpublished work) identified a novel co-regulated function of the KSHV latent-gene products: to orchestrate viral subversion of the host-cell senescence program. As described above, vCyclin expression triggers hyperproliferation and DDRS in infected cells, and the OIS response is activated; however, vFLIP effectively ablates the cellular senescence program by suppressing cellular autophagy (Leidal *et al*., unpublished work). As a result, KSHV-infected cells are resistant to senescence evoked by a viral oncogene. Moreover, KSHV subversion of the senescence response is further able to efficiently dampen this antiproliferative program in the presence of DNA damage evoked by exogenous genotoxic agents. This finding suggests that cellular senescence may be deleterious to latent KSHV infection and, perhaps, its tumourigenic potential. Interestingly, a recent study determined that DDRs and senescence are limiting to EBV-mediated oncogenesis, and that EBV encodes a regulator to attenuate infected-cell DDRs to promote infected-cell transformation (Nikitin *et al*., 2010). This accumulating evidence suggests that senescence plays an instrumental role in host-cell defence against oncogenic viruses, and that consequently viruses have evolved an arsenal of effector mechanisms to target this antiproliferative response.

It would be interesting to determine if vFLIP alone is able to subvert the senescence program in response to exogenous DNA-damaging agents, or if vFLIP can elicit bypass of OIS triggered by non-KSHV oncogenes such as $Hras^{V12}$. Furthermore, it would be important to confirm that etoposide treatment of KSHV-infected cells is eliciting DNA damage, and how the level of damage induced compares to that of control

cells. Finally, the senescence status of latently infected cells in a KS lesion would be important to address.

