To reactivate or not to reactivate: Control of KSHV lytic replication is essential for apoptosis in response to p53 restoration

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To my family

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I. **Sarek G**, Kurki S, Enbäck J, Iotzova G, Haas J, Laakkonen P, Laiho M, and Ojala PM. Reactivation of the p53 pathway as a novel treatment modality for KSHV-induced lymphomas. *J Clin Invest* 117:1019-1028.

II. **Sarek G**, Enbäck J, Järviluoma A, Haas J, Gessain A, Laakkonen P, and Ojala PM. p53 apoptotic response to MDM2 inhibition in primary effusion lymphomas is attenuated by KSHV reactivation. *Submitted.*

III. **Sarek G**, Järviluoma A, and Ojala PM. KSHV viral cyclin inactivates p27KIP1 through Ser10 and Thr187 phosphorylation in proliferating primary effusion lymphomas. *Blood* 107:725-732.

IV. **Sarek G**, Järviluoma A, Moore HM, Kurki S, Vartia S, Biberfeld P, Laiho M and Ojala PM. Nucleophosmin phosphorylation by v-cyclin-CDK6 controls KSHV latency. *PLoS Pathog* 6:e1000818.

Additional unpublished material is also presented.

ABBREVIATIONS

53BP1	p53-binding protein 1
aa	amino acid(s)
AIDS	acquired immunodeficiency syndrome
Ala	alanine
AML	acute myeloid leukemia
ARF	alternative reading frame
Asp	aspartic acid
ATM	ataxia telangiectasia mutated
Bcl-2	B-cell lymphoma 2
B-CLL	B-chronic lymphocytic leukemia
CAK	CDK-activating kinase
CBP	CREB-binding protein
CDK	cyclin-dependent kinase
CDKN	cyclin-dependent kinase inhibitor
cDNA	complementary deoxyribonucleic acid
CKI	CDK inhibitor
C-terminal	carboxyterminal
DE	delayed-early
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
EBV	Epstein-Barr virus
Gln	glutamine
Glu	glutamic acid
GSK	glycogen synthase kinase
HAART	highly active antiretroviral therapy
HAT	histone acetyl-transferase
HDAC	histone deacetylase
HIV	human immunodeficiency virus
i.p.	intraperitoneal
IE	immediate-early
IFN	interferon
IR	ionizing irradiation
kDa	kilodalton = 1.660 × 10-21 g
KO	knockout
KSHV	Kaposi’s sarcoma herpesvirus
L	late
LANA	latency-associated nuclear antigen
LCL	lymphoblastoid cell line
Leu	leucine
LT	latency transcript

MCD	multicentric Castleman's disease
MDM2	murine double minute-2
MEF	mouse embryo fibroblast
miRNA	microRNA
MMP	matrix metalloproteinase
mRNA	messenger RNA
NaB	sodium butyrate
NF-κB	nuclear factor kappa-B
NLS	nuclear localization signal
NOD	non-obsese diabetic
NPM	nucleophosmin
N-terminal	aminoterminal
ORC	origin recognition complex
ORF	open reading frame
OSCC	oral squamous cell carcinoma
PAGE	polyacrylamide gel electrophoresis
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFR	PDGF-receptor
PEL	primary effusion lymphoma
Phe	phenylalanine
PI3K	phosphoinositide-3-kinase
Pro	proline
Rb	retinoblastoma
RNA	ribonucleic acid
RNAi	RNA interference
rRNA	ribosomal RNA
RTA	replication transcriptional activator
s.c.	subcutaneous
SCID	severe combined immunodeficiency
Ser	serine
sh-RNA	short hairpin RNA
SUMO	small ubiquitin-related modifier
SV40	Simian virus 40
TA	transactivation
TGF-β	transforming growth factor-β
Thr	threonine
TNF-α	tumor necrosis factor-α
TP53	tumor protein 53
TPA	12-O-tetradecanoyl phorbol-13-acetate

Trp	tryptophan
UV	ultraviolet
v-cyclin	viral cyclin
VEGF	vascular endothelial growth factor
v-FLIP	viral FLICE-inhibitory protein
vGPCR	viral G protein-coupled receptor
vIRF	viral interferon regulatory factor
wt	wild type
XIAP	X-linked inhibitor of apoptosis

ABSTRACT

Kaposi's sarcoma herpesvirus (KSHV) is an oncogenic human virus and the causative agent of three human malignancies: Kaposi's sarcoma (KS), Multicentric Castleman's Disease (MCD), and primary effusion lymphoma (PEL). In tumors, KSHV establishes latent infection during which it produces no infectious particles. Latently infected cells can enter the lytic replication cycle, and upon provision of appropriate cellular signals, produce progeny virus. PEL, commonly described in patients with AIDS, represents a diffuse large-cell non-Hodgkin's lymphoma, with median survival time less than six months after diagnosis. As tumor suppressor gene *TP53* mutations occur rarely in PEL, the aim of this thesis was to investigate whether non-genotoxic activation of the p53 pathway can eradicate malignant PEL cells.

This thesis demonstrates that Nutlin-3, a small-molecule inhibitor of the p53-MDM2 interaction, efficiently restored p53 function in PEL cells, leading to cell cycle arrest and massive apoptosis. Furthermore, we found that KSHV infection activated DNA damage signaling, rendering the cells more sensitive to p53-dependent cell death. We also showed *in vivo* the therapeutic potential of p53 restoration that led to regression of subcutaneous and intraperitoneal PEL tumor xenografts without adversely affecting normal cells. Importantly, we demonstrated that in a small subset of intraperitoneal PEL tumors, spontaneous induction of viral reactivation dramatically impaired Nutlin-3-induced p53-mediated apoptosis. Accordingly, we found that elevated KSHV lytic transcripts correlated with PEL tumor burden in animals and that inhibition of viral reactivation *in vitro* restored cytotoxic activity of a small-molecule inhibitor of the p53-MDM2 interaction.

Latency provides a unique opportunity for KSHV to escape host immune surveillance and to establish persistent infections. However, to maintain viral reservoirs and spread to other hosts, KSHV must be reactivated from latency and enter into the lytic growth phase. We showed that phosphorylation of nucleolar phosphoprotein nucleophosmin (NPM) by viral cyclin-CDK6 is critical for establishment and maintenance of the KSHV latency.

In short, this study provides evidence that the switch between latent phase and lytic replication is a critical step that determines the outcome of viral infection and the pathogenesis of KSHV-induced malignancies. Our data may thus contribute to development of novel targeted therapies for intervention and treatment of KSHV-associated cancers.

REVIEW OF THE LITERATURE

1. Tumor suppressor protein p53

1.1 Discovery of p53

The SV40 DNA tumor virus became a popular research model for *in vitro* cell transformation studies in the 1970s. Several independent laboratories in 1979 reported the discovery of a 53-kDa protein interacting with the SV40 large T-antigen in transformed murine cells (Chang *et al.*, 1979; Kress *et al.*, 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Linzer *et al.*, 1979). This newly discovered protein was designated a cellular T-antigen and was regarded as an important target of SV40 large T-antigen. Further studies confirmed its high levels in a variety of transformed mouse cells as well in biopsies of cancer-affected patients (DeLeo *et al.*, 1979; Barque *et al.*, 1983). Additional work by Mercer and coworkers (1982) enhanced interest in this protein, since it was found to delay the S-phase of the cell cycle. Interestingly, the *TP53* gene was later cloned from neoplastic mouse and human cells and was found to possess oncogenic properties when expressed in rodent cells. In the late 1980s, however, it was discovered that researchers were investigating missense mutants instead of the wt gene. Starting from the early 1990s, *TP53* was generally recognized as a tumor suppressor gene, called “Guardian of the genome,” that was mutated or lost in approximately half of all human cancer cases (Hollstein *et al.*, 1991; Levine *et al.*, 1991; Lane, 1992). Not surprisingly, mice lacking *TP53* are predisposed to spontaneous tumorigenesis (Donehower *et al.*, 1992), and germ-line *TP53* mutations arise in individuals with the cancer-prone Li-Fraumeni syndrome (Srivastava *et al.*, 1990; Malkin *et al.*, 1992). With these data, the hunt for the accurate role of p53 as a tumor suppressor intensified, leading to an explosion of >50 000 scientific reports in the literature since the 1990s, all aimed at better understanding the function of p53 in tumorigenesis.

1.2 p53 structure

The *TP53* gene is localized in the short arm of chromosome 17 (17q13) and encodes a tetrameric transcription factor of approximately 400 amino acid (aa) residues in length (393 aa in humans) (Friedman *et al.*, 1993; Wang *et al.*, 1994). p53 consists of five distinct domains: a transactivation domain, a proline-rich region, a core domain, an oligomerization domain, and a C-terminal basic domain. The core, also known as a

DNA-binding domain, is responsible for binding to the sequence-specific DNA elements located in close proximity to the p53 target gene promoters (Cho *et al.*, 1994). In contrast to other transcription factors, p53 possesses a second DNA-binding domain located on its C-terminus (residues 323 to 393) and creating stable complexes with DNA in a non-specific manner. These sites include mismatched DNA and double- or single-stranded DNA (Bakalkin *et al.*, 1995; Lee *et al.*, 1995). Interestingly, the same region of the C-terminal domain controls the efficiency of p53 to function as a transcription factor. Its post-translational modifications and interactions with other proteins are involved in modulating of the stability of p53-specific DNA complexes (Ko and Prives, 1996; Vogelstein *et al.*, 2000). It is therefore possible that interactions between the core and the C-terminal domains of p53 may, upon its activation and stabilization, require allosteric reorganization of the p53 molecule (Hupp and Lane, 1994).

1.3 The p53 family

Two structural homologs of p53, called p63 and p73, were discovered in 1997 (Kaghad *et al.*, 1997; Schmale and Bamberger, 1997; Trink *et al.*, 1998; Yang *et al.*, 1998). Both, p63 and p73 display high levels of sequence similarity in the transactivation (TA), oligomerization, and highly conserved DNA-binding domain. This allows p63 and p73 to transactivate p53-responsive genes, and triggers cell cycle arrest and apoptosis. Due to their overall structure and sequence homology, p63, p73, and p53 form a family of transcription factors that evolved from a common predecessor (Yang *et al.*, 2002a). Nevertheless, they are not completely redundant, and the primary role of each p53 family member - as determined by transgenic knockout (KO) mice - shows that each member plays its own unique role. Whereas the *TP53* KO mice are viable and develop normally (Donehower *et al.*, 1992), studies on p63 KO mice reveal developmental failures in skin and epithelial tissues and show restricted viability up to a few days after birth (Mills *et al.*, 1999). The p73 KO mice are predisposed to inflammatory and neurological defects (Yang *et al.*, 1999).

Although p63 and p73 show an analogous domain structure, they are transcribed from two alternate promoters. This results either in the N-terminal transactivating forms TAp63 and TAp73 or the N-terminally truncated, transcriptionally impaired forms ΔNp63 and ΔNp73, which lack the TA domain and can exert dominant-negative effects on the TA forms (Stiewe, 2007). For instance, high expression of ΔNp63α can

promote survival of tumor cells by inactivating TAp73α (Rocco *et al.*, 2006). Furthermore, a study by Bourdon and coworkers (2005) revealed an alternative promoter in the *TP53* gene and identified multiple p53 splice variants. TAp63 and TAp73 activate a subset of p53 target genes implicated in cell cycle progression and apoptosis. They are also necessary for p53-dependent apoptosis in response to DNA damage and play a role in resistance to tumor formation (Tomasini *et al.*, 2008a; Tomasini *et al.*, 2008b). Interestingly, recent data indicate that p63 and p73 may suppress tumorigenesis by transcriptional regulation of genes involved in the DNA repair pathway (Lin *et al.*, 2009).

1.4 Regulation of p53

The p53 tumor suppressor protein is regulated at numerous levels, and new studies have further broadened our knowledge of how this protein is controlled. A broad range of negative and positive regulators acting in feedback loops exert their functions at three different levels: protein stability, protein activity, and subcellular localization. As it is beyond the scope of this thesis to review all possible aspects of p53 regulation, it will therefore focus on regulation of p53 protein stability, with special emphasis on mouse double minute protein 2 (MDM2) function and the regulation of p53 by phosphorylation.

1.4.1 Control of p53 stability by MDM2

The ubiquitin-mediated proteasomal degradation pathway plays a major role in the regulation of p53 protein levels in normal cells (Michael and Oren, 2003; Brooks and Gu, 2006). MDM2 possesses ubiquitin E3-ligase activity with great specificity towards p53 (Haupt *et al.*, 1997; Honda *et al.*, 1997; Kubbutat *et al.*, 1997). First, MDM2 binds p53 at its DNA-binding domain so that p53 cannot function as a transcription factor. Later, MDM2 labels p53 for degradation, and finally, MDM2 is responsible for the export of p53 from the nucleus to the cytoplasm, abolishing its transcriptional activity (Kruse and Gu, 2009). The significance of the p53 negative regulation by MDM2 was demonstrated by series of elegant experiments with MDM2 KO mice in which early embryonic lethality due to MDM2 deficiency was abolished in mice concurrently deficient in p53 (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995). Many human cancers overexpress MDM2, which seems adequate to inactivate p53 function without further need for p53 mutation (Momand *et al.*, 2000). Equally, failure in MDM2 expression causes massive *in vivo* apoptosis (de Rozieres *et al.*, 2000). Therefore,

regulation of p53 by MDM2 is based on the negative feedback loop, in which p53 induces the transcription of MDM2, whereas MDM2 promotes p53 turnover (Juven *et al.*, 1993; Haupt *et al.*, 1997; Kubbutat *et al.*, 1997).

Because MDM2 belongs to RING-finger ubiquitin ligases, it possess no intrinsic enzymatic activity (Fang *et al.*, 2000; Honda and Yasuda, 2000). It acts in concert with the ubiquitin-conjugating enzymes (E2) UbcH5B and UbcH5C to relocate ubiquitin directly on p53 (Saville *et al.*, 2004). Importantly, p53 oligomerization is fundamental for its ubiquitination and subsequent degradation by MDM2, since elimination of amino acids from the p53 C-terminus weakens its MDM2-dependent degradation (Kubbutat *et al.*, 1998). Several lysines operate as potential ubiquitination sites. Replacement between residues 370 to 386 impairs ubiquitination of p53 by MDM2 and impairs its responsiveness to MDM2-dependent degradation (Nakamura *et al.*, 2000; Rodriguez *et al.*, 2000). In contrast, the proline region protects p53 against MDM2-dependent degradation, and deletion of residues 62 to 91 from this region of p53 results in hypersensitivity to MDM2 (Berger *et al.*, 2001). Interestingly, the neighboring residues 92 to 112 are required for MDM2-dependent degradation of p53, which may suggest involvement of other proteins in the ubiquitination signaling (Gu *et al.*, 2000).

MDM2 causes mono- and poly-ubiquitination of p53, and these actions depend on MDM2 level. High levels of MDM2 induce p53 poly-ubiquitination, leading to degradation in the nucleus, whereas low levels trigger mono-ubiquitination with concomitant nuclear export of p53 (Xirodimas *et al.*, 2001; Li *et al.*, 2003). A prerequisite for poly-ubiquitination of p53 is the involvement of the transcriptional co-activator p300 (Grossman *et al.*, 2003). Mutants of MDM2 that do not bind p300 promote mono-ubiquitination of p53, but are unable to execute poly-ubiquitination and fail to target p53 for degradation (Zhu *et al.*, 2001; Grossman *et al.*, 2003). Ubiquitination of p53 takes place predominantly in the nucleus, despite some amount of p53 and MDM2 localized in the cytoplasm (Yu *et al.*, 2000). Disruption of the nuclear localization signal (NLS) of p53 prevents it from entering the nucleus, and also blocks the MDM2-dependent ubiquitination. Following poly-ubiquitination, p53 may be destroyed either in the nucleus or in the cytoplasm (Xirodimas *et al.*, 2001). Importantly, cytoplasmic degradation of p53 is tightly associated with transport of poly-ubiquitinated p53 that requires a C-terminal nuclear export sequence of p53

(Geyer *et al.*, 2000). Previous studies suggested mono-ubiquitination and cytoplasmic re-localization of p53 as an alternative mechanism to block nuclear function of p53. However, recent data suggest its role in apoptosis and autophagy (Tasdemir *et al.*, 2008). Shortly after its relocalization to the cytoplasm, p53 may enter the mitochondria where it triggers apoptosis (Marchenko and Moll, 2007). It interacts with Bcl-xL and Bcl-2, anti-apoptotic proteins of the Bcl family, and supports oligomerization of pro-apoptotic Bak and Bax. These events lead to formation of pores in mitochondria, to cytochrome c release, and to activation of other apoptotic factors (Mihara *et al.*, 2003; Chipuk *et al.*, 2004; Tomita *et al.*, 2006).

1.4.2 Role of ARF in p53 regulation

A key regulator of the MDM2-dependent degradation of p53 is alternative reading frame (ARF), a product of the cyclin-dependent kinase (CDK) inhibitor 2A gene (*CDKN2A*) (Sherr, 2001). ARF is a protein with tumor suppressor activity, and its loss results in a phenotype that is similar to p53 deficiency (Kamijo *et al.*, 1997). ARF binds to MDM2, represses its ubiquitin ligase activity, and as a consequence leads to p53 stabilization and the induction of apoptosis (Kamijo *et al.*, 1998; Pomerantz *et al.*, 1998). More precisely, ARF has been shown to be a nucleolar protein which sequesters MDM2 into the nucleolus and stabilizes nucleoplasmic p53 (Weber *et al.*, 1999). In addition, to its repressor function on MDM2, ARF controls and modifies activity of other E3 ubiquitin ligases. Accordingly, it is reported that ARF-BP1 ligase plays a role in the ARF p53-dependent activity (Chen *et al.*, 2005a).

1.4.3 MDM2-MDMX network in p53 regulation

Regulation of p53 is also mediated through proteins that directly interact with and modulate the inhibitory effects of MDM2. One important regulator of MDM2 function is MDMX (HDMX), known also as MDM4. These proteins are structurally similar and interact with each other through the C-terminal RING domains. MDMX stabilizes both MDM2 and p53, and, in addition also promotes the E3-ligase activity of MDM2 (Linares *et al.*, 2003; Poyurovsky *et al.*, 2007; Uldrijan *et al.*, 2007). Interestingly, even though MDMX is a RING-domain protein, it has no E3-ligase activity for p53 but rather inhibits p53 transcriptional activity (Marine *et al.*, 2005). Research on the functional network between p53, MDM2, and MDMX has provided evidence that MDMX predominantly represses p53 function, but does not trigger p53 degradation (Marine *et al.*, 2006; Wahl, 2006; Iwakuma and Lozano, 2007). It is possible that both

MDM2 and MDMX may repress transcription of p53 target genes by forming a protein complex with p53 on the promoters of specific p53-responsive genes. Importantly, acetylation counteracts down-regulation of p53 function by blocking the recruitment of both MDM2 and MDMX to p53-responsive promoters (Tang *et al.*, 2008).

Intriguingly, a study by Ringhausen and coworkers (2006) has shown that p53 may also be degraded in the MDM2-null cells. In addition, newly reported E3-ubiquitin ligases such as Arf-BP1, COP1, and Pirh2 have been confirmed to regulate p53 levels *in vitro* (Leng *et al.*, 2003; Dornan *et al.*, 2004; Chen *et al.*, 2005a). These results strongly suggest some MDM2-independent mechanisms that may also be involved in the control of p53 stability.

1.4.4 Regulation of p53 activity by phosphorylation

The switch of p53 from an inactive to an active state can be modulated by a variety of post-translational modifications. Phosphorylation is by far the most studied modification associated with the activation of p53 in response to cellular stress. One site constitutively phosphorylated on p53 is Ser33. GSK-3β and the CDK-activating kinase (CAK) complex (Cdk7-Mat1-cyclinH) phosphorylate this site in unstressed cells. This phosphorylation affects the association of p53 with other proteins interacting with the N-terminus, such as MDM2 and the TAFs (Ko *et al.*, 1997; Turenne and Price, 2001). In addition, p53 Thr55 is phosphorylated in quiescent cells by TAF1, and this phosphorylation enhances association of p53 with a protein promoting cytoplasmic translocation of p53, the nuclear export factor CRM1 (Gatti *et al.*, 2000; Li *et al.*, 2004a; Cai and Liu, 2008). Ser376 and Ser378 are also phosphorylated in proliferating cells by the CAK complex, but their physiological role is not fully understood (Lu *et al.*, 1997). The majority of the phosphorylated p53 appears in response to cellular stress, and one particular phosphorylation site depends on the inducer. Accordingly, Thr18 is primarily phosphorylated after ionizing irradiation (IR), whereas ultraviolet (UV) irradiation results in phosphorylation of Ser9, Ser33, and Ser37 (Saito *et al.*, 2003). In addition, after IR, phosphorylation of p53 peaks at 2 to 4 hours, whereas upon UV irradiation, it occurs between 8 and 24 hours. Several kinases involved in the DNA-damage response such as ATM, ATR, Chk1, and Chk2 have been determined to phosphorylate p53 (Siliciano *et al.*, 1997; Shieh *et al.*, 2000). Which one of these kinases will phosphorylate p53 depends on the cellular stress. IR activates phosphorylation of p53 by ATM and Chk2, whereas kinase

activity by ATR and Chk1 occurs upon UV irradiation or hypoxia (Bartek and Lukas, 2003). Both ATM and ATR kinases phosphorylate p53 protein at Ser15 (Ser18 in mice) to increase its transactivation activity (Banin *et al.*, 1998; Canman *et al.*, 1998; Nakagawa *et al.*, 1999; Tibbetts *et al.*, 1999). Moreover, ATM has been reported to bind and phosphorylate MDM2 and therefore to promote p53 stabilization and accumulation in cells (Khosravi *et al.*, 1999). Interestingly, ATM and ATR control p53 also through other kinases. ATM activates Chk2 kinase, which phosphorylates p53 at Ser20. This modification is essential for p53 stabilization, as it modulates binding between p53 and MDM2 (Chehab *et al.*, 1999). Phosphorylation of both Ser15 and Ser20 has been demonstrated as essential for the induction of p53-mediated apoptosis, which also requires p53 acetylation by p300 (Chao *et al.*, 2003; Sluss *et al.*, 2004).