A.4 Figures and Tables

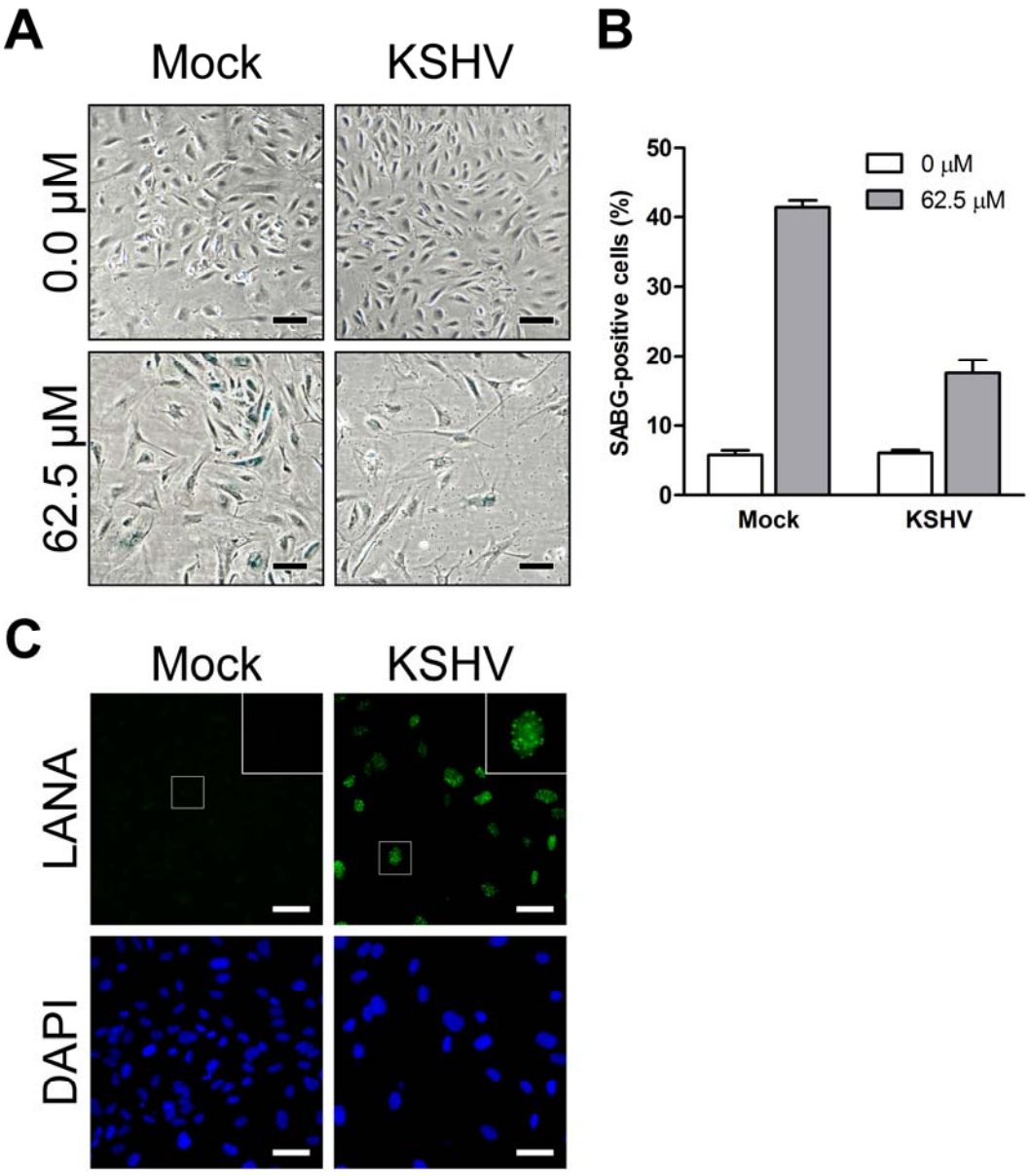

Figure A.1 KSHV infection suppresses etoposide-induced senescence in endothelial cells. TIME cells were infected with KSHV or were mock-infected, and at 48 h post-infection the cells were treated with 0 µM or 62.5 µM etoposide for 6 h. At 5 d post-treatment the cell monolayers were fixed and **(A)** stained for SA β-gal activity, and **(B)** positively stained cells were quantified and expressed as the mean per field ± SEM from one experiment. **(C)** Cell monolayers at 48 h post-infection were processed for immunofluorescence detection of LANA at 488 nm. Black scale bars, 100 µm; white scale bars, 40 µm.

APPENDIX B: INVESTIGATING SUBVERSION OF AUTOPHAGY BY KAPOSIN B

B.1 Introduction

Autophagy is a critical process that mediates the transition to senescence and has important implications in tumour suppression (Levine, 2007; Narita *et al*., 2009; Young *et al*., 2009). Given that autophagy and senescence may be detrimental to latent KSHV infection and/or pathogenesis, I hypothesized that KSHV may encode additional deregulators of cellular autophagy during latency to efficiently combat this antiviral and antiproliferative process. The latent kaposin B (kapB) protein was an ideal candidate based on its characterized role as a novel viral activator of mitogen-activated protein kinase (MAPK)-associated protein kinase 2 (MK2), as well as the upstream regulator p38 MAPK, evoking a robust pro-inflammatory cytokine secretion profile that is considered critical to KS development (McCormick and Ganem, 2005; 2006). Interestingly, MK2 may function as a negative regulator of autophagy based on recent reports that cells treated with rottlerin, a pharmacological inhibitor of MK2 (Davies *et al*., 2000), exhibit increased autophagic activity (Song *et al*., 2008; Balgi *et al*., 2009). p38 has also been directly found to function in autophagic control (Comes *et al*., 2007). MK2 may regulate autophagy by enhancing Akt activity (a negative autophagic regulator) through forming a molecular complex composed of heat shock protein 27 (HSP27), p38, MK2, and Akt (Rane *et al*., 2003; Zheng *et al*., 2006; Wu *et al*., 2007), or MK2 may coordinate with p38 to phosphorylate and inhibit the TSC2 subunit of TSC1/2, suppressing this negative regulator of mTOR (Li *et al*., 2002; 2003). I hypothesized that kapB may suppress autophagy by robustly activating MK2 and p38. Unfortunately, a complication with the clonal validity of our kapB-encoding expression vectors was encountered, and this, along with the necessary corrective measures, is described below.