1.5 Functions of p53

The p53 tumor suppressor protein responds to a variety of stresses, including DNA damage, hypoxia, cell cycle aberrations, oncogene activation, and virus infections. The exact molecular pathways activating p53 depend on type of stimuli and cell type. These determine the precise downstream effectors and cellular response such as growth arrest, apoptosis, or senescence (Fig. 1). The functional activation of the p53 pathway occurs either by transcriptional regulation of p53 target genes or by transcription-independent mechanisms (Mirza *et al.*, 2003; Harms *et al.*, 2004).

1.5.1 Role of p53 in cell cycle arrest

Growth inhibition occurring in the late G1 phase is crucial for the protection of genome stability, as it prevents the replication of damaged DNA. The p53 target gene *CDKN1A* (p21CIP1) plays a key role in the p53-dependent G1 arrest (el-Deiry *et al.*, 1993; el-Deiry *et al.*, 1994); p21CIP1 is a CDK inhibitor (CKI) and inhibits CDKs by binding to the cyclin-CDK complex, thus blocking S-phase entry (Harper *et al.*, 1993). Interestingly, p53 can also act during the DNA synthesis, and this activity is mediated by alternative isoforms of the p53 protein (Rohaly *et al.*, 2005) that participate in the induction of the 14-3-3σ and p21CIP1 proteins (Hermeking and Benzinger, 2006). The ability to inhibit the G2/M transition is essential to avoid segregation of damaged and partially replicated chromosomes. The block at this checkpoint occurs through inhibition of the Cyclin B/CDK1 complex (Peng *et al.*, 1997), and several p53-regulated target genes such as *GADD45*, *BTG2*, and *MCG10* participate in this process (Rouault *et al.*, 1996; Zhan *et al.*, 1998; Wang *et al.*, 1999; Zhu and Chen, 2000). In

addition, p53 has been demonstrated to associate with centrosomes in mitotic cells (Ciciarello *et al.*, 2001). Blockage of the assembly of the mitotic spindle by nocodazole leads to activation of p53 by ATM and in consequence to G1 arrest, with concomitant disruption of the p53 centrosomal localization(Ciciarello *et al.*, 2001).

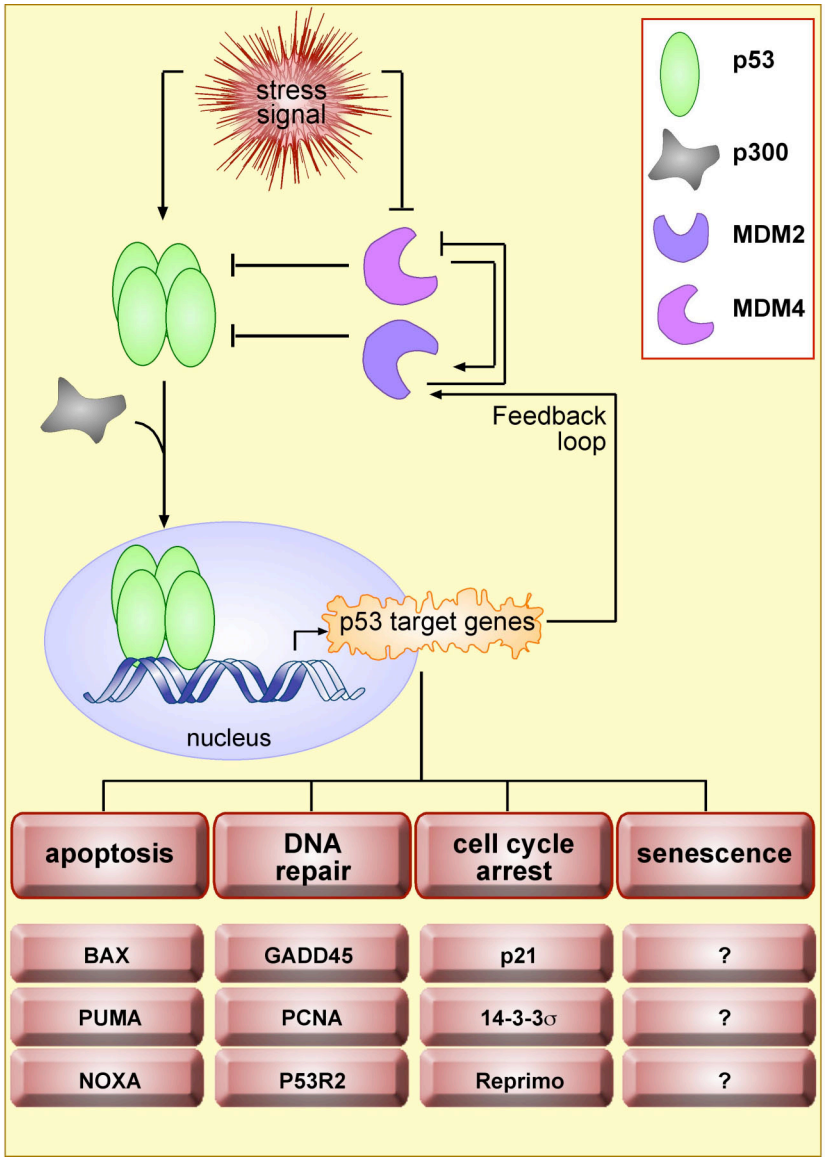

Figure 1. Simplified scheme of the regulatory network of the p53 pathway. In response to stress signals, p53 is activated through various only partly understood activation pathways. In conjunction with the coactivating acetyltransferases p300/CBP, p53 regulates the transcription of a variety of target genes, which determines cellular response. In a tightly controlled feedback loop, p53 also induces expression of its negative regulators, such as the E3 ubiquitin ligase MDM2, which binds to p53 and promotes its ubiquitination, followed by nuclear export and proteasomal degradation. This process, together with the action of other E3 ubiquitin ligases, keeps cellular p53 levels constitutively low. Moreover, MDM2 competes with p300/CBP in binding to the p53 N-terminus. Regulation of p53 activity also involves the MDM2 homolog MDM4, which acts in both distinct and synergistic ways. The complex p53 activation pathways either directly or indirectly disrupt this negative feedback loop (modified from Joerger and Fehrst, 2008).

1.5.2 Role of p53 in apoptosis

Activation of p53 can execute apoptosis through the transcriptional activation of pro-apoptotic target genes (Haupt *et al.*, 2003). These targets are members of intrinsic and extrinsic apoptotic pathways. The intrinsic p53 apoptotic pathway involves induction of the pro-apoptotic Bcl-2 family members Bax and the BH3-only proteins Bid, PUMA, and Noxa (Oda *et al.*, 2000; Nakano and Vousden, 2001; Sax *et al.*, 2002). They act as activators stimulating Bax/Bak oligomerization and induce apoptosis through dislocation of the anti-apoptotic proteins from the Bax-Bak complex (Vaseva and Moll, 2009). Importantly, the BH3 domain plays a key role in the pro-apoptotic activity of these proteins (Cory and Adams, 2002).

The first evidence of a direct role for p53 in mitochondrial apoptosis came from Marchenko and coworkers (2000), indicating that upon p53-dependent apoptosis, a small percentage of stabilized wt p53 promptly translocates to the outer membrane of the mitochondria. This translocation of p53 precedes modifications of mitochondrial membrane potential, cytochrome *c* release, and caspase activation (Marchenko *et al.*, 2000). After entering the mitochondria, p53 is promptly de-ubiquitinated by the HAUSP protein, which switches it to an active form (Li *et al.*, 2002). Moreover, binding of p53 to Bcl-2 and Bcl-xL is regulated by the p53 DNA-binding domain (Petros *et al.*, 2004). Interaction of p53 with the anti-apoptotic protein Bcl-xL releases the pro-apoptotic proteins Bax and Bid previously sequestered by Bcl-xL, enhancing their pro-apoptotic activity (Chipuk *et al.*, 2004; Chipuk and Green, 2006). Upon its activation by the cytoplasmic p53, the Bax protein changes its conformation, which assists in its translocation from the cytosol into mitochondria (Chipuk *et al.*, 2004; Schuler and Green, 2005). p53 binds to Bak in mitochondria and promotes its oligomerization and activation (Mihara *et al.*, 2003; Erster *et al.*, 2004; Schuler and Green, 2005). Moreover, p53 negatively regulates the anti-apoptotic protein called apoptosis repressor with caspase recruitment domain (ARC). Transcriptional repression of ARC by p53 leads to activation of the pro-apoptotic factors PUMA and Bad, and to initiation of the p53 apoptotic program (Li *et al.*, 2008a). Interestingly, a recent study has shown that p53 promotes release of cytochrome *c* by inducing the expression of the *OKL38* tumor suppressor gene. Loss of *OKL38* is reported to drive tumorigenesis, whereas its overproduction triggers apoptosis in several carcinoma cell lines *in vitro* (Yao *et al.*, 2008).

The extrinsic apoptotic response is initiated outside the cell and involves p53 to induce the expression of the death receptor Fas (CD95) and KILLER/DR5 (TRAIL receptor 2) (Haupt *et al.*, 2003). In addition, p53 activates expression of the TNFSF10 (TRAIL) death ligand, and the Fas ligand, TNFSF6 (FasL) (Maecker *et al.*, 2000; Kuribayashi *et al.*, 2008). Several lines of evidence indicate that p53 is also able to transactivate many other factors/genes of the apoptotic machinery, including *apaf-1*, *caspase 8*, and *caspase 6* (Haupt *et al.*, 2003), the apoptosis-enhancing nuclease implicated in DNA fragmentation (Kawase *et al.*, 2008), and *PIG3*, a gene involved in redox metabolism (Polyak *et al.*, 1997).

1.6 Restoration of p53 function

TP53 is the most frequently inactivated tumor suppressor gene in human malignancies, and its inactivation is advantageous for tumor survival. In about half of all human cancers, p53 is inactivated directly by mutations or deletions, whereas in the remaining half, p53 activity is suppressed due to malfunctions in the downstream pathways (Vogelstein *et al.*, 2000). Any appropriate strategy for pharmacological restoration of p53 function will therefore critically depend on p53 mutation status. Several approaches have been undertaken to restore p53 function in tumors, and these strategies can be classified into four groups: (a) design of inhibitors for negative regulators of p53 in tumors with wt p53 status (Vassilev *et al.*, 2004; Vassilev, 2005; Vassilev, 2007), (b) development of small molecules stabilizing and activating wt p53 (Issaeva *et al.*, 2004), (c) restoration of mutant p53 function with the ability to reverse the mutant p53 phenotype (Foster *et al.*, 1999; Bykov *et al.*, 2002a; Bykov *et al.*, 2002b; Bykov *et al.*, 2005), and (d) tumor therapy based on expression of exogenous p53, for instance through adenoviral gene transfer (Cai *et al.*, 1993; Rosenfeld *et al.*, 1995; Roth, 2006). Importantly, recent studies with transgenic mice, where p53 expression is reversibly switched on/off, have independently demonstrated that restoration of p53 function leads to apoptosis and tumor regression *in vivo*. These studies indicate that restoration of the p53 pathway is a promising therapeutic modality against cancer (Martins *et al.*, 2006; Ventura *et al.*, 2007).

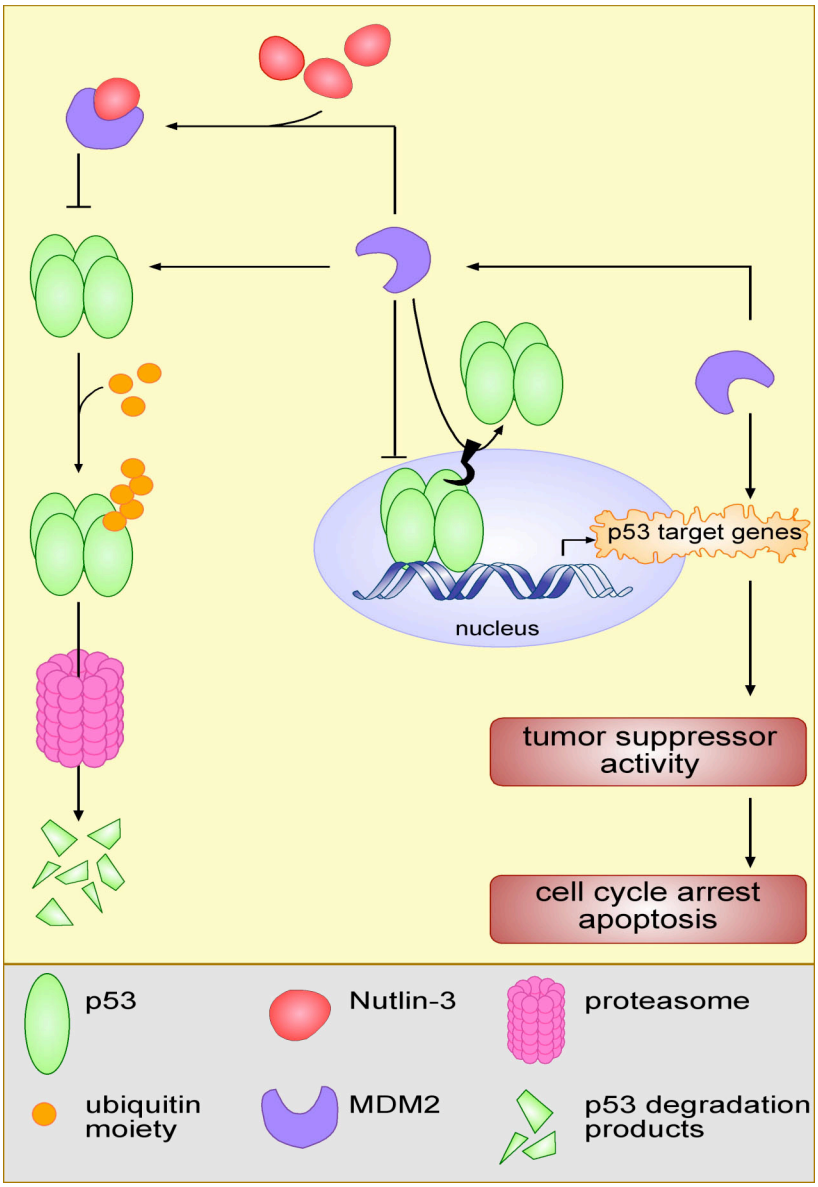

Figure 2. The negative p53-MDM2 regulatory feedback loop. Under physiological conditions, wt p53 has a very short half-life because of the autoregulatory negative feedback loop mediated by MDM2. First, wt p53 activates the transcription of the *MDM2* gene; subsequently the MDM2 protein, upon interaction with the p53 transactivation domain, impairs p53 transcriptional activity. Moreover, MDM2, through its E3 ubiquitin ligase activity, promotes proteasome-dependent p53 degradation and modulates nuclear export of p53. Small-molecule inhibitor (red), which can bind specifically to the p53-binding pocket on the surface of MDM2, disrupts the p53-MDM2 interaction and releases p53 from MDM2 control. Thus, by liberating p53 from MDM2 one might stabilize the tumor suppressor and activate the p53 pathway, leading to growth arrest and apoptosis. p53 continues to activate the *MDM2* promoter, and the level of MDM2 will rise, but it will be unable to bind to p53 because of the small-molecule antagonists that keep the p53 pocket occupied.

1.6.1 MDM2 antagonists

Cellular levels of p53 are strictly regulated through the E3 ubiquitin ligase MDM2 that sequesters p53 for proteasomal degradation via ubiquitination (Fig. 2, see also 1.4.1). In many human cancers, MDM2 is exceedingly over-expressed (Momand *et al.*, 1998), which efficiently abolishes p53 function regardless of p53 status. Therefore, MDM2

antagonists may present attractive targets of anti-cancer therapeutics for tumors with wt p53. In 2004, Vassiliev and coworkers (2004) identified a group of *cis*-imidazoline analogs termed Nutlins by screening a library of synthetic small-molecule compounds. These molecules disrupt the p53-MDM2 interaction through competition with p53 for MDM2. At cellular level, the consequences may result in cell cycle arrest in G1 and G2 phases or apoptosis, or both (Fig. 2). Nutlins interact with and bind to the highly hydrophobic p53-binding pocket in the N-terminal domain of MDM2, thus mimicking the binding mode of a short peptide derived from the N-terminus of p53. The interaction between p53 and MDM2 involves three major hydrophobic residues (Phe-19, Trp-23, and Leu-26) located in an amphipathic α-helix of p53 and in a deep hydrophobic p53-binding pocket in MDM2 (Kussie *et al.*, 1996; Schon *et al.*, 2002). Structural analysis reveals that in the MDM2-Nutlin complex an ethylether site of Nutlin resides in the Phe-19 binding pocket, whereas the bromophenyl groups are positioned in the Trp-23 and Leu-26 binding sites. The central imidazoline moiety operates as a scaffold for these groups (Vassilev *et al.*, 2004). Nutlins were soon proven to be highly potent and specific inhibitors of the p53-MDM2 interaction in a variety of cancer cell lines expressing wt p53, but they failed to restore p53 in cell lines expressing mutant p53 (Vassilev, 2004; Tovar *et al.*, 2006).

In addition to Nutlins, several other potent small molecule inhibitors disrupt the p53-MDM2 interactions. Benzodiazepinedione-based MDM2 antagonists were found through a series of screens with benzodiazepinediones and their derivatives (Grasberger *et al.*, 2005; Parks *et al.*, 2005). These inhibitors were further confirmed to stabilize p53 and trigger apoptosis in p53 wt hepatocellular carcinoma cells (Koblish *et al.*, 2006). High throughput screening of a large chemical library led to the discovery of non-peptidic MDM2 inhibitors based on a spiro-oxindole core (Ding *et al.*, 2005; Ding *et al.*, 2006). These inhibit growth of cancer cell lines with wt p53, and show approximately 50-fold weaker activity in cancer cell lines expressing mutant p53, further confirming the critical role of p53 status in cellular activity of the inhibitors. Interesting MDM2 inhibitors are α-helix mimetics of p53 (Kussie *et al.*, 1996), non-peptidic synthetic molecules based on the terphenyl scaffold. These compounds activate p53-dependent transcription and lead to strong cell cycle arrest in cells expressing wt p53 (Chen *et al.*, 2005b; Yin *et al.*, 2005). Alternative approaches to antagonize MDM2 in tumors include down-regulation of MDM2 expression with anti-sense oligonucleotides as well as development of small-molecule chemicals that

exclusively target the E3 ubiquitin ligase activity of MDM2 (Chen *et al.*, 1998; Tortora *et al.*, 2000; Prasad *et al.*, 2002; Zhang *et al.*, 2004b; Zhang *et al.*, 2005b). Moreover, approaches targeting p53-mediated protein-protein interactions are also plausible for other negative regulators of p53 such as MDMX (Michael and Oren, 2003). Although MDM2 and MDMX show structural similarities, Nutlin-3 does not inhibit MDMX (Hu *et al.*, 2006; Wade *et al.*, 2006). This may complicate the outcome of p53 restoration by MDM2 antagonists, especially in MDMX-overexpressing cancers (Toledo and Wahl, 2007). Collective inhibition of MDM2 and MDMX may therefore provide more effective anti-tumor activity for this kind of cancer (Toledo and Wahl, 2006; Hu *et al.*, 2007).