B.2 Results

B.2.1 Kaposin B Expression Vectors: Clonal Differences and Corrective Measures

The kaposin locus is composed of three primary overlapping open reading frames (ORFs), in the same and different reading frames, to generate kaposins A, B, and C, the

principal kaposin gene products (Figure B.1A; Sadler *et al*., 1999; Muralidhar *et al*., 2000). Indeed, one remarkable characteristic of the kaposin locus is that kapB and kapC are translated in different overlapping reading frames yet they both contain identical direct repeat (DR) regions, known as DR1 and DR2 (Figure B.1A). This phenomenon results from the presence of 23-nt repeats that are translated into 23-amino acid repeats of common sequence in all three reading frames (Figures B.1B and C; Sadler *et al*., 1999). These 23-nt repeats are not a multiple of 3; therefore, a ribosome can only translate 7 codons (21 nt) per repeat in one frame, and must then use the first nucleotide of the next repeat to translate the following codon, effectively shifting the reading frame by 1 nt (Figures B.1B and C). Following the translation of every three repeats, the ribosomes are back in the original reading frame, and, therefore, the resulting protein product consists of 23-codon repeats. Furthermore, a complex translation initiation program expands the coding potential of kaposins B and C to yield a number of secondary protein products (not shown).

During site-directed mutagenesis of the different internal start codons throughout the kapB ORF of our retroviral vector, DNA sequencing analysis revealed that our kapB construct did not perfectly agree with the putative kapB derived from a pulmonary KS isolate (Figure B.1D; reference sequence from D. Ganem, UCSF). Our kapB construct bears the N-terminal 37 nucleotides from kapC (Figure B.1D, bottom row; reference sequence from D. Ganem, UCSF), but the remainder of our construct aligns perfectly with the putative kapB (Figure B.1D, top row). This added N-terminal region results in our lab construct being read in the same frame as kapC, which is different than that of the putative kapB (Figures B.1A and D). Despite the different reading frames, our construct encodes the 23-amino acid DR1 and DR2 regions (Figure B.1E), domains that are critical for kapB function (McCormick and Ganem, 2006). Unfortunately, this alternative reading frame of our 'pseudo-kapB' would cause non-recognition of the putative termination codon, and may result in translation of vector DNA until the next possible stop codon is reached (~240 bp; Figures B.1A and E). It is difficult to determine what deleterious effects, if any, the additional amino acid content may have on function, as a result of possible effects on protein folding.

To construct a retroviral vector encoding a correct version of kapB, a DNA-sequencing screen of all lab pcDNA3-kaposin B vector clones was performed (data not shown). After identifying a clone that was 100% identical to the putative pulmonary KS isolate, the kapB ORF was subcloned into the pBMN-IP retroviral vector; indeed, excision of the newly generated pBMN-IP-kapB with *Bam*HI and *Eco*RI generated a 636-bp fragment (Figure B.2A, lanes 2 and 3), in contrast to the previous 'pseudo-kapB' construct that encodes the larger, incorrectly sized fragment (Figure B.2A, lanes 4 and 5). Furthermore, immunoblot analysis of HUVECs transduced with the validated kapB construct revealed the canonical 25-kDa and 50-kDa kapB bands, in addition to a number of other minor products (Figure B.2B, lane 1), whereas cells transduced with 'pseudo-kapB' yielded a major 30-kDa product (Figure B.2B, lane 2). I therefore characterized a fundamental problem with the lab clone of pBMN-IP-kapB, and then constructed and validated a new retroviral vector encoding the authentic putative kapB derived from a pulmonary KS isolate.

B.2.2 Kaposin B Does Not Modulate Starvation-Induced Autophagy

To investigate the modulation of autophagy by kapB we assayed for differential autophagic responses to nutrient deprivation, as changes in basal autophagy may be difficult to detect (Klionsky *et al*., 2008; Mizushima *et al*., 2010). To address this, primary HUVECs were transduced with retroviral vectors encoding empty vector (EV) control or kapB derived from a pulmonary KS isolate, and 2 d post-selection the medium was refreshed and 4 h later the cells were overlaid with EBM-2 and incubated for the indicated times. Immunoblot analysis revealed a sharp increase in LC3-II levels immediately after starvation was initiated for both EV- and kapB-transduced HUVECs; as expected, these LC3-II levels quickly decreased (Figure B.3A) and continued to gradually decline during prolonged nutrient deprivation (Figure B.3B). Although both HUVEC populations responded to starvation as expected (see Figure 3.2), no differences in LC3-II between EV- and kapB-expressing cells were detected throughout the starvation time course (Figures B.3A and B). Furthermore, EV- and kapB-HUVECs contained equal levels of LC3-II at time 0, indicating that baseline autophagic activity was comparable following 4 h in fresh growth medium (Figure B.3A). Together, these

data suggest that kapB does not modulate cellular autophagy during normal or starvation conditions in endothelial cells.