1.6.2 Nutlin-3 as an innovative therapeutic tool in hematological malignancies

Several hematological malignancies have retained wt p53, making them potential targets for MDM2 inhibitor-based therapy. Nutlin-3 effectively triggers apoptosis in acute myeloid leukemia (AML) and in B-chronic lymphocytic leukemia (B-CLL), as well as in multiple myeloma and mantle cell lymphoma (Kojima *et al.*, 2005; Stuhmer *et al.*, 2005; Coll-Mulet *et al.*, 2006; Kojima *et al.*, 2006; Secchiero *et al.*, 2006; Tabe *et al.*, 2009). Moreover, Nutlin-3 synergizes with doxorubicin and cytosine arabinoside in apoptosis of myeloblasts in AML and with doxorubicin, chlorambucil, and fludarabine in cell death of leukemic cells in B-CLL primary tumors (Kojima *et al.*, 2005; Coll-Mulet *et al.*, 2006; Kojima *et al.*, 2006; Secchiero *et al.*, 2006). Of note, treatments with Nutlin-3 alone or in combination with genotoxic agents are selective for cancer cells, as demonstrated by their low toxicity to peripheral blood mononuclear cells or bone marrow-derived hematopoietic progenitors and to bone marrow stromal epithelium cells (Stuhmer *et al.*, 2005; Secchiero *et al.*, 2006). Brummelkamp and coworkers (2006) identified the DNA damage protein p53-binding protein 1 (53BP1) as an essential mediator of Nutlin-3-induced cytotoxic effect. While Nutlin-3 is not itself genotoxic (Thompson *et al.*, 2004), studies with these compounds have strengthened the concept that activated DNA damage signaling occurs in cancer cells (Bartkova *et al.*, 2005; Gorgoulis *et al.*, 2005), and contributes to the cytotoxic activity of Nutlin-3. ATM protein kinase is a major regulator of p53 activity and a central mediator of cellular responses to DNA double-strand breaks (Jackson and Bartek, 2009). Interestingly, whereas low levels of ATM rendered fludarabine treatment ineffective, ATM did not influence Nutlin-3 activity. These data suggest that MDM2

inhibitors may sustain their activity in tumors with no upstream signals activating p53 (Kojima *et al.*, 2006).

An important factor contributing to chemoresistance in hematological malignancies is the X-linked inhibitor of apoptosis (XIAP) (Deveraux and Reed, 1999). Restoration of the p53 pathway synchronized with inhibition of XIAP greatly enhances activation of p53-dependent apoptosis both in AML cells and in chemoresistant primary blasts isolated from AML patients (Carter *et al.*, 2010). Interestingly, an alternative therapeutic approach to the induction of apoptosis is to promote terminal growth arrest and maturation of AML blasts (Gorin *et al.*, 2000). Intriguingly, Nutlin-3 promotes maturation not only in wt p53 myelocytic/monocytic lineages but also in the $p53^{-/-}$ leukemic cells (Secchiero *et al.*, 2007). These results suggest a new role for Nutlin-3 as an anti-neoplastic agent for certain types of AML.

Another interesting recent observation regarding chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia is that Nutlin-3 greatly enhances apoptosis induced by imatinib (a specific inhibitor of the BCR/ABL tyrosine kinase) by activation of Bax and caspase-3. These are normally inhibited in leukemias by over-expression of Bcl-xL (Kurosu *et al.*, 2010). Furthermore, Nutlin-3 also demonstrates autonomous biological activities distinct from cell death such as inhibition of vascular endothelial growth factor (LaRusch *et al.*, 2007) and stromal-derived factor-1/CXCL12 (Moskovits *et al.*, 2006). The therapeutic potential of Nutlin-3 may therefore involve two different, but possibly not mutually exclusive, mechanisms of action. One mechanism is based on a cytotoxic effect towards malignant cells and the second, an indirect effect, against tumor stromal and vascular cells.

2. Nucleophosmin (NPM), a multifunctional phosphoprotein

NPM, also termed B23, NO38, or numatrin was originally identified as an abundant nucleolar phosphoprotein located in granular regions of the nucleolus (Feuerstein and Mond, 1987; Schmidt-Zachmann *et al.*, 1987). It rapidly shuttles between the nucleus and cytoplasm and plays multiple roles in cell growth and proliferation (Borer *et al.*, 1989; Szebeni *et al.*, 1995). NPM function has been linked to ribosome biogenesis (Prestayko *et al.*, 1974; Spector *et al.*, 1984; Yung *et al.*, 1985), the synthesis and

processing of ribosomal RNA (rRNA) (Dumbar *et al.*, 1989; Herrera *et al.*, 1995; Savkur and Olson, 1998), and transport of rRNA and ribosomal proteins (Dutta *et al.*, 2001; Huang *et al.*, 2005). Elevated NPM levels are evident in proliferating cells as well as in cancer cells, probably due to an increased requirement for ribosomal synthesis. Fluctuations in NPM abundance and shuttling properties modify cellular status with respect to proliferation, differentiation, and apoptosis (Grisendi *et al.*, 2006).

2.1 NPM in centrosome duplication

NPM undergoes a variety of post-translational modifications such as phosphorylation, sumoylation, acetylation, ubiquitination, and poly(ADP-ribosyl)ation all of which direct its cellular functions (Okuwaki, 2008). NPM also plays an important role in cell cycle regulation. In interphase, the majority of NPM protein localizes to the nucleolus, whereas in mitosis it distributes itself throughout the nucleoplasm and associates with condensed chromosomes (Ochs *et al.*, 1983). NPM is attached to single, unreplicated centrosomes in late G1 (Fig. 3), and undergoes phosphorylation at Thr199 by cyclin E-CDK2 (Fig. 3), which causes NPM dissociation from the centrosome and its subsequent duplication (Okuda *et al.*, 2000; Tokuyama *et al.*, 2001; Okuda, 2002; Tarapore *et al.*, 2002). A closer look at the centrosomal association with NPM reveals that NPM dissociates from centrosomes prior to the initiation of centrosome duplication (Fig. 3), implying a role for NPM in the pairing of centrioles (Shinmura *et al.*, 2005). Meraldi and coworkers (1999) have shown that cyclin A-CDK2 may also play an important role in centrosome duplication. Whereas cyclin E-CDK2 kinase activity is low during the S phase, due to the instability of cyclin E protein (Dulic *et al.*, 1992; Koff *et al.*, 1992), the amount of cyclin A increases during the S phase and remains elevated throughout the G2 phase (Pines and Hunter, 1990; Marraccino *et al.*, 1992; Pagano *et al.*, 1992). Importantly, NPM is also phosphorylated on Thr199 by cyclin A-CDK2 *in vitro* (Tokuyama *et al.*, 2001), making it possible that the continuous activity of cyclin A-CDK2 may be responsible for preventing NPM from re-associating with centrosomes during the S and G2 phases. NPM re-associates with the centrosomes only in mitosis (Zatsepina *et al.*, 1999; Okuda *et al.*, 2000). Cha and coworkers (2004) report that the cyclin B-CDK1 complex, as a key controller of the M phase, phosphorylates the Thr234 and Thr237 sites of NPM and allows NPM to target the centrosome during mitosis.

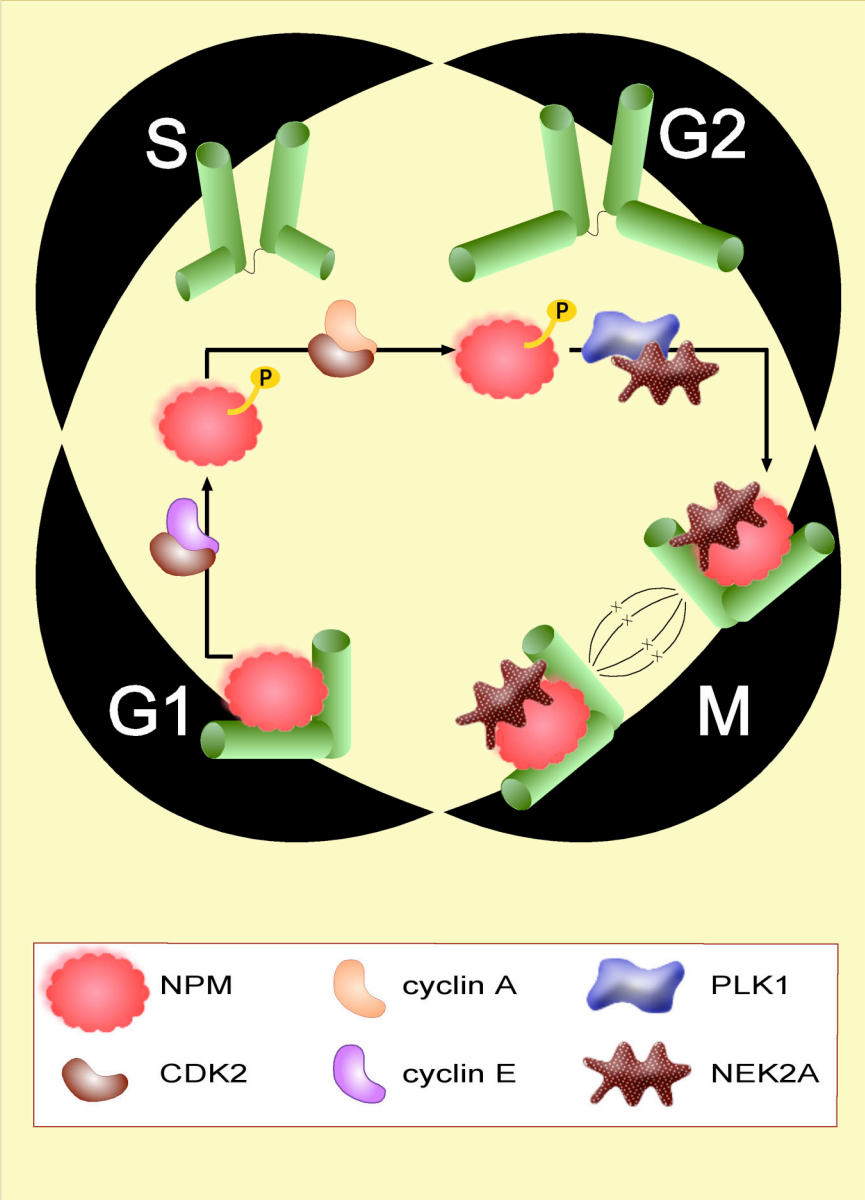

Figure 3. Schematic model of NPM involvement in the control of centrosome duplication and mitosis. In early G1, centrosome-bound NPM dissociates from centrosomes after phosphorylation on Thr199 by cyclin E-CDK2. This phosphorylation leads to centriole separation and the initiation of centrosome replication. During centrosome duplication and maturation (S and G2 phases), cytoplasmic NPM is prevented from re-associating with centrosomes, an event that is probably mediated by the phosphorylation activity of cyclin A-CDK2. During mitosis, the re-association of NPM with the centrosome at the mitotic spindle depends on the phosphorylation activity of polo-like kinase 1 (PLK1) and never in mitosis gene A-related kinase 2 (NEK2A) mitotic kinases, the functions of which have been involved in the control of correct spindle formation and chromosome segregation (Adapted and modified from Grisendi *et al.*, 2006).

Of interest, phosphorylation of NPM on Ser4 by PLK1 may play a role in centrosome homeostasis and in cytokinesis (Zhang *et al.*, 2004a). Furthermore, nuclear export of NPM by the Ran–Crm1 complex may also been involved in the NPM-driven induction of centrosome duplication. When NPM nuclear export signal mutants are over-expressed or mouse skin fibroblasts are treated with leptomycin B, an inhibitor of

Crm1-mediated nuclear export, this effectively hampers NPM export. This then results in NPM accumulation in the nucleus, with subsequent dissociation from the centrosome (Shinmura *et al.*, 2005; Wang *et al.*, 2005b). In this regard, NPM acts as a suppressor of centrosome duplication. Indeed, NPM inactivation results in unrestricted centrosome duplication and aneuploidy (Grisendi *et al.*, 2005), suggesting that NPM is essential for embryonic development and for the maintenance of genomic integrity. Furthermore, aberrant numbers of centrosomes have appeared upon dysregulation of cyclin E-dependent kinase activity and excessive phosphorylation of NPM (Saavedra *et al.*, 2003; Zhang *et al.*, 2004a). NPM may therefore serve as a tumor suppressor, since abrogation of its function results in tumorigenic phenotypes (Lim and Wang, 2006). In contrast, NPM also controls cell proliferation and upon over-expression may show oncogenic potential. Importantly, one clinical study has shown a correlation between level of NPM and progression from early to advanced grades of ovarian cancer and of oral squamous cell carcinoma (OSCC) (Zhang, 2004; Shandilya *et al.*, 2009), implying that NPM functions as a positive regulator of cell proliferation during tumorigenesis (Lim and Wang, 2006).

2.2 NPM involvement in transcriptional regulation

NPM is a histone chaperone identified in *Xenopus* egg extracts as a factor that binds histones and loads them onto DNA (Laskey *et al.*, 1978; Laskey and Earnshaw, 1980). NPM possesses chaperone activity for both nucleic acids and proteins, with its N-terminal core domain being responsible for this activity (Szebeni and Olson, 1999; Hingorani *et al.*, 2000; Okuwaki *et al.*, 2001b). NPM can bind to histones H3, H4, and H2B, and assemble nucleosomes *in vitro*, like other members of the nucleoplasmin family (Okuwaki *et al.*, 2001b; Namboodiri *et al.*, 2004). Molecular chaperones including NPM are supposed to function by a dual mechanism either by aiming directly at chromatin remodeling through histone modifications or by modulating transcription through interaction with transcriptional regulatory partners. Murano and coworkers (2008) demonstrated that NPM associates with the rRNA gene chromatin, with the level of rRNA gene transcription proportional to the amount of NPM. Further experiments reveal that NPM inhibits histone-modifying enzymes such as GCN5 and therefore blocks GCN5-dependent acetylation of free and nucleosomal histones (Zou *et al.*, 2008). Interestingly, NPM is also recruited to join the promoters of certain retinoic acid-responsive genes such as *p120*, heat-shock protein 60 gene (*Hsp60*), or *b-Myb*, and NPM acts as a negative co-regulator of retinoic acid signaling (Liu *et al.*,

2007a). Furthermore, NPM binds to c-Myc and is recruited to c-Myc target gene promoters such as *eIF4E* and *tert*, thereby controlling c-Myc transcriptional activity (Li *et al.*, 2008b). As a co-regulator for the transforming activity of c-Myc, NPM enhances c-Myc-driven cell proliferation and transformation.

Importantly, NPM protein undergoes other post-translational modifications in addition to phosphorylation. NPM is acetylated by the histone acetyltransferase p300 and is the only known chaperone to become acetylated with a functional consequence. Swaminathan and coworkers (2005) reported that NPM acetylation enhances its histone chaperone activity, and modulates *in vitro* transcription from chromatin templates by RNA Pol II in the presence of p300. In addition, acetylation of NPM results in an increase in p53-responsive synthetic reporter gene transcription. Importantly, acetylated NPM has a higher affinity for acetylated histones, which may facilitate histone exchange at the active gene promoters and in chromatin remodeling. Furthermore, high levels of acetylated NPM occur in OSCC isolated from patients (Shandilya *et al.*, 2009). NPM has been shown to occupy the TNF-α promoter in conjunction with active RNAP II and p300 and to enhance gene expression. Intriguingly, acetylation of NPM is markedly higher in later stages of OSCC, suggesting that NPM acetylation status contributes during carcinogenesis to its oncogenic potential (Shandilya *et al.*, 2009).

Interestingly, one level of cell proliferation control at which NPM is involved comprises interactions with a number of transcription factors such us ARF, IRF-1, YY1, and NF-κB (Inouye and Seto, 1994; Kondo *et al.*, 1997; Dhar *et al.*, 2004; Chan *et al.*, 2005; Korgaonkar *et al.*, 2005). NPM can form a complex with YY1 and reverse the YY1 transcription repression function (Inouye and Seto, 1994). Later, NPM was found to play a pivotal role in the regulation of proliferating cellular nuclear antigen (PCNA) promoter through YY1 (Weng and Yung, 2005). In addition, NPM also controls the transcriptional activity of IRF-1 and p53 (Kondo *et al.*, 1997; Colombo *et al.*, 2002). Interestingly, Dhar and coworkers (2004) demonstrated one role of NPM as an NF-κB co-activator in controlling the expression of the human superoxide dismutase 2 (*SOD2*) gene. Consistent with this role, an increase in NPM results in amplification of *SOD2* transcription in a dose-dependent manner. Moreover, NPM is identified as a novel transcriptional regulator of E2F1 promoter by modulating the association of NF-κB, E2F1, and pRB, essential to activate this promoter (Lin *et al.*,

2006). Recently, Leotoing and coworkers (2008) suggested that NPM may alter the activity of androgen receptor by regulating its binding to the androgen response element. In this regard, NPM may be involved in tumor progression through the androgen receptor signaling pathway.

2.3 Biological role of NPM in tumorigenesis

The exact role of NPM in cancer is multifaceted, as it seems to suppress the early stages of tumor formation but promote the growth of established cancer cells (Kondo *et al.*, 1997; Colombo *et al.*, 2002; Bertwistle *et al.*, 2004; Kurki *et al.*, 2004; Korgaonkar *et al.*, 2005). The ability of NPM to suppress tumorigenesis at early stages may be related to its function in maintaining the DNA integrity and stability of the genome (Colombo *et al.*, 2005; Grisendi *et al.*, 2005). Loss of NPM leads to constitutive activation of the DNA damage response, because NPM-null mouse embryo fibroblasts (MEFs) show elevated levels of γ-H2AX, an indicator of DNA double-strand breaks (Colombo *et al.*, 2002). This is in line with other findings that link NPM with the DNA repair process after UV radiation by up-regulating PCNA or GADD45, essential components of the DNA repair machinery (Wu *et al.*, 2002; Gao *et al.*, 2005). Upon UV exposure, NPM is up-regulated and directly binds to p53, and affects its transcriptional activity and checkpoint control (Wang *et al.*, 1999; Colombo *et al.*, 2002; Yang *et al.*, 2002b; Maiguel *et al.*, 2004). In regard to genome integrity, NPM therefore acts as a tumor suppressor by preventing DNA damage.

NPM over-expression has been shown in many human cancers such as colon (Nozawa *et al.*, 1996), ovarian (Shields *et al.*, 1997), prostate (Subong *et al.*, 1999), and gastric (Tanaka *et al.*, 1992) cancers. In addition to interacting with p53, NPM binds to MDM2 (Kurki *et al.*, 2004) and ARF (Itahana *et al.*, 2003; Bertwistle *et al.*, 2004; Brady *et al.*, 2004) and regulates cell growth through the activities of these proteins. NPM interacts with the tumor suppressor ARF, a positive regulator of p53 (Bertwistle *et al.*, 2004; Korgaonkar *et al.*, 2005). ARF prevents the degradation of p53 by interacting with MDM2 to inhibit MDM2-mediated ubiquitination of p53. This leads to increased expression of p53 target genes responsible for cell cycle arrest or apoptosis (Gjerset and Bandyopadhyay, 2006). Because both NPM and MDM2 are able to bind to the N-terminus of ARF, NPM thus competes with MDM2 for binding to ARF. In consequence, NPM sequesters ARF to the nucleolus and inhibits its function (Korgaonkar *et al.*, 2005). Interestingly, NPM acts as a nuclear PI(3,4,5)P3

receptor and binds to active CAD/DFF40 to inhibit apoptotic DNA fragmentation (Ahn *et al.*, 2005). Moreover, several studies have shown NPM to be a co-activator for the transcription of survival genes such as *Bcl-2*, known as an oncogene from follicular lymphoma of B-cell origin (Bakhshi *et al.*, 1985; Tsujimoto and Croce, 1986). NPM blocks mitochondrial p53 and prevents p53-mediated apoptosis (Dhar and St Clair, 2009). NPM has also been shown to bind tumor suppressor IRF-1, a transcriptional activator in the interferon (IFN) system (Harada *et al.*, 1989; Harada *et al.*, 1993). NPM interaction with IRF-1-response elements leads to inactivation of IRF-1 transcriptional activity and manifestation of oncogenic properties (Kondo *et al.*, 1997; Hsu and Yung, 1998).

Previous studies have shown NPM to be a direct transcriptional target of the c-Myc proto-oncogene, and expression of c-Myc correlates with the NPM levels (Guo *et al.*, 2000; Kim *et al.*, 2000; Neiman *et al.*, 2001; Zeller *et al.*, 2001). A recent report by Li and coworkers (2008b) confirms that both endogenous and exogenous NPM directly interact with c-Myc and control the expression of endogenous c-Myc target genes at the promoter. This finding strengthens the notion that NPM, being a special target of the c-Myc pathway, plays an important role in the neoplastic growth of human cancers. Altogether, these data implicate NPM as an anti-apoptotic protein that suppresses either p53-dependent or -independent apoptosis. It has been suggested that the anti-apoptotic potential of NPM depends on its expression level and subcellular localization (Kondo *et al.*, 1997; Colombo *et al.*, 2002; Bertwistle *et al.*, 2004; Korgaonkar *et al.*, 2005). Actively dividing cells expressing high levels of NPM are resistant to apoptosis induced by UV damage or hypoxia (Nozawa *et al.*, 1996; Subong *et al.*, 1999; Wu and Yung, 2002; Li *et al.*, 2004b). Similar to that in other proto-oncogenes, over-expression of NPM is primarily linked to cell proliferation (Itahana *et al.*, 2003), suggesting that NPM as a proto-oncogene promotes cell survival.