B.3 Discussion

B.3.1 Summary

We have previously demonstrated that cells latently infected with KSHV are resistant to senescence, and that viral subversion of autophagy by vFLIP is necessary to confer this effect (Leidal *et al*., unpublished work). Considering the importance of autophagic activity in mediating the cellular transition to senescence, as well as the fundamental tumour-suppressive nature of the autophagic process, the potential of the other latent KSHV gene products to impact host autophagy was examined. Here, we report that kaposin B, a potent activator of p38 and MK2 signalling, does not modulate basal levels or starvation-induced autophagy in our system.

B.3.2 Does Kaposin B Modulate Autophagy via Paracrine Mechanisms?

A significant portion of my research was dedicated to characterizing possible effects of kapB on cellular autophagy. After spending considerable time examining miniscule, variable autophagic responses in kapB-expressing cells, an unrelated sequencing analysis revealed that our kapB-expression vector was compromised. Upon correcting the clonal impropriety, no detectible change in the basal level of autophagy, or in the autophagic response to nutrient deprivation, in kapB-expressing primary endothelial cells was observed. Although the kapB-mediated activation of p38 and MK2 has been well characterized (McCormick and Ganem, 2005; 2006), these activation events were not directly examined, and therefore it is possible that kapB may not elicit the same robust stimulation in these cells.

Regulation of autophagy by secreted products such as cytokines and growth factors has been extensively studied (Lipinski *et al*., 2010). By stabilizing the normally labile mRNAs that encode cytokines and growth factors, the kapB secretory profile significantly contributes to the extracellular environment, and is believed to play a critical role in KS development; importantly, these secreted factors may be able to subvert autophagy in neighbouring cells. Interestingly, the nature of the experimental setup may

have diminished any paracrine effects that kapB might have on the autophagic machinery. By overlaying the cells with fresh culture medium prior to harvest of cell lysates, any contribution of the kapB secretory profile to the extracellular environment may have been effectively depleted, diminishing any paracrine-mediated deregulation of autophagy. This predicament may be difficult to reconcile because cells display significantly high basal levels of autophagy if the culture medium is not refreshed prior to harvesting cell lysates. Further investigation is needed to completely understand the potential for kapB-mediated subversion of autophagy. Additionally, it would be interesting to determine if the other kaposin proteins, kaposins A and C, evoke any changes in autophagic activity, as this may reveal novel mechanisms that underlie KSHV pathogenesis and better our understanding of KS development.