Mutations and rearrangements of the *NPM1* gene located on human chromosome 5q35 occur in a variety of hematological disorders (Morris *et al.*, 1994; Redner *et al.*, 1996; Falini *et al.*, 2005; Grisendi and Pandolfi, 2005). These rearrangements lead to formation of NPM fusion proteins such as NPM-RARα in acute promyelocytic leukemia (Redner *et al.*, 1996), NPM-MLF1 in AML (Yoneda-Kato *et al.*, 1996), and NPM-ALK in non-Hodgkin's lymphoma (Morris *et al.*, 1994). In AML, alterations in the C-terminus of NPM appear in about 35% of adult patients and result in

translocation of NPM to the cytoplasm (Falini *et al.*, 2005). These mutations seem to represent tumor-initiating lesions, since they appear before the other AML-associated genetic alterations are detectable (den Besten *et al.*, 2005).

2.4 NPM and viruses

NPM has been involved in the replication cycle of several various viruses. In cells infected by adenoviruses, NPM interacts with adenoviral core proteins and relocalizes from the nucleolus to speckle-patterned structures representing viral DNA replication foci (Walton *et al.*, 1989; Matthews, 2001). Okuwaki and coworkers (2001a) showed that NPM is involved in the *in vitro* replication of the adenovirus DNA. Further experiments by Samad and coworkers (2007) confirmed that NPM interacts with adenovirus basic core proteins and functions as a chaperone for viral chromatin assembly at the late stage of infection. Interestingly, that NPM binds to replication proteins of adeno-associated viruses suggests it may play a role in virus amplification and virion assembly (Bevington *et al.*, 2007). Several lines of evidence indicate that NPM binds to the hepatitis delta antigen, to HTLV-1 Rex, and to HIV-1 Rev proteins (Fankhauser *et al.*, 1991; Adachi *et al.*, 1993; Szebeni *et al.*, 1997; Huang *et al.*, 2001). A recent study by Lee and coworkers (2009b) identified NPM as a host factor that interacts with HBV core protein 149 (Cpl49). Moreover, phosphorylation by caseine kinase 2 (CK2) enhances NPM affinity to the NLS sequences derived from the SV40 large T antigen and the HIV Rev protein (Szebeni *et al.*, 1995; Szebeni *et al.*, 1997), as well as modulating its molecular chaperoning activity, especially in its interaction with target proteins (Szebeni *et al.*, 2003). The chaperone activity of NPM may therefore be essential for proper assembly of the replication machinery and for transport of ribonucleoprotein particles during viral RNA replication. Interestingly, a recent report by Caporale and coworkers (2009) has demonstrated that NPM may be directly involved in the shuttling of envelope glycoprotein (JSE-SP) of the Jaagsiekte sheep retrovirus. Interaction of NPM with JSE-SP has been suggested to enhance the nuclear export of full-length viral RNA and to increase viral particle production by acting at a post-translational step in the viral replication cycle.

3. Kaposi's sarcoma herpesvirus (KSHV)

KSHV, or human herpesvirus-8, is the most recently discovered human herpesvirus. This virus emerged in a representational difference analysis, which identified unique viral DNA sequences in KS biopsies (Chang *et al.*, 1994). KSHV occurs in all subtypes of KS (see below) and also represents the etiological agent of lymphoproliferative disorders including PEL and MCD (Ablashi *et al.*, 2002). KSHV belongs to the double-stranded DNA (dsDNA) viruses of the gammaherpesvirinae subfamily from the same genus as rhadinovirus. It shares sequence homologies with a number of other rhadinoviruses (Fig. 4) such as herpesvirus saimiri, rhesus monkey rhadinovirus, and murine gamma herpesvirus 68 (Neipel *et al.*, 1997; Jung *et al.*, 1999; Searles *et al.*, 1999; Alexander *et al.*, 2000). Similar to other herpesviruses, KSHV exists in one of the two life-cycle programs known as latency or lytic replication. In the latent infection phase, KSHV persists as a nuclear multicopy episomal DNA (Cesarman *et al.*, 1995b; Moore *et al.*, 1996a; Renne *et al.*, 1996a), expressing a small subset of genes that allows the virus to maintain its permanent infection (Zhong *et al.*, 1996). During lytic replication, the complete set of viral genes is expressed, resulting in the assembly of new infectious viral particles (Renne *et al.*, 1996b; Miller *et al.*, 1997). Both latently and lytically infected cells coexist in KS tumors and PEL, with latency as their predominant mode of infection (Decker *et al.*, 1996; Zhong *et al.*, 1996; Staskus *et al.*, 1997). Therefore, the KSHV proteins expressed in both phases contribute substantially to persistent infections, as well as to KSHV pathobiology.

3.1 KSHV-associated malignancies

3.1.1 Kaposi's sarcoma (KS)

In 1872, the famed Austro-Hungarian dermatologist Moritz Kaposi (known as Moritz Kohn before he converted from Judaism to Catholicism) described in *Archiv für Dermatologie und Syphilissequences* a skin disorder that affected elderly men. Since the neoplasms consisted of red and purple plaques or nodules, he expressed his finding as a "sarcoma idiopathicum multiplex haemorrhagicum." Later, KS has been classified accordingly to several distinct clinical-epidemiologic settings (Antman and Chang, 2000). Classic KS is a non-aggressive disorder usually affecting elderly men from the Mediterranean region (DiGiovanna and Safai, 1981). This malignancy is not associated with HIV co-infection and presents on the lower extremities, usually without disease dissemination. Endemic KS affects individuals in sub-Saharan Africa. Although

endemic KS is not linked to HIV infection, it is a more aggressive disease than classic KS (Taylor *et al.*, 1971; Bayley, 1984; Stein *et al.*, 1994). Iatrogenic KS has been described among immunosuppressed patients undergoing solid-organ transplantation (Farge, 1993; Margolius *et al.*, 1994; Parravicini *et al.*, 1997b; Shepherd *et al.*, 1997; Qunibi *et al.*, 1998; Farge *et al.*, 1999; Cattani *et al.*, 2000). Finally, AIDS-associated KS, or "epidemic" KS, is the most common malignancy in patients affected with HIV infection. The prevalence of AIDS-associated KS is about 20-fold higher in homosexual men than in other HIV-infected risk groups (Kedes *et al.*, 1997a; Hermans, 1998). It is generally a highly disseminated cutaneous disease, and the advanced stages involve the oral mucosa and visceral organs including the gastrointestinal tract and lungs (Beral *et al.*, 1990; Biggar and Rabkin, 1996; Hermans *et al.*, 1998).

Early-stage KS lesions, described as patch lesions, are composed of small and irregular endothelial cells located around the normal blood vessels. These cells are accompanied by an infiltration of inflammatory cells. As the lesions grow, the vascular process expands through the skin, and at further stages, spindle-shaped cells appear and form vascular channels containing red blood cells (plaque stage). Late-stage KS is recognizable, as the nodular stage comprises multiple layers of spindle cells and slit-like vascular spaces (Ganem, 2006). The spindle cell is considered the KS tumor cell, and is thought to be of endothelial origin (Rutgers *et al.*, 1986). Inflammatory and spindle cells in KS lesions express cytokines (IFN-γ, TNF-α, IL-1, IL-6), chemokines (MCP-1, IL-8) and angiogenic factors (βFGF, VEGF, PDGF) (Weninger *et al.*, 1999; Ensoli *et al.*, 2001). In addition, high levels of TGF-β, PDGF-β, CCR5, and their receptors (TGF-βR, PDGFR-β) or ligand (CCL5), are reported in spindle cells of KS nodular lesions, when compared to normal skin (Wang *et al.*, 2004a).

3.1.2 Primary effusion lymphoma (PEL)

Primary effusion lymphoma (PEL) is a rare B-cell-non-Hodgkin lymphoma commonly found in KSHV- and HIV-positive individuals with advanced AIDS and KS (Cesarman *et al.*, 1995a; Ansari *et al.*, 1996; Carbone *et al.*, 1996; Nador *et al.*, 1996). PEL can also be found in HIV-negative individuals and may affect patients after solid organ transplantation (Jones *et al.*, 1998; Dotti *et al.*, 1999). This type of malignant lymphoma predominantly occurs in the peritoneal, pericardial, or pleural body cavities as neoplastic effusions without associated solid tumor masses (Carbone and Gaidano,

1997; Cesarman and Mesri, 1999; Cesarman, 2002). That most PEL cells are also co-infected by Epstein-Barr virus (EBV) (Cesarman *et al.*, 1995b; Nador *et al.*, 1996), suggests that EBV is an important pathogenic co-factor. EBV infection in PEL is of monoclonal origin, and the virus establishes the restricted latency I program (Fassone *et al.*, 2000). Symptoms in PEL generally result from accumulation of the malignant effusion in a certain body cavity. Patients frequently suffer from dyspnea (as a result of pleural or pericardial disease) or abdominal swelling (peritoneal disease). No optimal therapy for PEL currently exists, and although some cases respond to cytotoxic chemotherapy regimens, the tumor is often resistant to conventional treatment, with the majority of the patients dying within a few months (Boulanger *et al.*, 2003; Boulanger *et al.*, 2004; Waddington and Aboulafia, 2004; Boulanger *et al.*, 2005). Consequently, new therapeutic approaches for PEL with potential clinical applicability are urgently needed.

Several studies have provided evidence for rearrangements of immunoglobulin genes and somatic hypermutation of the *BCL-6* gene in PEL (Drexler *et al.*, 1998; Matolcsy *et al.*, 1998; Fais *et al.*, 1999; Gaidano *et al.*, 2000). In addition, PEL cells typically express the post-germinal center B-cell marker CD138/syndecan-1 (Gaidano *et al.*, 1996; Carbone *et al.*, 1997), suggesting that they are in a pre-terminal stage of B-cell differentiation close to that of plasma cells. PEL cells usually lack CD20, a marker of B-cell differentiation, but display on their surface activation markers such as CD30, CD38, CD71, and the epithelial membrane antigen (Carbone *et al.*, 1998; Gaidano and Carbone, 2001). Furthermore, expression of several plasma cell markers such as VS38c and MUM-1/IRF-4 has also been reported (Carbone *et al.*, 2001).

Interestingly, gene-expression profiling studies have suggested that PEL displays attributes bridging immunoblastic and anaplastic large-cell lymphomas (Klein *et al.*, 2003). These results suggest that PEL tumors consist of a malignant counterpart of B-cells that have matured but have not reached terminal plasma cell differentiation. Other expression profile analyses obtained from PEL have demonstrated overexpression of genes responsible for inflammation, cell adhesion, and invasion (Jenner *et al.*, 2003). Cytogenetic studies of PEL cells have revealed complex karyotypes but no common chromosomal aberrations (Boulanger *et al.*, 2001). However, few cases have presented recurrent anomaly such as trisomy 7, trisomy 12, or chromosomal aberrations of bands 1q21–25 (Gaidano *et al.*, 1999). Similarly to KS lesions, the pattern of KSHV gene

expression in PEL is predominantly latent (Zhong *et al.*, 1996; Dupin *et al.*, 1999; Katano *et al.*, 2000; Parravicini *et al.*, 2000).

3.1.3 Other KSHV-induced disorders

MCD is another devastating lymphoproliferative syndrome, manifested by fever, anemia, lymphadenopathy, and pulmonary symptoms such as cough and dyspnea (Oksenhendler *et al.*, 1996). Evidence shows that a majority of MCD cases are KSHV-positive, including all HIV-seropositive patients and about half of all HIV-negative individuals (Soulier *et al.*, 1995; Gessain *et al.*, 1996; Dupin *et al.*, 1999; Casper, 2005). The two most important pathologic categories of MCD, the hyaline vascular and the plasma-cell types, are based on histologic characteristics of the affected lymph nodes (Hall *et al.*, 1989). In contrast to PEL, a majority of MCD lesions are EBV-negative, and clinical manifestations are in part due cytokine dysregulation. Several studies have shown high levels of vIL-6 (Parravicini *et al.*, 1997a; Aoki *et al.*, 2001), which controls B-cell differentiation and promotes the expansion of B-cell malignancies (Du *et al.*, 2001). Moreover, high expression of IL-10, and of C-reactive protein, together with elevated KSHV reactivation, may have implications in the MCD's rapid progression and high fatality (Oksenhendler *et al.*, 2000).

In addition to KS, PEL, and MCD, some evidence also exists for KSHV-positive lymphoadenopathies with different clinical, histological, and immunophenotypic characteristics in both HIV-positive and -negative patients. Interestingly, a KSHV- and EBV-associated disease called germinotropic lymphoproliferative disorder involves plasmablasts from the germinal centers of lymph nodes (Du *et al.*, 2002). Dupin and coworkers (2000) found a rare disorder called MCD-associated plasmablastic lymphoma, characterized by large B-cells with one or two prominent nucleoli. These plasmablasts show signs of light chain restriction and may belong to a monotypic cell population, a fact which supports the appearance of plasmablastic lymphomas in MCD lesions (Dupin *et al.*, 2000). Moreover, KSHV infection is also present in HIV-associated solid plasmablastic lymphomas which may represent a tissue-based variant of PEL (Deloose *et al.*, 2005). The literature also reports several unverified or controversial links between KSHV and other lymphomas including multiple myeloma and angioimmunoblastic lymphoma (Schulz, 1999; Ablashi *et al.*, 2002).

3.1.4 Treatment modalities against KSHV-associated diseases

Regardless of considerable improvements in understanding of the pathobiology of KSHV-associated diseases over the last 16 years, treatment of these disorders at present is both toxic and only partially successful. Current data indicate a failure rate up to 22% for the highly active anti-retroviral therapy (HAART) in KS (primarily due to non-compliance), and suggest that KS represents a continuing health problem for years to come (Dittmer *et al.*, 2005). Patients affected by KS, MCD, or PEL are usually co-infected with HIV, and control of HIV viral loads is a necessary element of their care. Currently, no evidence exists for promising responses in patients with their PEL or MCD treated only with HAART therapy, suggesting that in cases of advanced KS as well as of MCD and PEL, treatment with HAART alone is insufficient. Patients in need of adjunct treatment along with HAART currently receive traditional chemotherapy. To treat KS, anthracyclines, anti-mitotic agents, microtubule stabilizers, or other chemotherapeutic agents alone or in combination in small clinical trials have produced responses ranging from 25 to 90% (Vanni *et al.*, 2006). Overall responses depend on tumor burden, associated diseases, and control of primary immunodeficiency. Notably a smaller amount of data is available as to treatment of PEL and MCD. Treatment of these diseases with regular chemotherapy has been restricted by short-lived responses and high mortality (Boulanger *et al.*, 2005; Casper, 2005). The poor outcome and high toxicity of standard combinatorial chemotherapy regimens have prompted researchers to investigate novel therapeutic approaches for the treatment of KSHV-associated malignancies.

3.1.4.1 Anti-viral therapies

The viral load of KSHV in blood has been one of the predictors for progress of KS (Smith *et al.*, 1997; Alagiozoglou *et al.*, 2003; Laney *et al.*, 2004; Laney *et al.*, 2007). Additionally, findings of one *in vitro* study suggest that a small amount of lytic KSHV infection is essential for the induction and maintenance of KS tumors (Grundhoff and Ganem, 2004). MCD is characterized by periodic KSHV lytic replication with high viremia in the peripheral blood and a lytic viral gene program (Katano *et al.*, 2000; Oksenhendler *et al.*, 2000). PEL usually has a more latent infection program than does KS or MCD (Katano *et al.*, 2000), implying that in the prevention of or in therapies against KSHV-associated malignancies, an anti-viral treatment designed to abolish KSHV lytic replication may represent a viable option. To this end, a series of DNA synthesis inhibitors have been developed with variable activity against human

herpesviruses (Naesens and De Clercq, 2001). Activity of these drugs is based on the ability of a nucleoside analogue to incorporate itself into a growing viral DNA chain and thereby terminate the elongation of DNA synthesis. Anti-herpetic inhibitors differ in their mechanism of action. Aciclovir and ganciclovir are phosphorylated by the herpesvirus thymidine kinase and by UL97 phosphotransferase and act directly on the pyrophosphate-binding site of the DNA polymerase. Cidofovir and foscarnet work independently of the herpesvirus thymidine kinase and UL97 (Mercorelli *et al.*, 2008). Promising therapeutic outcomes in PEL patients have been reported with ganciclovir and cidofovir alone or in combination with chemotherapy or HAART (Pastore *et al.*, 2000; Hocqueloux *et al.*, 2001; Luppi *et al.*, 2005; Crum-Cianflone *et al.*, 2006). Intracavitary administration of cidofovir, as well as IFN-α (Boulanger *et al.*, 2003), may represent a rational option in patients refractory to standard chemotherapy or in patients unable to tolerate more toxic systemic treatments.

Effects comparable to those of PEL have also appeared in MCD patients treated with ganciclovir (Casper *et al.*, 2004; Valencia *et al.*, 2005), but not with cidofovir (Berezne *et al.*, 2004). Due to the fact that the majority of anti-herpes drugs are most efficient against the lytic phase, it has been thought that the effectiveness of anti-viral treatment could be enhanced through induction of lytic replication with subsequent blockage of virus production. *In vitro* data suggest that induction of viral reactivation with the proteasome inhibitor bortezomib, or with valproic or glycyrrhizic acid may render tumor cells more sensitive to the anti-viral therapy (Brown *et al.*, 2005; Curreli *et al.*, 2005; Klass and Offermann, 2005).

3.1.4.2 Therapies under investigation

KS is a highly vascularized tumor, and expression of the human vascular endothelial growth factor (VEGF) is crucial for KS tumorigenesis (Cornali *et al.*, 1996; Masood *et al.*, 1997; Nakamura *et al.*, 1997). Clinical studies with the first approved VEGF inhibitors reveal their moderate toxicity and minimal efficacy in KS tumors (Pluda *et al.*, 1993; Eckhardt *et al.*, 1996). Intriguingly, later work on an innovative anti-sense oligonucleotide to VEGF (VEGF-AS) has led to an impressive decrease in VEGF plasma levels and a total remission of KS in one patient refractory to HAART and to chemotherapy (Levine *et al.*, 2006). Despite the fact that targeting VEGF is a promising therapeutic modality, inhibition of other signaling pathways involved in KS has also been under exploration. Platelet-derived growth factor receptor (PDGFR) and

c-kit proto-oncogene are both reported to be overexpressed in KS (Werner *et al.*, 1990; Sturzl *et al.*, 1992; Pistritto *et al.*, 1994; Moses *et al.*, 2002). Imatinib, a tyrosine kinase inhibitor, inhibits activity of multiple tyrosine kinases such as Bcr-Abl, PDGFR, and c-kit (Druker *et al.*, 1996; Heinrich *et al.*, 2000). To assess the efficacy of imatinib for KS, patients with advanced AIDS-associated KS were treated with the drug. Intriguingly, among 10 individuals, a clinical response occurred in four (Koon *et al.*, 2005). An additional promising therapeutic strategy for KS may be the inhibition of matrix metalloproteinases (MMPs), endopeptidases involved in angiogenesis through degradation of extracellular matrix (Egeblad and Werb, 2002). MMPs are also highly expressed in KS lesions, suggesting that their role in tumorigenesis most likely is in facilitating the invasion of tumor cells into the basement membrane (Benelli *et al.*, 1994; Blankaert *et al.*, 1998). Clinical trials in KS patients with MMP inhibitors similar in structure to tetracycline have resulted in a 40% response rate. Importantly, these inhibitors were also well tolerated and had an anti-tumor potential in patients with AIDS-associated KS (Dezube *et al.*, 2006).

One line of therapeutic approaches with great potential for KSHV-induced diseases perhaps could rely on targeting the NF-κB pathway through proteasome inhibition. NF-κB is constitutively active in those PEL cells (Keller *et al.*, 2000; Guasparri *et al.*, 2004) sensitive to BAY-117082-mediated blockade of NF-κB signaling (Keller *et al.*, 2000; Keller *et al.*, 2006). Interestingly, bortezomib, an inhibitor of the NF-κB pathway, induces apoptosis in PEL cell lines *in vitro* (An *et al.*, 2004a). Whether these findings can be extended to a clinical setting remains to be seen. As NF-κB has a crucial anti-apoptotic role in cells, it is possible that its pharmacological inhibition with the currently available inhibitors may lead to increased apoptosis in normal tissues or to unacceptable toxicities due to its inhibition of signaling pathways other than NF-κB (Graham and Gibson, 2005). PEL tumors in NOD/SCID mice have also been treated with an anti-viral therapy combined with IFN-α. Azidothymidine and IFN-α treatment delays tumorigenesis but does not rescue PEL-engrafted SCID mice (Wu *et al.*, 2005). Moreover, the pro-apoptotic effect observed for azidothymidine and IFN-α in PEL cells occurs through the concomitant activation of TRAIL and the blockade of NF-κB (Toomey *et al.*, 2001; Ghosh *et al.*, 2003), which may enhance the possibility of their non-specific apoptotic effect.