B.4 Figures and Tables

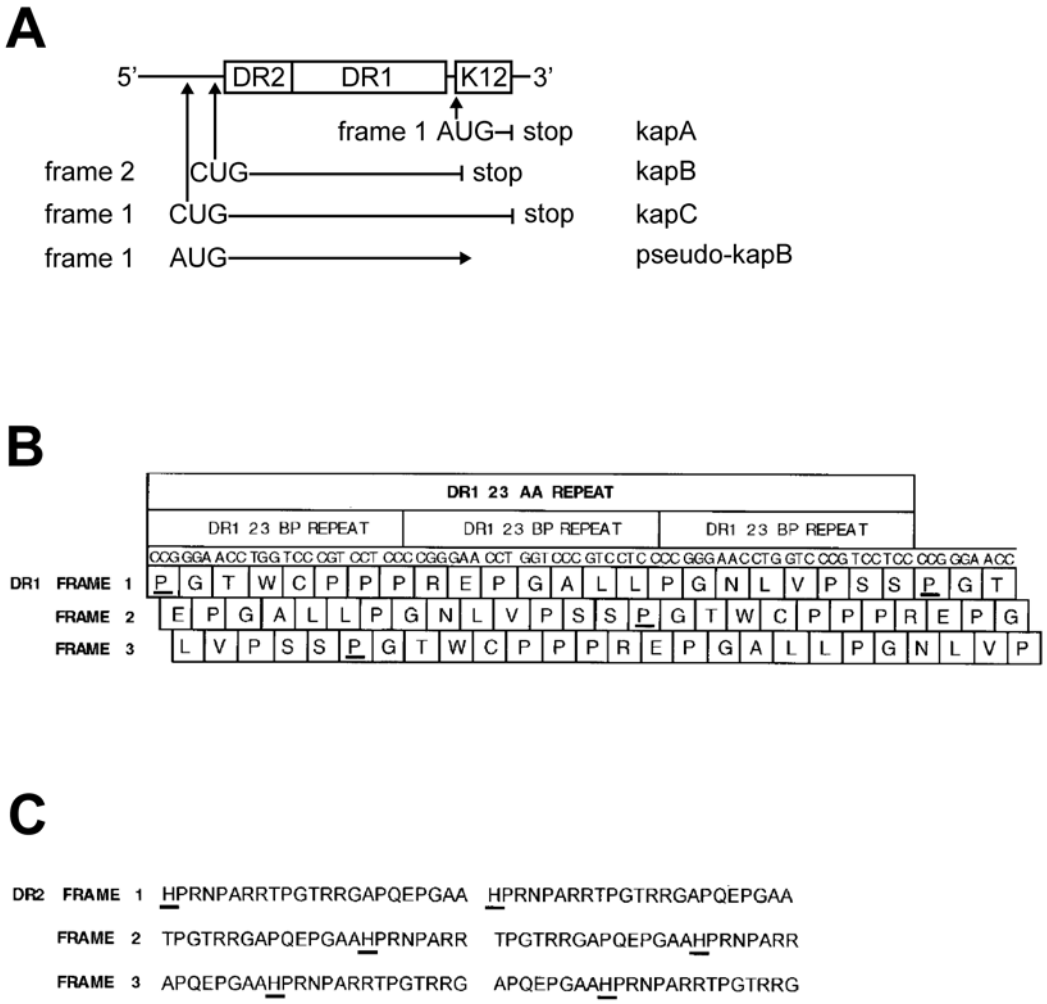

Figure B.1 Sequence analysis of kaposin B clones.

D

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---------- ---------- ---------- -------ATG CGAACCCTGC (1-13)
ATGGCACACC CCAGGAACCA GGTAGTACCC CCGAACCCTG CGAACCCTGC (1-50)

GAACCCTGCA GTACCCCGGC GCG....CCT CCTCCCTCCT CACTCCAATC (14-613)
GAACCCTGCA GTACCCCGGC GCG....CCT CCTCCCTCCT CACTCCAATC (51-650)

CGAATGCATG GATAGAGGCT TAA (614-636)
CGAATGCATG GATAGAGGCT TAA (651-673)
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E

```
-------------MRTLRTLQYPGAVPPAP  ← unique N-term
MAHPRNQVVPPNPANPANPAVPRRGPPRTP

-------RTPGTRRGAPQEPGAA HPRNPARRTPGTRRGAPQEPGAA
HPRNPARRTPGTRRGAPQEPGAA HPRNPARRTPGTRRGAPQEPGAA   DR2 repeats

HPRNPARRTPGTRRGAPQEPGAA HPRNPARRTPA
HPRNPARRTPGTRRGAPQEPGAA HP--------S

--------SSPGTWCPPPREPGA LLPGNLVPSSPGTWCPPPREPGA
LLPGNLVPSSPGTWCPPPREPGA LLPGNLVPSSPGTWCPPPREPGA   DR1 repeats

LLPGNLVPSSPGTWCPPPREPGA LLPGNLVPSSPGTWCPPPREPGA
LLPGNLVPSSPGTWCPPPREPGA LLPGNLVPSSPGTWCPPPREPGA

LLPGNLVPSSPGTWCPPPREPGA LLP
LLPGNLVPSSPGTWCPPP----- ---

PHSNPNAWIEA*
SSLQSECMDRGL…  ← unique C-term
```