Several studies have suggested that an Akt-signaling downstream effector termed tuberous sclerosis complex-mammalian target of rapamycin (TSC-mTOR), is a key intracellular pathway controlling pathological angiogenesis and tumor development of endothelial origin (Montaner *et al.*, 2001; Phung *et al.*, 2006; Sodhi *et al.*, 2006). Activation of the PI3K/Akt/mTOR pathway by the KSHV lytic protein vGPCR (viral G protein-coupled receptor) plays a fundamental role in KS development. Interestingly, a highly potent and specific inhibitor from the rapamycin family can inactivate vGPCR oncogenesis *in vitro* and *in vivo* (Sodhi *et al.*, 2006). Rapamycin (sirolimus) has been used for transplant recipients affected by iatrogenic KS (Campistol and Schena, 2007), as well as for patients with the classic form of the disease (Guenova *et al.*, 2008; Merimsky *et al.*, 2008). Interestingly, Sin and coworkers (2006) have demonstrated that rapamycin inhibits PEL growth *in vitro* and *in vivo*. Another report has suggested that rapamycin induces only a preliminary response; it does not eradicate PEL. The hypoxic environment of the body cavity is implicated in initiation of cytokine-mediated escape mechanisms leading to disease development (Gasperini and Tosato, 2009).

3.2 KSHV latency program

KSHV has a large, double-stranded DNA genome that forms a closed circular episome upon entry into the nucleus. During productive replication, viral progeny DNA is ultimately synthesized as linear, single-unit genomes destined for incorporation into independent virions (Renne *et al.*, 1996a). During latent infection, KSHV encodes only a minority of the nearly 100 genes in the genome (Zhong *et al.*, 1996). Among the viral proteins expressed in latently infected cells are: (a) a latency-associated nuclear antigen (LANA), (b) a viral homolog of cyclin D2 (viral cyclin, v-cyclin), and (c) an anti-apoptotic viral FLICE-inhibitory protein (v-FLIP). These proteins are encoded by the open reading frames *ORF73*, *ORF72*, and *ORF71*, respectively (Fig. 4), and they belong to a common polycistronic transcriptional unit, identified as the latency transcript (LT) cluster (Dittmer *et al.*, 1998; Sarid *et al.*, 1998; Talbot *et al.*, 1999). In latent infection, genes in the LT cluster are transcribed from a constitutively active promoter. This results in an unspliced, full-length 5.8-kb transcript or an alternatively spliced 5.4-kb mRNA containing *ORF71*, *ORF72*, and *ORF73*. Additionally, a short 1.7-kb transcript is formed by mRNA splicing and contains only *ORF71* and *ORF72*. The major translation product of the longer (tricistronic) mRNA is LANA, whereas v-cyclin and v-FLIP are primarily translated from the shorter (bicistronic) transcript,

the latter by utilizing an internal ribosome entry site at the end of *ORF72* (Bieleski and Talbot, 2001; Grundhoff and Ganem, 2001; Low *et al.*, 2001). Moreover, a rare splice variant, a short 1.1-kb mRNA that contains exclusively *ORF71*, is detectable in PEL cells (Grundhoff and Ganem, 2001).

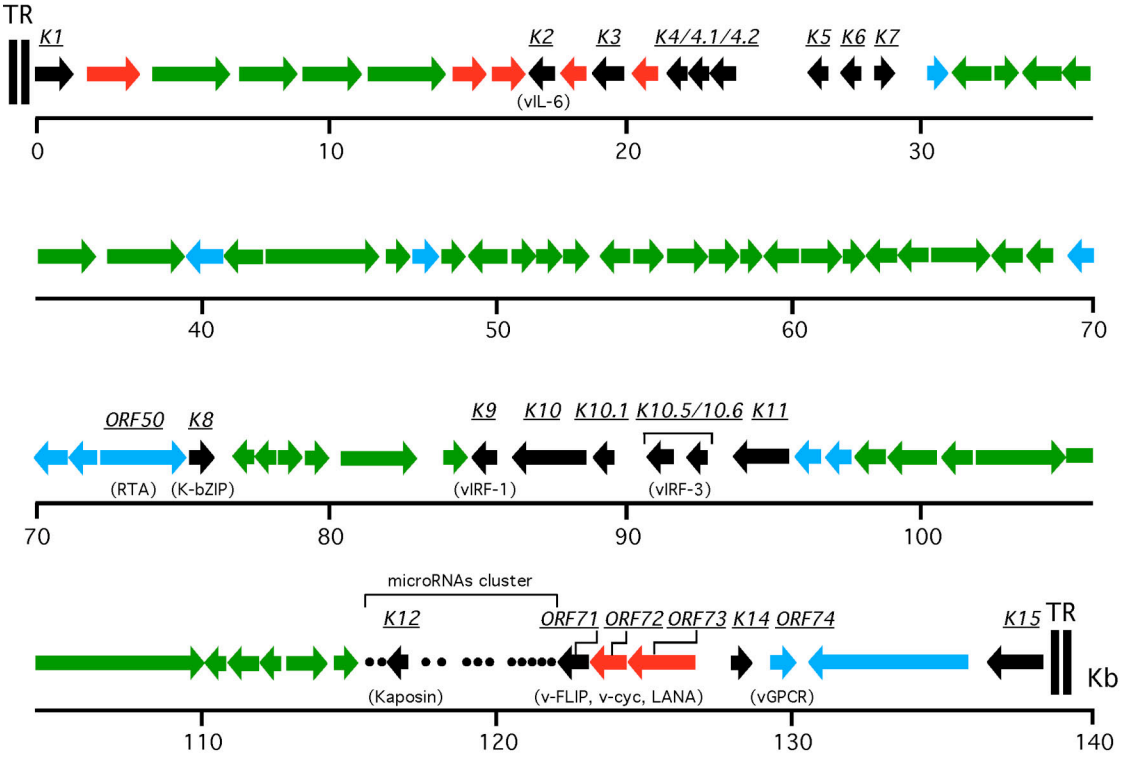

Figure 4. KSHV genomic open reading frames (ORFs). Arrows represent individual ORFs, and the dots represent the KSHV-encoded microRNAs. ORFs unique to KSHV are indicated by black arrows. ORFs common to rhadinoviruses are indicated by red arrows, ORFs common to other γ-herpesviruses by light blue arrows, and ORFs common to most herpesviruses by green arrows. TR denotes terminal repeats. Alternative names mentioned in this thesis for some of these unique KSHV genes appear in parentheses (Adapted and modified from Wen and Damania, 2010).

During the latent phase of KSHV infection, other latent genes are also expressed from the viral episome. The *K12* gene, encoding Kaposin (Muralidhar *et al.*, 1998; Sadler *et al.*, 1999), is expressed at low levels during latency. *K12* is separated from the LT cluster by a 4-kb intergenic sequence (Fig. 4), which is the biggest coding gap in the unique region of the KSHV genome. Interestingly, 10 of the 12 recently discovered KSHV microRNAs (miRNAs, Fig. 4) are positioned in this intergenic region (Cai *et al.*, 2005; Pfeffer *et al.*, 2005; Samols *et al.*, 2005). The two other miRNAs, miR-K10 and miR-K12, are located within the K12 coding region and within the 3'-UTR of K12 (Fig. 4), respectively (Grundhoff *et al.*, 2006). Viral interferon regulatory factor 3 (vIRF-3, LANA2), encoded by the *K10.5* gene, is another protein classified as a latent KSHV gene product (Lubyova and Pitha, 2000), and its expression seems to be

restricted to KSHV-infected hematopoietic tissues, including PEL and MCD; vIRF-3 is not detectable in KS lesions.

3.2.1 KSHV latency-associated nuclear antigen

LANA, initially termed latent nuclear antigen, is one of the major proteins expressed during KSHV latency (Gao *et al.*, 1996a; Kedes *et al.*, 1997b). It is a multi-domain protein that possesses multifunctional properties. The LANA protein is 222 to 234 kDa in size, consisting of 1,162 aa. It produces a typical speckled nuclear signal upon indirect immunofluorescence with LANA-specific antibodies (Gao *et al.*, 1996a; Gao *et al.*, 1996b; Kedes *et al.*, 1996). LANA consists of three domains: (a) an N-terminal domain rich in Ser/Thr, Pro, and basic residues, (b) an internal repeat domain, which is highly polymorphic and abundant in Glu, Asp, Gln, and Leu, and (c) a C-terminal domain where charged and hydrophobic residues are located (Gao *et al.*, 1999; Zhang *et al.*, 2000).

3.2.1.1 LANA and episome maintenance

Due to the fact that the KSHV genome encodes no homologs of centromeres, there exists a need for an alternative mode of episome replication, segregation, and latency maintenance in the daughter cells. LANA has been shown to bind episomes and colocalize with KSHV (Ballestas *et al.*, 1999; Komatsu *et al.*, 2004; Tetsuka *et al.*, 2004), and to tether the KSHV episome to the host cell chromosomes (Ballestas *et al.*, 1999; Cotter and Robertson, 1999; Szekely *et al.*, 1999; Mattsson *et al.*, 2002; Shinohara *et al.*, 2002; Barbera *et al.*, 2006). This further shows LANA to be a key player in episomal persistence. Additionally, expression of LANA has been demonstrated as sufficient for replication and segregation of viral minigenomes, *i.e.* plasmids with terminal repeat elements (Garber *et al.*, 2001; Hu *et al.*, 2002; Grundhoff and Ganem, 2003). Episomal segregation in mitosis, which guarantees that identical numbers of KSHV episomes are divided into every daughter cell, is also LANA-dependent (Ballestas *et al.*, 1999; Hu *et al.*, 2002; Grundhoff and Ganem, 2003). Consequently, a KSHV mutant, BAC36-ΔLNA, with its LANA disrupted by transposon-based mutagenesis with a KSHV BAC clone, BAC36, is inefficient in the establishment and maintenance of latency in mammalian cells (Ye *et al.*, 2004).

LANA interacts with the core histones H2A and H2B to tether episomes to the nucleosome during mitosis and interphase (Barbera *et al.*, 2006). Both N- and

C-terminal LANA domains bind to host chromosomes. However, the C-terminus is insufficient to maintain episome persistence alone in the N-terminus mutants (Piolot *et al.*, 2001; Krithivas *et al.*, 2002; Shinohara *et al.*, 2002; Barbera *et al.*, 2004; Barbera *et al.*, 2006). On the other hand, the LANA C-terminus is essential for its oligomerization, and current data imply that efficient tethering of the KSHV episome depends on LANA oligomerization (Schwam *et al.*, 2000; Viejo-Borbolla *et al.*, 2005; Verma *et al.*, 2006). Moreover, to generate the best possible conditions for episomal replication, LANA also interacts with multiple chromosome-binding and origin recognition complex (ORCs) proteins such as Brd2/RING3, ORC2, HBO1, CBP, and also cohesins (Stedman *et al.*, 2004; Verma *et al.*, 2006; Stedman *et al.*, 2008). Of note, a particular 32-bp sequence upstream of LANA binding site 1, referred to as replication element, associates with ORC2. Ectopic expression of LANA multiplies the number of copies of chromatin-bound DNA of replication elements, suggesting that LANA is essential for the recruitment of ORCs and may contribute to the stabilization of the replication protein complexes at the replication element site (Verma *et al.*, 2007). These data clearly indicate that LANA not only tethers the episome to the chromosomes but also actively takes control of cellular proteins to support the virus in its episomal replication process.

3.2.1.2 LANA and regulation of viral and cellular gene transcription

Additional to its role in episomal maintenance and segregation, LANA also modulates cell proliferation, transformation, apoptosis, and immune responses. LANA takes action as a direct or indirect transcriptional repressor or even sporadically as a transcriptional activator contributing to latent infection. A study by Radkov and coworkers (2000) showed that LANA with *H*ras may induce transformation in primary rat fibroblasts. Moreover, LANA overexpression enhances the lifespan and cellular proliferation of primary HUVEC cells (Watanabe *et al.*, 2003). Importantly, sole overexpression of LANA is unable to initiate anchorage-independent growth of NIH3T3 cells, demonstrating the importance of other viral genes and factors for KSHV-induced malignancies (Watanabe *et al.*, 2003).

The actual mechanism of LANA-mediated cellular transformation has been subjected to extensive research. These studies have revealed that LANA acts as a transcriptional co-factor and targets the retinoblastoma protein-E2F transcriptional regulatory pathway (Radkov *et al.*, 2000). Moreover, LANA activates c-Myc-dependent

transcription by promoting its phosphorylation (Liu *et al.*, 2007b). It also interacts with Sp1 transcription factor, thus regulating the expression of telomerase (Verma *et al.*, 2004). Finally, LANA also interacts with and inhibits p53 transcriptional activity and p53-dependent apoptosis (Friborg *et al.*, 1999). Suppression of p53 activity by LANA has also been linked with genomic instability (Si and Robertson, 2006). Moreover, enhanced multinucleation, abnormal establishment of centrosomes, and creation of mitotic bridges are reported in mammalian cells with a stable expression of LANA (Si and Robertson, 2006).

KS and PEL express high levels of β-catenin (Fujimuro and Hayward, 2003; Fujimuro *et al.*, 2003; Fujimuro *et al.*, 2005), which should normally be directed toward degradation in the cytoplasm by its negative regulator GSK3β. LANA binds β-catenin and subsequently translocates GSK3β to the nucleus, thus preventing negative regulation of β -catenin. β -catenin then targets gene products that have essential functions in cell cycle progression and cell proliferation such as Cyclin D1, Myc, and Jun (Fujimuro *et al.*, 2003). Moreover, high expression of LANA promotes the expression of IL-6 by activating the AP-1 pathway (An *et al.*, 2002). This may lead to consequent activation of STAT3 signaling, and LANA ability to interact with STAT3 enhances STAT3-induced transcription (Muromoto *et al.*, 2006). Furthermore, LANA activates the AP-1 pathway by itself, through binding to Jun to facilitate the assembly of Jun-Fos heterodimers (An *et al.*, 2004b).

Regulation and control of transcription by LANA may involve multiple mechanisms. LANA may repress transcription *in vitro* through either the C- or the N-terminus domain when fused to a heterologous DNA-binding motif (Schwam *et al.*, 2000). Another possible mechanism of transcriptional repression is recruitment and binding to members of the mSin3 transcriptional repressor complex (Krithivas *et al.*, 2000). Additionally, LANA actively represses the transcriptional activity of the ATF4/CREB complex (Lim *et al.*, 2000). Another finding is that LANA represses both: CBP transcriptional activation as well as histone acetyltransferase (HAT) activity (Lim *et al.*, 2001). LANA is responsible for enhanced expression of Id-1 (Tang *et al.*, 2003), which plays an important role in cell cycle regulation and differentiation (Benezra *et al.*, 1990). LANA also regulates transcriptional repressor KLIP1 and alleviates its transcriptional repression effects (Zhang *et al.*, 2001; Pan *et al.*, 2003). Lastly, LANA

also has the ability to auto-regulate its own promoter and to activate its own transcription (Jeong *et al.*, 2004).

3.2.1.3 LANA and repression of lytic replication

As a final point, LANA-mediated transcriptional control has strong implications for viral latency. LANA interacts with RBP-Jk or Sp1 transcription factors and modulates the expression of replication transcriptional activator (RTA) (Verma *et al.*, 2004; Lan *et al.*, 2005b). Notably, LANA may interact directly with RTA, and it suppresses activation of the RTA promoter (Lan *et al.*, 2004; Lan *et al.*, 2005a). Interestingly, this ability is antagonized by Pim-1 and Pim-3 kinases (Cheng *et al.*, 2009). Furthermore, LANA regulates viral gene expression through epigenetic modifications of the viral genome (Lu *et al.*, 2006). Viral genes have been regulated in a LANA-dependent manner via its interaction with SUV39H1 and its recruitment of HP-1. When histone-H3 is being methylated by SUV39H1, HP-1 is recruited and forms inactive heterochromatin (Sakakibara *et al.*, 2004). These events lead to condensation of the viral episome, and as a result, to transcriptional supression of all viral genes except those within the latency locus (Sakakibara *et al.*, 2004).

3.2.2 KSHV-encoded cyclin (v-cyclin)

The KSHV gene *ORF72* encodes a protein of 257 aa with an approximate molecular weight of 29 kDa. The product of this gene is called v-cyclin, K-cyclin, or KSHV-cyclin, and has 32% identity and 54% similarity to mammalian cyclin D2 (Li *et al.*, 1997). The v-cyclin preferably binds to and activates CDK6 and only weakly associates with CDK2, CDK3, CDK4, CDK5, and CDK9 (Godden-Kent *et al.*, 1997; Li *et al.*, 1997; Platt *et al.*, 2000; Chang and Li, 2008). Interaction of v-cyclin with its catalytic subunit CDK6 is a prerequisite to form a powerful, constitutively active kinase complex (Van Dross *et al.*, 2005), which is not regulated by the CDK-activating kinase (Kaldis *et al.*, 2001). This kinase complex phosphorylates a broad range of substrates including histone H1 and retinoblastoma protein Rb (Godden-Kent *et al.*, 1997; Li *et al.*, 1997), p27KIP1 (Swanton *et al.*, 1997; Ellis *et al.*, 1999; Mann *et al.*, 1999), p21CIP1 (Jarviluoma *et al.*, 2006), Id-2 and Cdc25a (Mann *et al.*, 1999), Cdc6 and ORC1 (Laman *et al.*, 2001), Bcl-2 (Ojala *et al.*, 2000), NPM (Cuomo *et al.*, 2008), and caldesmon (Cuomo *et al.*, 2005).

Rb operates as a cell-cycle checkpoint protein by binding to the transactivation domain of E2F transcription factor to prevent cell cycle progression (Helin *et al.*, 1993; Dunaief *et al.*, 1994). Importantly, v-cyclin-CDK6 phosphorylates and inactivates Rb, leading to suppression of the Rb-induced cell cycle arrest (Chang *et al.*, 1996; Godden-Kent *et al.*, 1997). Cell cycle progression is regulated by the consequent activation and inactivation of cyclin-dependent kinases (CDKs). Many signals arrest the cell cycle through the suppression of CDKs by CKI; v-cyclin-CDK6 overcomes the p27KIP1-mediated growth arrest (Swanton *et al.*, 1997; Ellis *et al.*, 1999; Mann *et al.*, 1999). Overexpression of v-cyclin leads to phosphorylation of p27KIP1 on Thr187 by the binary complex v-cyclin-CDK6 and subsequent degradation of p27KIP1 via the proteasome-dependent pathway (Ellis *et al.*, 1999; Mann *et al.*, 1999). This may nevertheless represent a simpler situation than under *in vivo* conditions. In contrast to other lymphomas, PEL cells express high levels of p27KIP1, and yet proliferate actively (Carbone *et al.*, 2000; Jarviluoma *et al.*, 2004). The v-cyclin-CDK6 complex prevents p27KIP1 from accomplishing its anti-proliferative function by formation of inactive complexes (Jarviluoma *et al.*, 2004). Furthermore, during latent infection, phosphorylation of Ser10 on p27KIP1 by the v-cyclin-CDK6 complex leads to translocation of p27KIP1 into the cytoplasm and allows PEL cells to proliferate despite highly abundant p27KIP1 (III). Of interest, we have found that upon lytic replication, v-cyclin-CDK6 directs p27KIP1 to degradation through phosphorylation of Thr187 (III). In addition to p27KIP1, v-cyclin-CDK6 has been resistant to other CKIs such as p21CIP1 and p16INK4 (Swanton *et al.*, 1997; Jarviluoma *et al.*, 2006).

v-cyclin not only has been linked to deregulation of cellular gene expression, it has also been shown to suppress STAT3-mediated gene activation (Lundquist *et al.*, 2003) and upregulate pro-proliferative genes such as the cyclin A (Duro *et al.*, 1999). Similar to many other oncogenes, v-cyclin can also lead to apoptosis or induce cell cycle arrest under certain circumstances. It induces apoptosis in the presence of high levels of CDK6 (Ojala *et al.*, 1999; Ojala *et al.*, 2000), or stress signals (Verschuren *et al.*, 2002). To promote apoptosis, v-cyclin-CDK6 phosphorylates and inactivates cellular anti-apoptotic protein Bcl-2 (Ojala *et al.*, 2000). Additionally, v-cyclin causes a DNA damage response and senescence in endothelial cells (Koopal *et al.*, 2007).

Apoptosis and abnormal cell proliferation triggered by v-cyclin are closely related to p53 (Verschuren *et al.*, 2002; Verschuren *et al.*, 2004). Transient expression of v-

cyclin in MEFs causes p53 accumulation, which contributes to v-cyclin-induced cell cycle arrest and apoptosis in MEFs (Verschuren *et al.*, 2002). In a transgenic mouse model (Eμ-K-cyclin mice), v-cyclin accelerates lymphomagenesis in $p53^{-/-}$ background (Verschuren *et al.*, 2004). These data indicate the existence of a relationship between v-cyclin and p53 during tumorigenesis. Additionally, v-cyclin overexpression raises MDM2 levels and supports complex formation between MDM2-NPM and p53-NPM (Kurki *et al.*, 2004). Interestingly, recent data have suggested CDK9 as a novel v-cyclin-interacting partner and indicated that the v-cyclin-CDK9 kinase complex phosphorylates p53 on Ser33 (Chang and Li, 2008). Any functional consequences of this phosphorylation need further investigation, however.

3.3 Molecular events involved in the switch from latency to lytic KSHV replication

A fundamental question concerning the viral life cycle focuses upon the physiological stimuli that direct the switch from latent infection to lytic replication, *i.e.* viral reactivation. This switch can be described as a two-event route where: (a) upstream actions result in de-repression and induction of the activator genes to initiate the lytic cycle and (b) downstream events resulting from a multitude of actions executed by the products of the lytic cycle activator genes. Several chemicals and biological stimuli can activate the upstream events of viral reactivation. These include phorbol esters, which are protein kinase C agonists, histone deacetylase (HDAC) inhibitors, DNA methyltransferase inhibitors, anti-immunoglobulins, hypoxia, and also the presence of other viruses (Renne *et al.*, 1996b; Miller *et al.*, 1997; Chen *et al.*, 2001; Davis *et al.*, 2001; Vieira *et al.*, 2001).

3.3.1 Lytic replication phase

The viral lytic program develops through a sequentially regulated cascade of gene expression. These lytic cycle-specific genes (Table 1) can be grouped into three categories based on their expression kinetics: immediate-early (IE), delayed-early (DE), and late genes (L). After primary infection or upon reactivation from latency, expression of IE genes begins instantly. These genes are critical for the start of viral transcription and usually encode for regulatory proteins. RTA is the gene product of *ORF50*, the major IE transcript of KSHV, which is necessary and sufficient to force the switch from latency to lytic gene expression (Sun *et al.*, 1998). Expression of the RTA mRNA is insensitive to protein synthesis inhibitors like cycloheximide,

indicating that *de novo* protein production is not required for reactivation (Gradoville *et al.*, 2000; Ye *et al.*, 2007). DE genes are expressed after the IE, but prior to viral DNA synthesis. Inhibitors of viral DNA synthesis such as phosphonoacetic acid or ganciclovir (GCV) do not affect expression of these genes. Late lytic genes whose expression is inhibited by phosphonoacetic acid or GCV usually appear after 30 hours post-induction (Sun *et al.*, 1999; Zhu and Yuan, 2003). Examples of genes from these groups are in Table 1.

Table 1. Classification of KSHV lytic genes

Group	Gene and gene description	Reference
IE	*ORF50*/RTA (R transcriptional activator)	(Sun *et al.*, 1999)
IE	*ORF45* (virion phosphoprotein)	(Zhu *et al.*, 1999)
IE	*ORF29* (putative ATPase subunit of terminase)	(Saveliev *et al.*, 2002)
IE	*ORF4.2/*KCP (complement control protein)	(Zhu *et al.*, 1999)
IE	*ORF K3* (E3 ubiquitin ligase)	(Sun *et al.*, 1999)
IE	*ORF K5* (E3 ubiquitin ligase)	(Sun *et al.*, 1999)
DE	*ORF16*/vBcl-2 (bcl-2-like inhibitor of apoptosis)	(Sun *et al.*, 1999)
DE	*ORF49* (c-Jun-mediated transcriptional activator)	(Gonzalez *et al.*, 2006)
DE	*ORF57 (*DNA polymerase catalytic subunit)	(Lukac *et al.*, 2001)
DE	*ORF59* (DNA polymerase processivity subunit)	(Chan and Chandran, 2000)
DE	*ORF74*/vGPCR (viral G protein-coupled receptor)	(Kirshner *et al.*, 1999)
DE	*ORF K1* (ITAM signal-transducing membrane glycoprotein)	(Jenner *et al.*, 2001)
DE	*ORF K2*/v-IL6 (interleukin-6)	(Sun *et al.*, 1999)
DE	*ORF K4*/vCCL-2 (interleukin 8-like CC chemokine)	(Sun *et al.*, 1999)
DE	*ORF K8* (K-bZIP transcription factor)	(Lukac *et al.*, 2001)
DE	*ORF K9*/vIRF1 (interferon regulatory factor)	(Chen *et al.*, 2000)
DE	*ORF K15* (signal-transducing membrane protein)	(Choi and Nicholas, 2010)
L	*ORF65/*sVCA (small viral capsid antigen)	(Lin *et al.*, 1997)
L	*ORF51*/K8.1 (envelope glycoprotein)	(Chandran *et al.*, 1998)
L	*ORF25/*MCP (major capsid protein)	(Ascherl *et al.*, 1999)
L	*ORF17* (minor capsid scaffold protein)	(Unal *et al.*, 1997)

3.3.2 ORF50/RTA: master regulator of the latent-lytic switch

The KSHV latent-lytic switch is triggered by a number of factors that stimulate the expression of the major lytic switch protein, RTA. Expression of RTA is absolutely required to execute the lytic program resulting in tightly regulated expression of viral proteins, replication of viral DNA, assembly and release of viral particles, and host cell death (Lukac *et al.*, 1998; Sun *et al.*, 1998; Lukac *et al.*, 1999). During the latency, only a few genes are transcribed, whereas expression of RTA is highly repressed (Katano *et al.*, 2001a). Several lines of evidence suggest that epigenetic modifications and chromatin remodeling are actively involved in this repression (Lu *et al.*, 2003). Epigenetic modifications including DNA methylation control gene expression during development and play important roles in cancer by transcriptional silencing of essential growth controllers (Baylin, 2005). It has been suggested that hypermethylation of the RTA promoter controls its expression and subsequently regulates the KSHV latent-lytic switch (Chen *et al.*, 2001). Moreover, high methylation at the RTA promoter region in latency promotes the association of transcriptional repressors and HDACs (Shamay *et al.*, 2006). Additionally, other chromatin modifications such as acetylation, and modifications in the chromatin-binding proteins (Lu *et al.*, 2003) influence chromatin structure and, together with DNA methylation, may also control transcription of the *ORF50* gene.

Viral reactivation can be induced by chemicals including sodium butyrate (NaB) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Renne *et al.*, 1996b; Miller *et al.*, 1997). NaB is an HDAC inhibitor (Boffa *et al.*, 1978), whereas TPA is an inducer of HAT (Masumi *et al.*, 1999). Deacetylation reverses the acetylation process and leads to establishment of strongly packaged nucleosomes, which are inaccessible to transcription factors (Yang *et al.*, 1996). Therefore, both NaB and TPA affect the acetylation status of the RTA promoter, and thus KSHV lytic replication, by distinct mechanisms. TPA induces the KSHV lytic cycle by activating a variety of transcription factors and enhancing their DNA-binding activity (Wang *et al.*, 2004b), whereas successful activation by NaB critically depends on binding of the Sp1 transcription factor to the butyrate-responsive element in the promoter of ORF50 (Ye *et al.*, 2005). Subsequently, treatment with NaB enhances recruitment of Sp1 with its co-activator CBP, which possesses HAT activity and physically interacts with RTA (Gwack *et al.*, 2001; Lu *et al.*, 2003). Following the initial activation, RTA is recruited to its responsive elements through direct interaction with the Notch signaling pathway

transcription factor RBP-Jk (Liang and Ganem, 2004; Chang *et al.*, 2005a; Chang *et al.*, 2005b).

RTA-mediated control of RBP-Jk activity from its repressed state to its activation is critical for further lytic gene expression (Liang and Ganem, 2003). To this end, RTA recruits the chromatin-remodeling complex SWI/SNF and the TRAP/mediator coactivator to its downstream viral promoters, a recruitment essential for RTA-dependent viral gene expression (Gwack *et al.*, 2003). As reviewed in 3.2.1, LANA takes control of RTA activity by direct binding to the ORF50 promoter as well as through protein-protein interactions. Treatment of PEL cells with NaB leads to an increase in the lysine acetylation of LANA and its dissociation from the ORF50 promoter. Subsequent removal of LANA from Sp1 allows Sp1 and ORF50 to interact, and leads to transcriptional activation (Lu *et al.*, 2006). These findings indicate that regulation of the latent-lytic switch depends on an intimate interplay between viral RTA, cellular HAT, and HDAC activities, plus a variety of host factors that regulate chromatin methylation and acetylation.

3.3.2.1 Downstream targets of RTA

RTA has also been demonstrated to control and transactivate a number of downstream lytic genes such as *K1* (Bowser *et al.*, 2002; Bowser *et al.*, 2006), *K2*/vIL-6 (Deng *et al.*, 2002), *K3* (Chang *et al.*, 2005a), *K5* (Haque *et al.*, 2000), *K9*/vIRF-1 (Ueda *et al.*, 2002), *K8* (Lukac *et al.*, 1999), *K15* (Wong and Damania, 2006), *ORF57* (Lukac *et al.*, 1999), *ORF74*/vGPCR (Jeong *et al.*, 2001), and others. RTA downstream targets are activated through two distinctive strategies: RTA either directly recognizes RTA-responsive elements in the promoters of its target genes, or it interacts with cellular or viral factors bound to the promoters (Song *et al.*, 2003; Chang *et al.*, 2005a; Chang *et al.*, 2005d). Although no significant homology has emerged among the RTA-responsive viral promoters, an evaluation of the *K8* RTA-responsive elements with other viral RTA-responsive elements confirms a striking pattern of A/T triplets located within a frequency of 10 to 20 bp (Liao *et al.*, 2003). The multiplicity of RTA-binding sites suggests that the activation of RTA target gene promoters is a consequence of RTA interaction with factors that mediate the DNA-transcriptional complex interaction. These cellular factors may involve NF-κB (Roan *et al.*, 2002) or TATA-binding protein, as has been demonstrated for the *K1* promoter (Bowser *et al.*, 2006).

RTA also regulates *ORF74* (vGPCR) and *K14* (vOX2) genes by an IFN-stimulated response element-like sequence in the promoter region (Zhang *et al.*, 2005a).

3.3.3 Diverse cellular factors involved in KSHV reactivation

Many factors regulate the expression of RTA and imply KSHV lytic replication. Recognition of cellular factors that cooperate with RTA in the switch from KSHV latency to lytic replication is a prerequisite in understanding the virus life cycle and its pathobiology. KSHV lytic reactivation has been reported to occur as a response to a variety of stimuli such as TPA (Renne *et al.*, 1996b; Sarid *et al.*, 1998), NaB (Miller *et al.*, 1997), ionomycin (Lukac *et al.*, 1999), 5-azacytidine (Chen *et al.*, 2001), IL-6 (Chang *et al.*, 2000; Chatterjee *et al.*, 2002), IFN-γ (Blackbourn *et al.*, 2000), hypoxia (Davis *et al.*, 2001), and co-infection with other viruses (Harrington *et al.*, 1997; Vieira *et al.*, 2001). Induction of viral reactivation by TPA involves protein kinase C signaling and the calcium-dependent calcineurin pathway (Zoeteweij *et al.*, 2001; Deutsch *et al.*, 2004). It has also been shown that epinephrine and norepinephrine are capable of reactivating KSHV from latency through activation of protein kinase A signaling (Chang *et al.*, 2005c). MEK/ERK, JNK, and p38 MAP kinase pathways have also been suggested to be involved in the latent-lytic switch (Cohen *et al.*, 2006; Ford *et al.*, 2006; Pan *et al.*, 2006). These results have been further confirmed, and in addition all three MAPK pathways are demonstrated to mediate KSHV reactivation through AP-1, which associates with the RTA promoter, leading to expression of RTA protein, and to activation of the lytic program (Xie *et al.*, 2008). Moreover, AP-1, the cellular complex of c-Jun and c-Fos, mediates the transcription of MAPK target genes (Wang *et al.*, 2004b). Both the RTA promoter and the origin of lytic replication have AP-1 binding sites (Wang *et al.*, 2004b), which allow the virus to reactivate promptly if cellular conditions are unfavorable for the latency. Intriguingly, KSHV utilizes dopamine receptors with the associated MAPK pathways to detect and transmit stress signals for reactivation (Lee *et al.*, 2008). By using a gain-of-function kinome screen to identify novel kinases inducing viral reactivation, a recent study has revealed that Ser/Thr kinase Pim-1 induces KSHV reactivation (Varjosalo *et al.*, 2008). This finding was further extended to Pim-3, and phosphorylation of LANA by these kinases was established as a mechanism that disables LANA-dependent repression of viral transcription (Cheng *et al.*, 2009). Recently, inhibition of the phosphatidylinositol 3-kinase-(PI3K)-Akt pathway has been reported to enhance KSHV lytic replication and facilitate reactivation from latency (Peng *et al.*, 2010).

The primary target of the Notch signaling pathway RBP-Jk mediates RTA-dependent activation of KSHV lytic genes (Liang *et al.*, 2002). Interestingly, high expression of intracellular-activated Notch1 reactivates KSHV from latency in PEL cells (Lan *et al.*, 2006). The CCAAT/enhancer-binding protein-α (C/EBPα) was the first discovered member of the leucine zipper family of transcription factors, which comprises c-Jun, c-Fos, ATF, and CREB (Landschulz *et al.*, 1988). Notably, C/EBPα is expressed at the early stages of KSHV reactivation (Wang *et al.*, 2003a), and its overexpression has been confirmed to activate the RTA promoter in reporter gene assays (Wang *et al.*, 2003b). Another basic leucine zipper transcription factor, the X-box binding protein 1, has also been identified as inducing lytic replication in PEL cells through transactivation of the RTA promoter (Wilson *et al.*, 2007; Yu *et al.*, 2007). Moreover, X-box binding protein 1 has recently been shown to cooperate with hypoxia-inducible factor 1α to induce RTA expression, leading to the production of infectious viral particles under hypoxia (Dalton-Griffin *et al.*, 2009).

The role of the NF-κB signaling pathway in the KSHV lytic replication program is rather controversial. NF-κB is constitutively active in PEL cells and promotes their survival (Keller *et al.*, 2000). One report by Brown and coworkers (2003) has shown that inhibition of NF-κB results in elevated lytic protein synthesis in both PEL cells and *de novo* KSHV-infected epithelial cells. In contrast, Sgarbanti and coworkers (Sgarbanti *et al.*, 2004) have demonstrated that induction of NF-κB signaling is required for the production of new infectious virions from TPA-induced PEL cells. The initial host defense against viral pathogens is based on the innate immune system. This includes a variety of cellular elements and humoral factors in which the type I IFN (IFN-α/β) plays a fundamental role (Muller *et al.*, 1994). Wang and coworkers (2005a) have demonstrated that the cellular IRF-7 antagonizes KSHV lytic replication by competing with RTA for binding to the RTA-responsive element in the ORF57 promoter. Subsequently, RTA, which possesses E3-ubiquitin ligase activity, targets IRF-7 for proteasome-mediated degradation (Yu *et al.*, 2005). Inflammatory cytokines including IFN-γ and oncostatin M have also been demonstrated to induce viral lytic replication (Monini *et al.*, 1999; Blackbourn *et al.*, 2000; Chang *et al.*, 2000; Mercader *et al.*, 2000), the detailed mechanism remains unresolved, however.

3.4 Key aspects of lytic replication for KSHV pathogenesis

Although latency plays an important role in persistent viral infection, lytic replication has also been suggested as a key event in the development and progression of KSHV-induced malignancies. Indeed, KS expansion from the asymptomatic to the fully established disease has been linked with increased KSHV viral loads in peripheral blood mononuclear cells (Moore *et al.*, 1996b). Moreover, high viral loads are detectable upon appearance of new KS lesions and are associated with disease severity (Whitby *et al.*, 1995; Brambilla *et al.*, 1996; Campbell *et al.*, 2000; Pellet *et al.*, 2002; Quinlivan *et al.*, 2002; Campbell *et al.*, 2003; Engels *et al.*, 2003; Song *et al.*, 2004; Guttman-Yassky *et al.*, 2007; Laney *et al.*, 2007; Mancuso *et al.*, 2008). Furthermore, treatment of patients dually infected with KSHV and HIV-1 with GCV, which is active against KSHV lytic replication, results in a decrease in AIDS-related KS incidence (Martin *et al.*, 1999).

Infected cells are predominantly latent; however, a minority of tumor cells undergo spontaneous lytic reactivation (Zhong *et al.*, 1996; Staskus *et al.*, 1997; Reed *et al.*, 1998; Staskus *et al.*, 1999). Importantly, one *in vitro* study has suggested that a small amount of lytic KSHV infection is essential for the induction and maintenance of KS tumors (Grundhoff and Ganem, 2004). Several lines of evidence have illustrated how lytic replication is associated with expression of a number of viral-encoded cellular homologs with identifiable cytokine and cytokine-receptor signal transduction properties. These molecules may possibly mediate autocrine and paracrine signaling during tumor development. One of these homologs, termed vGPCR increases endothelial cell proliferation and promotes survival by activating MAPK, PI3-kinase, and p38 MAP pathways (Sodhi *et al.*, 2000; Smit *et al.*, 2002), as well as by stimulating NF-κB signaling (Schwarz and Murphy, 2001). Therefore, vGPCR acts as an important factor in promoting endothelial cell growth and transformation (Arvanitakis *et al.*, 1997; Bais *et al.*, 1998; Couty *et al.*, 2001; Pati *et al.*, 2001; Grisotto *et al.*, 2006). Interestingly, ectopic expression of vGPCR in endothelial cells leads to activation of VEGF receptors and to cell immortalization. These cells actively proliferate and form foci in culture as well as forming tumors in nude mice independently of VEGF stimulation (Bais *et al.*, 2003; Grisotto *et al.*, 2006). Furthermore, mice expressing the vGPCR transgene develop highly angioproliferative tumors resembling KS lesions (Yang *et al.*, 2000; Jensen *et al.*, 2005). vGPCR also directly enhances expression of a variety of other cytokines and growth factors

including IL-4 and GM-CSF (Pati *et al.*, 2003), IL-6 and TNF-α (Schwarz and Murphy, 2001), Gro-α (Montaner *et al.*, 2004), and CCL-2 (Choi and Nicholas). These results suggest that vGPCR contributes to tumorigenesis through autocrine and paracrine mechanisms, which are essential for tumor cell proliferation, neoangiogenesis, and inflammation.

The KSHV-encoded homolog of IL-6 also promotes cell survival and proliferation (Nicholas *et al.*, 1997; Aoki *et al.*, 1999; Hideshima *et al.*, 2000). Moreover, vIL-6 stimulates production of VEGF and cellular IL-6, thus playing an important role in neoangiogenesis and hematopoiesis (Mori *et al.*, 2000; Liu *et al.*, 2001). Interestingly, it modulates the host's immune response and protects PEL cells from IFN-α-induced antiviral defence (Chatterjee *et al.*, 2002). Finally, it may also be the case that expression of viral lytic homologs of human anti-apoptotic proteins plays an important role in the development of KSHV-induced malignancies. Being a homolog of the human anti-apoptotic protein Bcl-2, vBcl-2 inhibits apoptosis (Cheng *et al.*, 1997; Matta and Chaudhary, 2004). Moreover, it occurs at high levels in both monocytes and spindle cells in KS lesions (Sarid *et al.*, 1997; Widmer *et al.*, 2002). Additionally, the *K7* gene encodes a viral lytic inhibitor of apoptosis (vIAP), a homolog of cellular protein survivin-DeltaEx3 (Wang *et al.*, 2002), which prevents apoptosis by bridging the mitochondrial protein Bcl-2 and caspase-3 to suppress caspase-3 function (Wang *et al.*, 2002). In addition, it promotes cytosolic Ca^{2+} entry and protects against mitochondrial damage (Feng *et al.*, 2002). Taken together, control of the latent-to-lytic switch in KSHV-infected cells, and the expression of lytic gene products may both play fundamental roles in the pathogenesis of KSHV-induced diseases.

AIMS OF THE STUDY

Unlike in many other human cancers, in KSHV-induced lymphomas, *TP53* gene mutations are rare, and the majority of these tumors have retained wt p53. This study was undertaken to elucidate the functional consequences and mechanisms of p53 restoration by the small-molecule inhibitor of MDM2 in KSHV lymphomas (PELs) *in vitro, ex vivo,* and *in vivo*. Subsequently, an intriguing and unexpected finding of compromised apoptotic response in some of the mice redirected our attention toward discovery of the mechanism behind this phenomenon. As a second aim, we sought to identify novel *in vivo* interaction partners for the KSHV latent gene product v-cyclin, and to reveal biological consequences of such an interactions.