Figure B.1 cont'd **(A)** Schematic map of the kaposin locus. Translation of DR1 **(B)** and DR2 **(C)** yields a repeating 23-amino acid peptide in all three reading frames. **(B)** The amino acid content of DR1 is depicted below each of the appropriate reading frames of the repeating DNA content. The underlined proline residue (P) has been assigned as the start of each repeat unit in each reading frame. **(C)** The amino acid content of each reading frame of DR2 is depicted. The underlined histidine residue (H) has been assigned as the start of each repeat unit in each reading frame. Comparison of the kaposin B derived from a pulmonary KS isolate of KSHV (wt; top rows) to the lab clone, or mutant, kaposin B (bottom rows) by **(D)** nucleotide and **(E)** amino acid sequence alignments. **(D)** The mutant clone of kapB (673 bp) reads through frame 1 and wt kapB (636 bp) reads through frame 2. The resulting codons and the nucleotide numbers are indicated. **(E)** Primary amino acid sequences are aligned to highlight direct-repeat elements. DR2 repeats, red; DR1 repeats, green. Stop codon is indicated by *. Panel A is adapted from McCormick and Ganem (2005); panels B and C are reproduced from Sadler *et al*., (1999); panel E is adapted from McCormick and Ganem (2006).

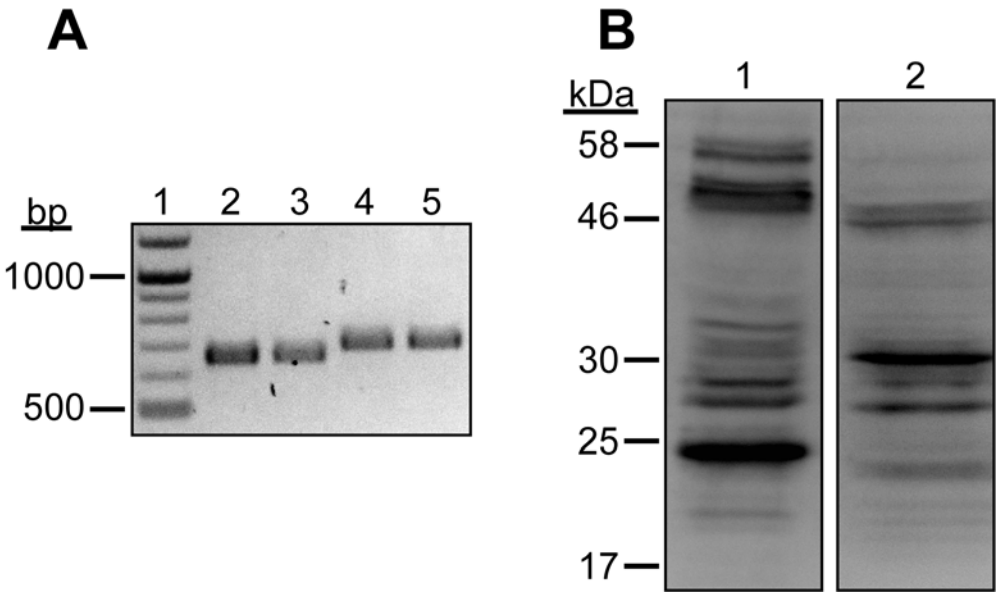

Figure B.2 Construction and verification of pBMN-IP encoding kaposin B derived from a pulmonary KS isolate. (A) wt kapB coding sequence was subcloned into pBMN-IP, and subsequently both pBMN-IP-kapB (lanes 2 and 3) and pBMN-IP bearing the mutant kapB (lanes 4 and 5) were restriction digested with *Eco*RI and *Xho*I and resolved by agarose-gel electrophoresis to yield 636-bp and 673-bp fragments, respectively. Lane 1 contains 1-kb DNA ladder (Invitrogen). **(B)** HUVECs were transduced with retroviruses encoding wt kapB (lane 1) or mutant kapB (lane 2) and selected in puromycin. At 2 d post-selection, cell monolayers were harvested, and portions of the lysates were resolved by SDS-PAGE and subjected to immunoblot analysis of kaposin B. wt kapB yields the canonical 25- and 50-kDa protein products, whereas the mutant kapB yields a predominantly 31-kDa product. The sizes of the prestained protein molecular-weight markers are indicated.