MATERIALS AND METHODS

The materials and methods of this study are listed below, with detailed descriptions in the original publications, here referred to by Roman numerals.

Table 2. Cell lines used in this study

Cell line	Description	Source or reference	Used in
BC-1	Human primary effusion lymphoma cell line (PEL); $KSHV^+/EBV^+$; wt p53	ATCC	I, III, IV
BC-3	Human primary effusion lymphoma cell line (PEL); $KSHV^+/EBV^-$; wt p53	Dr. Ethel Cesarman	I, II, III, IV
BC-3/NF-kB luc	Human primary effusion lymphoma cell line (PEL) carrying an NF-κB-regulated luciferase reporter gene; $KSHV^+/EBV^-$; wt p53	Dr. Ethel Cesarman	II
BCBL-1	Human primary effusion lymphoma cell line heterozygous for the M246I mutation in one of the copies of the *TP53* gene (PEL); $KSHV^+/EBV^-$	Dr. Ethel Cesarman	I, III, IV
CZE	Lymphoblastoid cell line (LCL); $KSHV^-/EBV^+$; wt p53	This study	I, II
DG-75	Burkitt lymphoma cell line; $KSHV^-/EBV^-$; mutant p53	ATCC	I, II
EA.hy926	Endothelial cell line derived from fusing human umbilical vein endothelial cells with the human lung carcinoma cell line A594; $KSHV^-/EBV^-$; wt p53	Prof. Kari Alitalo (Edgell *et al.*, 1983)	I, IV
HL-60	Human promyelocytic leukemia cell line; $KSHV^-/EBV^-$; p53 null	ATCC	I
IHE	Lymphoblastoid cell line (LCL); $KSHV^-/EBV^+$; wt p53	This study	I, II, IV
IHH	Lymphoblastoid cell line (LCL); $KSHV^+/EBV^-$; wt p53	This study	I, IV
JOK-1	Human hairy-cell leukemia cell line; $KSHV^-/EBV^-$; mutant p53	Dr. Leif Andersson	III
JSC-1	Human primary effusion lymphoma cell line (PEL); $KSHV^+/EBV^+$; wt p53	ATCC	II, III, IV
SLK	Human endothelial cell line originally established from a mucosal Kaposi's sarcoma lesion of an HIV-negative kidney transplant patient; $KSHV^-/EBV^-$; mutant p53 (D. Dittmer, personal communication)	Prof. T. Schulz (Herndier *et al.*, 1994)	IV
U2OS	Human osteosarcoma cell line; $KSHV^-/EBV^-$; wt p53	ATCC	I, II, III, IV

Table 3. Mouse lines used in this study

Mouse line	Description	Source or reference	Used in
BALB/c Nude Mouse	Immunodeficient athymic nude mice	Taconic	I
SCID	Severe combined immunodeficient mice lacking both T and B lymphocytes	Taconic	II
NOD/SCID	Non-obese diabetic SCID mice	Taconic	II

Table 4. Expression vectors used in this study

Vector	Description	Source or reference	Used in
Lentiviral vectors			
pLKO.1-shNPM	targets human NPM	Open Biosystem	IV
pDSL_hpUGIH shCDK4	targets human CDK4	(Koopal *et al.*, 2007)	IV
pDSL_hpUGIH shCDK6	targets human CDK6	(Koopal *et al.*, 2007)	IV
pLenti6/V5-DEST-GFP	Encodes GFP	Dr. Juergen Haas	II, IV
pLenti6/V5-DEST-LANA	Encodes LANA	Dr. Juergen Haas	II, IV
Retroviral vectors			
v-FLIP-pBMN	Encodes v-FLIP	Dr. Johanna Furuhjelm	IV
v-cyclin-pBMN (KpBMN)	Encodes v-cyclin	Dr. Emmy Verschuren	IV
pSUPER.retro sh-v-cyclin	Targets v-cyclin	Dr. Rene Medema (Koopal *et al.*, 2007)	IV
Mammalian expression vectors			
Myc-v-cyclin/ pcDNA3	Encodes myc-tagged v-cyclin	Dr. Sibylle Mittnacht	III
Myc-p27/ pcDNA3	Encodes myc-tagged human p27KIP1	Dr. Sibylle Mittnacht	III
Myc-p27S10A/ pcDNA3	Encodes myc-tagged human p27KIP1 with a Ser-to-Ala point mutation S10A	Dr. Sibylle Mittnacht	III
pEGFP-C1-B23.1	Encodes EGFP-tagged human NPM	Dr. Kyosuke Nagata	IV
pEGFP-C1-B23.1-T214/234/237A	Encodes EGFP-tagged human NPM with Thr-to-Ala point mutations of codons 214, 234, and 233 (T3A)	Dr. Kyosuke Nagata	IV
pEGFP-C1-B23.1-T199/214/234/237A	Encodes EGFP-tagged human NPM with Thr-to-Ala point mutations of codons 199, 214, 234, and 233 (T4A)	Dr. Kyosuke Nagata	IV

Table 5. Primary antibodies used in this study

Antibody	Description	Source or reference	Used in
53BP1	Rabbit polyclonal antibody against 53BP1	Novus Biologicals	I
Acetyl-lysine (06-933)	Rabbit polyclonal antibody against acetyl-lysine	Upstate Biotechnology	IV
Actin (C-2)	Mouse monoclonal antibody against actin	Santa Cruz Biotechnology	I, II, III, IV
Active caspase-3	Rabbit polyclonal antibody against cleaved caspase-3	Cell Signaling Technology	II
Bax (B-9)	Mouse monoclonal antibody against Bax	Santa Cruz Biotechnology	I
BrdU (Bu20a)	Mouse monoclonal antibody against BrdU	DakoCytomation	I
CDK2 (M2)	Rabbit polyclonal antibody against CDK2	Santa Cruz Biotechnology	III
CDK4 (DCS-35)	Mouse monoclonal antibody against CDK4	NeoMarkers	III
CDK4 (H-22)	Rabbit polyclonal antibody against CDK4	Santa Cruz Biotechnology	IV
CDK6 (Ab-3)	Mouse monoclonal antibody against CDK6	NeoMarkers	III
CDK6 (C-21)	Rabbit polyclonal antibody against CDK6	Santa Cruz Biotechnology	III, IV
Chk2 (DCS-270)	Mouse monoclonal antibody against Chk2	Cell Signaling Technology	I
Cyclin A (C-19)	Rabbit polyclonal antibody against cyclin A	Santa Cruz Biotechnology	IV
Fibrillarin (ab5821)	Rabbit polyclonal antibody against fibrillarin	Abcam	IV
GFP (TP401)	Rabbit polyclonal antibody against GFP	Torrey Pines Biolabs	IV
HDAC1 (06-720)	Rabbit polyclonal antibody against HDAC1	Upstate Biotechnology	IV
K8.1 A/B	Mouse monoclonal antibody against K8.1	ABI Biotechnologies	IV
LANA	Rabbit polyclonal antibody against LANA	(Sharma-Walia *et al.*, 2010)	IV
LANA (HHV8-ORF73)	Rat monoclonal antibody against LANA	ABI Biotechnologies	I, II, IV
MDM2 (2A10)	Mouse monoclonal antibody against MDM2	Santa Cruz Biotechnology	I, II
MDM2 (IF-2)	Mouse monoclonal antibody against MDM2	Oncogene Sciences	I, II
MDM2 (SMP14)	Mouse monoclonal antibody against MDM2	Santa Cruz Biotechnology	I, II
Myc (9E10)	Mouse monoclonal antibody against Myc-epitope	Babco	III, IV
NPM (32-5200)	Mouse monoclonal antibody against NPM	Zymed	IV
ORF59	Mouse monoclonal antibody against ORF59	(Chan *et al.*, 1998)	II, III, IV
p21 (C-19G)	Rabbit polyclonal antibody against p21CIP1	Santa Cruz Biotechnology	II

p21 (SX118)	Mouse monoclonal antibody against p21CIP1	BD Biosciences-Pharmingen	I
p27 KIP1	Mouse monoclonal antibody against p27KIP1	Upstate Biotechnology	III
p27KIP1 (C-19)	Rabbit polyclonal antibody against p27KIP1	Santa Cruz Biotechnology	III
p300 (C-20)	Rabbit polyclonal antibody against p300	Santa Cruz Biotechnology	IV
p53 (DO-1)	Mouse monoclonal antibody against p53	Santa Cruz Biotechnology	I, II
p53 (FL-393)	Rabbit polyclonal antibody against p53	Santa Cruz Biotechnology	I, II
p-Chk2 (Thr68)	Rabbit polyclonal antibody against phosphorylated Chk2 (Thr68)	Cell Signaling Technology	I
p-H2AX (Ser139) (JBW301)	Mouse monoclonal antibody against the phosphorylated form of histone H2AX (Ser139)	Upstate Biotechnology	I
Pim-1 (12H8)	Mouse monoclonal antibody against Pim-1	Santa Cruz Biotechnology	IV
p-NPM (Thr199)	Rabbit polyclonal antibody against phosphorylated NPM (Thr199)	Cell Signaling Technology	IV
p-p27 (Ser10)	Rabbit polyclonal antibody against phosphorylated p27KIP1 (Ser10)	Santa Cruz Biotechnology	III
p-p27 (Thr187)	Rabbit polyclonal antibody against phosphorylated p27KIP1 (Thr187)	Zymed	III
p-p53 (Ser15)	Rabbit polyclonal antibody against phosphorylated p53 (Ser15)	Cell Signaling Technology	I
Sp1 (PEP-2)	Rabbit polyclonal antibody against Sp1	Santa Cruz Biotechnology	III
Tubulin (5H1)	Mouse monoclonal antibody against tubulin	BD Biosciences-Pharmingen	II, IV
v-cyclin	Rabbit polyclonal antibody against v-cyclin	This study	III, IV
vIL-6	Rabbit polyclonal antibody against vIL-6	ABI Biotechnologies	II, IV
vIRF-3 (CM-A807)	Mouse monoclonal antibody against vIRF-3	Novus Biologicals	IV
β-tubulin (D-10)	Mouse monoclonal antibody against β-tubulin	Santa Cruz Biotechnology	III

Table 6. Methods used in this study

Methods	Used in
Bioluminescence imaging	II
BrdU incorporation assay	I
Cytocentrifugation	I, II, III, IV
Flow cytometry	I, IV
Gel filtration chromatography	I, III, IV
Gene knockdown by RNA interference	IV
Immunofluorescence microscopy	I, II, III, IV
Immunohistochemistry	IV
Immunoprecipitation	I, III, IV
Implantation of tumor cells into mice	I, II
In vitro kinase assay	III, IV
Lenti- and retroviral production	IV
Mammalian cell culture	I, II, III, IV
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Cell culture, virus reactivation and drug treatments

PEL cell lines, KSHV-negative LCL cells, JOK-1, DG-75, and HL-60 were cultured in a humidified 5% CO_2 atmosphere at 37°C in RPMI 1640 medium supplemented with 15% FCS (Invitrogen), 100 U/ml penicillin G, and 100 μg/ml streptomycin. U2OS human osteosarcoma, EA.hy926, and SLK endothelial cells were grown in DMEM supplemented with 10% FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. To induce KSHV lytic replication, cells were treated with 20 ng/ml TPA (Sigma) or 1 mM NaB (Sigma). Inhibition of lytic gene expression was carried out with 5 μM of ganciclovir (Sigma). Inhibition of proteasome activity was performed with MG132. Treatment of PEL cells with MG132 in hypoxia or normoxia was first titrated to non-toxic doses of 1 μM (data not shown). Hypoxia studies were performed by incubating cells in a 37°C incubator containing 1% O_2 and 5% CO_2 (INVIVO 400 Workstation; Ruskinn Technology Ltd, UK). The oxygen level was regulated by infusing nitrogen into a chamber controlled by a Gas Mixer Q controller (Ruskinn Technology Ltd).

PEL cells, EBV-transformed LCL cells, and KSHV-infected LCL cells, suspended at 2×10^5 cells/ml, were incubated with 7 μM Nutlin-3 (Cayman) or a comparable amount of the solvent (vehicle; 0.1% DMSO) for the indicated time. rKSHV-infected and non-infected parental U2OS and EA.hy 926 cells or U2OS cells stably expressing GFP or LANA were plated at a density of 0.5×10^5 cells per well in 24-well plates, and after 48 hours the cells were treated with 7 μM Nutlin-3. Cell viability was determined by trypan blue exclusion (Sigma-Aldrich). The control treatment with DMSO was always included, and the relative survival in each assay was calculated as the percentage of live cells relative to the live cell population in the control. To inhibit the ATM-Chk2 pathway in the KSHV-infected PEL cell line BC-1, KSHV-infected LCL IHH, or EBV-transformed LCL IHE, the cells were pretreated with 2 mM caffeine (Sigma-Aldrich) for 24 hours before the Nutlin-3 treatment. Caffeine was kept constant during exposure to Nutlin-3.

Production of anti-v-cyclin antibody

In order to produce recombinant v-cyclin fusion protein (GST-v-cyclin), the pCool vector (a modified version of pGEX-2T from Amersham Pharmacia Biotech) was expressed in *Escherichia coli* (Kaldis *et al.*, 2001). The fusion protein was then bound to glutathione 4B sepharose beads. The beads were washed twice with cold phosphate-buffered saline (PBS) to remove protease inhibitors, followed by incubation with

thrombin to remove the GST (1 mM $CaCl_2$; 50 mM Tris, pH 8.0; 5 U/ml thrombin; Sigma, Steinheim, Germany) for 18 hours at 14°C. Rabbit polyclonal immunoglobulin G (IgG) antibodies directed against the thrombin-cleaved recombinant v-cyclin were produced by BIOTREND Chemikalien (Köln, Germany).

Gel filtration chromatography

Cell lysates prepared in ELB (150 mM NaCl; 50 mM HEPES, pH 7.4; 0.1% Igepal; 5 mM EDTA; 2 mM DTT) or NET (50 mM Tris-HCl, pH 7.5; 0.2% Ipegal; 1 mM EDTA; 150 mM NaCl) lysis buffers in the presence of phosphatase inhibitor cocktail tablets (PhosphoSTOP, Roche) and protease inhibitors (Complete tablets, Roche) were passed through a 0.22-μm pore size MILLEX-GS filter (Millipore) and fractionated on a Superdex 200 HR column by a fast protein liquid chromatography (FPLC) system (Pharmacia Biotech). Samples were loaded onto the column and separated in a gel filtration buffer (50 mM HEPES, pH 7.5; 150 mM NaCl) at a flow rate of 0.3 ml/min. The molecular mass standards (Sigma) used to calibrate the column were blue dextran (2000 kDa), thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (BSA; 66 kDa), and carbonic anhydrase (29 kDa). For each fractionation, 30 fractions of 0.5 ml were collected. Fifty microliters of each fraction served for immunoblotting and 450 μl of each fraction for immunoprecipitation experiments.

***In vitro* kinase assay**

Whole-cell extracts from PEL cells or fractions from gel filtration chromatography were incubated for 2 hours at 4°C with the rabbit polyclonal anti-v-cyclin antibody. Immunocomplexes were coupled to protein A sepharose beads for an additional 2 hours at 4°C and washed three times with the lysis buffer followed by two washes with the kinase buffer (20 mM Tris, pH 7.5; 50 mM KCl; 7.5 mM $MgCl_2$; 10 mM $MnCl_2$ 1 mM DTT; 25 mM β-glycerophosphate; 2 μg/ml leupeptin; 2 μg/ml pepstatin; and 1.5 μg/ml aprotinin). Kinase reactions were performed by incubating the immunocomplexes with 10 μl kinase buffer containing 2 μCi (0.074 MBq) [^{32}P] adenosine triphosphate (ATP) for 20 minutes at 30°C. The *in vitro* kinase activity was measured toward v-cyclin coimmunoprecipitated proteins or Histone H1 (Roche Diagnostics), GST-pRb, or GST-p27KIP1. To this end, 2 μg Histone H1 and GST-pRb or 0.5 μg GST-p27KIP1 were added to the kinase reactions as

substrates. Reactions were terminated by the addition of 5 × SDS-PAGE sample buffer, were resolved in SDS-PAGE, and analyzed by autoradiography. The extent of [^{32}P] incorporation into the substrates was quantified from the autoradiographs by the Typhoon 9400 Image Quant analyzer (Amersham Biosciences).

Establishment of EBV-transformed and KSHV-infected LCL cells

PBMCs were isolated from EDTA-treated blood of two healthy individuals by discontinuous gradient centrifugation (Lymphoflot; Biotest). To induce the expression of lytic viral proteins in EBV-positive B95-8 or KSHV-positive BCBL-1 cells, cells were treated with either TPA (Sigma-Aldrich) or 3 mM NaB (Sigma-Aldrich) for 24 hours. Supernatants of the B95-8 or BCBL-1 cells grown at densities greater than 5×10^5/ml were filtered through 0.4-μm filters and serially diluted in flat-bottomed 96-well microtiter plates. Subsequently, PBMCs were added at a density of 10^4 cells per well. The culture medium used to generate LCL cells contained RPMI, 20% heat-inactivated FCS, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate (Invitrogen), 20 mM bathocuproine disulfonic acid, and 50 μm/ml α-thioglycerol (Sigma-Aldrich).

rKSHV.219 production and infection of target cells

Vero cells latently infected with a GFP-expressing rKSHV (rKSHV.219), were used to produce infectious virus as described (Vieira and O'Hearn, 2004). In brief, 80% to 90% confluent rKSHV.219 Vero cells in six-well plates were reactivated by adding 400 μl/well of a recombinant baculovirus (BacK50) expressing the KSHV lytic activator ORF 50 (RTA; a gift from J. Vieira) for 2 hours, removed, and replaced with complete media (DMEM:RPMI; 40:60) containing 1.25 mM sodium butyrate (Sigma-Aldrich). Supernatants containing rKSHV.219 were collected 72 hours later. U2OS, EA.hy926, and SLK cells were plated at a density of 2×10^5 cells per well in a six-well dish and were infected the next day with 1.0 ml/well of rKSHV.219 virus supernatant in the presence of 8 μg/ml polybrene to enhance their infectivity. The plates were spin-transduced by centrifugation at 1,050 *g* (Heraeus Multifuge) for 30 minutes at RT. Cells were returned to 37°C at 5% CO_2 for 2 hours, after which the rKSHV.219 supernatant was replaced with complete DMEM media. Cells were routinely cultured in a humidified 5% CO_2 atmosphere at 37°C in DMEM containing 10% (w/v) FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin in the presence of 1 μg/ml puromycin, which was included 2 days after infection. Infected cells were

grown in the presence of puromycin for about one week to obtain a 100% infected stable cell line.

Lenti- and retroviral production and transduction

Production of retro- and lentiviruses and CDK silencing in EA.hy926 and SLK cells were performed as described (Koopal *et al.*, 2007). Briefly, lentiviral supernatants were generated by transfection of Invitrogen ViraPower™ viral packaging plasmids pLP1, pLP2, and pLP/VSVG, together with the lentiviral expression plasmid into 293FT cells (ATCC) by use of the Lipofectamine™ 2000 reagent (Invitrogen). Transfections were performed with 4 x 10^6 293FT cells per 10-cm cell culture dish (Reverse transfection procedure, Invitrogen). Packaging plasmids (pLP1 : pLP2 : pLP-VSVG) were used in the ratio of 1 : 0.47 : 0.65 (2.83μg : 1.33μg : 1.84μg). Amphotropic retroviruses were produced by transfection of Phoenix-Ampho retrovirus–producing cells with retroviral vectors by use of Lipofectamine 2000 reagent. Viral supernatants were harvested 72 hours after transfection through sterile 0.22-μm-pore-size filters (Millipore), aliquoted, and stored at -80°C until further use.

To silence the CDKs and NPM, the PEL cells were seeded at density 5 × 10^5/ml and transduced in a 50-ml culture flask with 2 ml lentiviral supernatants in the presence of 8 μg/ml polybrene (Sigma); 24 hours after transduction, the culture was replenished with fresh media, and cells were kept for 48 hours, after which they were subjected to selection with 3.5 μg/ml puromycin (silencing of NPM) or 300 μg/ml hygromycin (silencing of CDKs).

For an acute depletion of v-cyclin expression in the rKSHV.219-SLK cells, the cells were spin-transduced (2500 rpm; Heraeus Multifuge) for 30 min at RT with fresh amphotropic retroviruses expressing control sh-RNA (sh-Scr) or sh-RNA against v-cyclin (sh-v-cyclin) in the presence of 8 μg/ml polybrene. Cells were then returned to 37°C in 5% CO_2, and after 24 hours of incubation, the viral supernatant was removed and replaced with fresh complete media. Cells were harvested for analysis 48 hours post-transduction.