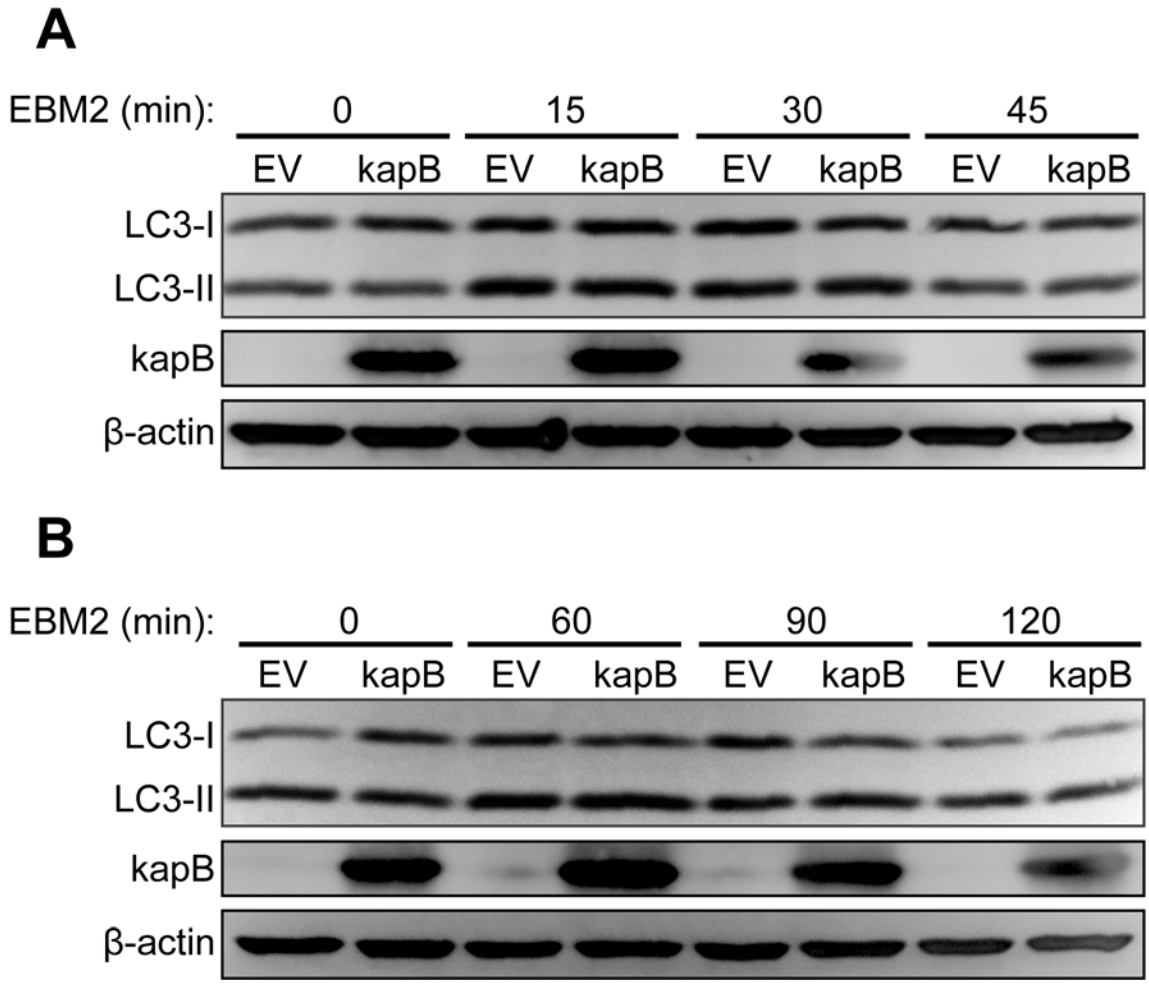

Figure B.3 Kaposin B does not modulate starvation-induced autophagy in HUVECs. HUVECs were transduced with EV or a retroviral vector encoding kaposin B (kapB) and selected in puromycin. At 2 d post-selection the culture medium was refreshed, 4 h later cells were washed and overlaid with EBM-2, and at the **(A)** early or **(B)** late indicated times the cell monolayers were harvested. Portions of the lysates were resolved by SDS-PAGE and subjected to immunoblot analysis of LC3. β-actin served as a protein loading control.