U2OS osteosarcoma cells were spin-transduced as described above with GFP- or LANA-expressing lentiviruses in the presence of 8 μg/ml polybrene, and the cells were incubated for 48 hours. Thereafter, LANA and GFP-expressing cells were cultured in the presence of 5 μg/ml of blasticidin (Sigma) for at least two weeks. Stable expression

of the transduced proteins was assessed by immunofluorescence and Western blotting using anti-GFP or anti-LANA antibodies.

Measurement of cell proliferation and detection of apoptosis

The proportion of cells at the S phase was determined by measuring incorporation of BrdU and PI into the DNA. Cells were grown at a density of 2×10^5 cells/ml 24 hours prior to the treatment with Nutlin-3. The cells were pulse-labeled with 25 μM BrdU (Sigma-Aldrich) for 30 minutes and fixed in ice-cold 70% ethanol. After fixation, the cells were washed in PBS and treated with 3.5 N HCl for 30 minutes at room temperature. After being washed in a neutralizing washing buffer (0.1% BSA/PBS), the cells were incubated with an anti-BrdU antibody (Dako; 1:50) for 45 minutes. Alexa Fluor 488-conjugated (Invitrogen) secondary antibody served for detection. Finally, the cells were stained with 30 μg/ml PI (Invitrogen) in PBS supplemented with 50 μg/ml RNase (Sigma-Aldrich) for 30 minutes at 37°C. Apoptosis was measured by dual-labeling with the Annexin V-FITC Apoptosis Detection kit I (Pharmingen) according to manufacturer's instructions and analyzed by flow cytometry. Labeled cells were acquired by a BD-LSR Flow Cytometer (BD Biosciences), and the cell populations were analyzed by CellQuest software (BD Biosciences).

Apoptotic cells were detected by an *in situ* cell death detection kit (TMR red; Roche Applied Science), according to manufacturer's instructions. This assay measures DNA fragmentation by immunofluorescence using TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) method at the single-cell level. Per sample, 500 cells were counted to quantitate the percentage of apoptotic PEL cells.

***In vivo* studies**

Female Balb/c nude mice (4 to 6 weeks old, Taconic) were maintained under specific pathogen-free conditions in a temperature- and humidity-controlled environment. Mice under anesthesia were injected subcutaneously with 6×10^6 BC-3 cells in 50% Matrigel (BD Biosciences). Treatment was started intraperitoneally after the tumors were established (*i.e.*, palpable). Nutlin-3 (20 mg/kg) or the vehicle control was administered every second day for a total of 2 weeks (7 doses). Tumor volumes were measured with a caliper and calculated according to the formula V = width × height × depth/2, derived from the formula for the volume of an ellipsoid. In order to monitor the health of the animals, the mice were weighed once a week.

Intraperitoneal PEL tumors using BC-3/NF-κB-luc cells in female SCID and NOD/SCID mice (Taconic Europe) were established as previously described (Keller *et al.*, 2006). To monitor tumor growth, D-luciferin (SYNCHEM OHG, Germany) in PBS (100 mg/kg) was injected i.p., and mice were imaged by the IVIS Imaging system (Xenogen). Data were analyzed with The Living Image software (Xenogen). For quantitative analysis, a constant region-of-interest (ROI) was drawn over the tumor region, and the intensity of the signal was measured as total photon/s/cm^2/steradian (p/s/cm^2/sr). Every second day, 20 to 40 mg/kg Nutlin-3 (Cayman Chemical, MI) or the vehicle control (DMSO) were administered intraperitoneally.

DNA isolation and quantitative genome copy-number analysis

Prior to DNA extraction, rKSHV-EA.hy926 and -SLK cells were washed with PBS and stored at −70°C as a dry pellet of 2×10^6 cells. DNA was extracted according to standard protocol by proteinase K digestion and isopropanol precipitation. Briefly, the cell pellet was resuspended in 300 µl of lysis buffer (200 mM NaCl; 40 mM Tris-HCl, pH 8.0; 20 mM EDTA; 0.5% sodium dodecyl sulphate; 0.5% β-mercaptoethanol; 1 mg of proteinase K per ml) and incubated 10 min at RT. The cell lysate was subjected to DNA extraction with isopropanol, and DNA was precipitated by centrifugation (13,300 g for 15 min). DNA was then washed twice with 70% ethanol, dried, and resuspended in 100 µl of TE buffer. Quantitative real-time PCR with LANA primers to measure the copies of KSHV genomes was performed as detailed previously (Lallemand *et al.*, 2000; Cai *et al.*, 2005).

RESULTS AND DISCUSSION

1. Restoration of p53 function by MDM2 inhibition as a novel therapeutic modality against PEL cells (I)

1.1 Inhibition of MDM2 leads to cell cycle arrest and apoptosis in PEL cells

There exists currently no optimal therapy for PEL, and new therapeutic approaches for PEL with potential clinical applicability are urgently needed. Alterations in the *p53* gene (*TP53*) in KSHV-associated malignancies are very rare (Nador *et al.*, 1996; Carbone *et al.*, 1998; Katano *et al.*, 2001b; Chadburn *et al.*, 2004). Since the p53 pathway in PEL cells is functional in response to a common cytotoxic drug, doxorubicin (Petre *et al.*, 2007), we investigated the outcome of non-genotoxic activation of the p53 pathway by the small-molecule inhibitor Nutlin-3 (I).

First, we performed experiments using a panel of patient-derived, naturally KSHV-infected PEL cell lines: BC-1, BC-3, and BCBL-1. Asynchronously growing PEL cells were treated with 7 μM Nutlin-3 for 12 hours. Whole-cell lysates were subjected to SDS-PAGE, followed by immunoblotting with anti-p53 antibody. The analysis revealed rapid accumulation of p53 in BC-1 and BC-3 cells (Fig. 1 in I). However, the BCBL-1 cell line, shown to be heterozygous for the M246I mutation in one of the copies of the *TP53* gene (Katano *et al.*, 2001b; Petre *et al.*, 2007), displayed a delayed induction of p53 (Fig. 1 in I). Subsequently, the samples were analyzed for the induction of p53 targets that mediate cell cycle arrest and apoptosis. The immunoblotting analysis, using antibodies against p21CIP1 and Bax, revealed a robust induction of both targets in BC-1 and BC-3 cell lines (Fig. 1 in I).

Secondly, we assessed the effect of Nutlin-3 on cell cycle progression. BC-1, BC-3, and BCBL-1 cells exposed to Nutlin-3 for 12, 24, or 48 hours were labeled with bromodeoxyuridine (BrdU) and propidium iodide (PI), and analyzed by flow cytometry (Fig. 2A in I). Typically, within 12 to 24 hours of Nutlin-3 treatment, we observed a strong G1 arrest and a dramatic decrease in the accumulation of all PEL cells in the S phase (Fig. 2A in I). Interestingly, prolonged exposure to Nutlin-3 resulted in a further increase in the cells at G1, and an increase in the number of cells in the sub-G1 phase, suggesting elevated levels of cell death (Fig. 2A in I). We thus aimed to determine whether the sub-G1 phase cells were apoptotic. To this end, we treated BC-1 cells with 7 μM Nutlin-3 for up 120 hours, whereas BC-3 and BCBL-1

cells were incubated with the same Nutlin-3 dose for 96 hours. Subsequently, cells were stained with Annexin V/PI and subjected to flow cytometric analysis. We found a massive apoptosis in all PEL cell lines tested (Fig. 3B-C in I). Interestingly, the pronounced apoptosis observed in BC-1 cells between 48 and 72 hrs (Fig. 3B in I) overlaps the time course of Bax expression upon Nutlin-3 treatment in this cell line, as we have shown in a later report (Sarek and Ojala, 2007), supporting the importance of Bax in p53-mediated apoptosis in PEL cells. Following the Nutlin-3 treatment, at all time-points analyzed (6 to 72 hours), we further revealed high expression of p21CIP1 in BC-1 cells. Collectively, this shows that PEL cells have a functional p53, and that restoration of its activity by MDM2 inhibition results in massive apoptosis.

It has been suggested that the cell cycle arrest mediated by induction of p21CIP1 may be an early event in the cascade by which activation of p53 leads to apoptosis (Meikrantz and Schlegel, 1995; Kang *et al.*, 1999; Poole *et al.*, 2004). On the other hand, high expression of p21CIP1 may protect cells against apoptosis (Suzuki *et al.*, 1998; Asada *et al.*, 1999), and act as a critical determinant for the non-apoptotic cellular response to Nutlin-3 observed in colon cancers (Donner *et al.*, 2010). An interesting recent study by Enge and coworkers (2009) has revealed that the p53-reactivating compound RITA releases MDM2 from p53, and this promotes degradation of both p21CIP1 and the transcriptional cofactor hnRNP K required for p53-mediated induction of the *CDKN1* gene. As a consequence, that p21CIP1 was unable to execute its growth-suppressive function favored p53-dependent apoptosis in the HCT-116 colon carcinoma cell line (Enge *et al.*, 2009). In contrast, treatment with Nutlin-3 induced binding of hnRNP K to the upstream site of the *CDKN1A* promoter, leading instead of to apoptosis, to p21CIP1 induction and cell-cycle arrest (Enge *et al.*, 2009). Therefore, regulation of p21CIP1 expression may represent a major switch between growth arrest and apoptotic response to p53 restoration. We have however, observed a massive apoptotic response to Nutlin-3 in the PEL cells (I), despite high p21CIP1 induction (Sarek and Ojala, 2007). If p21CIP1 represents the major determinant of the non-apoptotic response, how can this apparent discrepancy be explained? As v-cyclin inhibits the cell cycle-arresting activity of p21CIP1 by phosphorylation on Ser130 (Swanton *et al.*, 1997; Jarviluoma *et al.*, 2006), it is possible that v-cyclin is at least partially involved in directing the response towards apoptosis. However, this theory requires further experimentation.

1.2 Restoration of the p53 pathway selectively kills KSHV-infected cells

To assess whether restoration of the p53 pathway by Nutlin-3 selectively targets KSHV-infected lymphoblastoid cells for apoptosis, we examined the response to Nutlin-3 treatment in two KSHV-negative, EBV-immortalized lymphoblastoid cell lines (LCL; CZE and IHE) which express wt p53 (I). CZE and IHE were treated with 7 μM Nutlin-3 for 12 hours and analyzed by immunoblotting using anti-p53 and anti-p21CIP1 antibodies. Similar to PEL cells (see 1.1 and Fig. 1 in I), the KSHV-negative LCL cells displayed a strong induction of p53 and its target p21CIP1 (Fig. 1 in I). Next, we examined whether KSHV-negative LCL cells undergo cell cycle arrest in response to Nutlin-3 treatment. CZE and IHE cells were exposed to Nutlin-3 for 24 and 48 hours, labeled with BrdU and PI, and analyzed by flow cytometry. The results revealed that LCL cells, similar to PEL cells (see 1.1 and Fig. 2A in I), can undergo an efficient G1 arrest in response to Nutlin-3 (Fig. 2B in I).

Next, we assessed the cytotoxicity of Nutlin-3 in three PEL cell lines (BC-1, BC-3, and BCBL-1), in the KSHV-infected lymphoblastoid cell line IHH, and in KSHV-negative LCL cells (CZE and IHE). To this end, all cells were treated with 7 μM Nutlin-3 for 5 days, and their viability was determined by trypan blue exclusion. We observed a 60% to 90% reduction in survival of all KSHV-infected cells after 5 days of p53 restoration (Fig. 3A in I). However, during the treatment period, viability of the KSHV-negative LCL cells declined only minimally (Fig. 3A in I). To confirm the lack of apoptotic response in the KSHV-negative LCL cells upon Nutlin-3 treatment, we performed AnnexinV/PI flow cytometric analysis, analogous to that described above for PEL cells (see 1.1). In contrast to PEL cells (see 1.1 and Fig. 3B in I), the KSHV-negative LCL cells did not undergo apoptosis (Fig. 3C in I). All together, these results suggest that restoration of p53 function selectively induces apoptosis in KSHV-infected lymphoblastoid cells, but not in the KSHV-negative LCL cells.

The notable increase in the p53-dependent apoptosis in both PEL cells and the KSHV-positive lymphoblastoid cell line IHH over the KSHV-negative LCL cells (I) suggests that KSHV infection may somehow enhance cytotoxic effects upon Nutlin-3. To further examine this possibility, we used U2OS osteosarcoma and EA.hy926 endothelial cells (Edgell *et al.*, 1983) that have been remarkably resistant to Nutlin-3-induced apoptosis (Tovar *et al.*, 2006; Shen *et al.*, 2008). We *de novo* infected these cell lines with recombinant KSHV (rKSHV.219; (Vieira and O'Hearn, 2004), and

subsequently treated them with 7 μM Nutlin-3 for 24, 48, and 96 hours (I). Parental non-infected U2OS and EA.hy926 served as a control, and the cytotoxic effect of Nutlin-3 was determined by trypan blue exclusion. Following 96 hours of Nutlin-3 treatment, we observed about a four-fold increase in cell death in KSHV-infected U2OS and EA.hy926 cells when compared to their non-infected parental cells (Fig. 4A in I). A similar observation was reported also in regards to human dermal microvascular endothelial cells (Koopal *et al.*, 2007). Collectively, these data confirmed that MDM2 inhibition induced cytotoxic activity selectively in KSHV-infected cells.

1.2.1 Active DNA-damage signaling in PEL cells augments cytotoxicity of Nutlin-3

The selective cytotoxicity of Nutlin-3 towards KSHV-infected cells prompted us to examine the underlying mechanisms. Stimulated by discovery of a recently described property of cancer cells to activate DNA damage signaling (Bartkova *et al.*, 2005; Gorgoulis *et al.*, 2005), and the role of 53BP1 in promoting the cellular cytotoxicity of Nutlin-3 (Brummelkamp *et al.*, 2006), we evaluated whether the DNA damage response could contribute to the selectivity of Nutlin-3 in PEL cells (I). Cellular DNA damage is marked by the presence of nuclear foci, which contain phosphorylated histone H2AX (γH2AX) and 53BP1 (Rogakou *et al.*, 1998; Chehab *et al.*, 2000; DiTullio *et al.*, 2002; Gorgoulis *et al.*, 2005). To explore activation of the DNA damage signaling in PEL cells we performed immunostaining with an antibody against γH2AX, and quantified the γH2AX foci in BC-1, BC-3, and BCBL-1, and in the KSHV-negative LCL cells (CZE and IHE) (Fig. 5A-B in I). We found that 50% to 70% of PEL cells showed accumulation of the DNA damage response signal, whereas only 20% of the control LCL cells were affected (Fig. 5A-B in I). Notably, the lower number of foci in the control LCL cells was not due to a defect in DNA damage detection or impaired signaling, because these cells remained fully capable of forming γH2AX foci in response to IR (data not shown in I).

To investigate the involvement of DNA damage checkpoint activation in Nutlin-3-mediated cytotoxicity, we pretreated KSHV-infected BC-1 and IHH cell lines with 2 mM caffeine, an inhibitor of the ATM-Chk2 DNA damage pathway, for 24 hours, followed by Nutlin-3 treatment (Fig. 5D in I). We observed that the caffeine treatment greatly impaired the Nutlin-3-induced apoptosis in KSHV-infected cells (Fig. 5D in I).

This demonstrated that activated DNA damage signaling indeed contributes to the Nutlin-3-induced cell death in PEL cells.

A synergistic effect between cytotoxic drugs and Nutlin-3 enhances apoptosis in multiple myeloma, in mantle cell lymphoma, and in a broad panel of leukemia cell lines (Kojima *et al.*, 2005; Stuhmer *et al.*, 2005; Coll-Mulet *et al.*, 2006; Secchiero *et al.*, 2006; Tabe *et al.*, 2009). This strongly supports our findings here that DNA damage signaling is essential for the Nutlin-3-induced cytotoxicity in PEL cells (I). Because v-cyclin induces strong DNA damage in human primary and immortalized dermal microvascular endothelial cells (Koopal *et al.*, 2007), the activated DNA damage response observed in PEL cells (I) may result from the constitutive expression of v-cyclin.

1.2.2 Disruption of the p53-MDM2-LANA complex sensitizes PEL cells to apoptosis upon p53 restoration

As restoration of the p53 function with Nutlin-3 leads to inhibition of the p53-MDM2 interaction and to consequent activation of the p53 signaling pathway (Vassilev, 2004; Vassilev *et al.*, 2004; Vassilev, 2005), we investigated whether the major latency-associated nuclear antigen LANA would form a complex with p53 and MDM2 (I). We focused on LANA, since it interacts with p53, inhibits p53-mediated apoptosis (Friborg *et al.*, 1999), and represses the p53-driven transcription of the pro-apoptotic protein Bax (Wong *et al.*, 2004).

To examine whether LANA could form complexes with p53 and MDM2, we subjected BC-3 cell extracts to gel filtration chromatography followed by reciprocal immunoprecipitation with anti-p53 and anti-MDM2 antibodies (Fig. 6A-B in I). The immunocomplexes were resolved by SDS-PAGE and analyzed by Western blotting using antibodies against p53, MDM2, and LANA. Immunoblot analysis revealed LANA as a component of the p53-MDM2 complex in BC-3 cells (Fig. 6B in I).

Next, we examined whether treatment of PEL cells with Nutlin-3 disrupts the p53-MDM2-LANA ternary complex. To this end, BC-3 cells were treated with 7 μM Nutlin-3 for 12 hours. The cell lysates were fractionated by gel filtration chromatography and subjected to immunoprecipitation using anti-p53 and anti-MDM2 antibodies. The resulting co-precipitates were analyzed by Western blotting using

antibodies directed against p53, MDM2, and LANA (Fig. 6B in I). We found that treatment of BC-3 cells with Nutlin-3 abolished the interaction of LANA with both p53 and MDM2 (Fig. 6B in I). Thus, the release of both p53 and MDM-2 from LANA may contribute to the specificity of Nutlin-3-induced apoptosis in PEL cells.

Very recently, Chen and coworkers (2010) confirmed our findings by purifying LANA complexes from PEL cells. Moreover, the authors suggested that only a minor, phosphorylated fraction of LANA is able to bind to MDM2, and further speculated that MDM2 may either directly contribute to the p53-LANA interaction or may change the conformational state of p53 to allow its interaction with LANA (Chen *et al.*, 2010). In the light of these findings, it would be of great importance to investigate whether MDM2 associates with LANA in a direct or in a p53-mediated manner.

1.2.3 High levels of MDM2 and co-infection with EBV may sensitize PEL cells to Nutlin-3-induced cytotoxicity

As demonstrated above, KSHV-infection, intrinsic DNA damage signaling, and disruption of the p53-MDM2-LANA complex may all contribute to Nutlin-3-induced cytotoxicity in PEL cells. We thus aimed to identify other potential factors able to influence the cytotoxicity mediated by Nutlin-3 (I).

As elevated expression of MDM2 in cancer cells can promote selective cytotoxicity of Nutlin-3 (Patton *et al.*, 2006; Tovar *et al.*, 2006), we first wanted to examine whether MDM2 levels in PEL cells correlate with Nutlin-3-induced cytotoxicity. To this end, PEL cells (BC-1, BC-3, and BCBL-1) and KSHV-negative LCL cells (CZE and IHE) were lysed and subjected to immunoblotting with anti-MDM2 antibody (Fig. 1 in I). The results revealed markedly higher levels of MDM2 in PEL cells than in KSHV-negative LCL cells (Fig. 1 in I). This is in line with previous findings demonstrating that among over 60 B-cell tumors only PEL display highly elevated MDM2 transcripts (Petre *et al.*, 2007).

Another factor that could theoretically sensitize cells to Nutlin-3-induced cytotoxicity is coinfection with EBV. Treatment of wt p53-bearing Burkitt's lymphoma (BL) cells with Nutlin-3 restores their p53 pathway regardless of their EBV status (Renouf *et al.*, 2009). Apoptosis in response to Nutlin-3 occurs in BL cells expressing the EBV latency program I, whereas BL cells in EBV latency program III undergo strong cell

cycle arrest but no apoptosis (Renouf *et al.*, 2009). Importantly, the majority of PEL cells, including the BC-1 cell line, are dually infected with KSHV and EBV (Cesarman *et al.*, 1995a; Renne *et al.*, 1996b; Carbone *et al.*, 1998), which may influence their response to restoration of the p53 pathway. Dually infected PEL cells display the restricted EBV latency program I (Horenstein *et al.*, 1997; Szekely *et al.*, 1998), which is in accordance with our results demonstrating that the most efficient apoptotic response occured in the dually infected BC-1 cell line (Fig 3A-B in I). Furthermore, lymphoblastoid cells exhibit the type III latency program (Lewin *et al.*, 1995; Szekely *et al.*, 1998), supporting our finding that the type III latency LCL cells (CZE and IHE) underwent growth arrest without apoptosis (Fig. 2B in I). To conclude, EBV coinfection may predispose PEL cells to Nutlin-3-induced cell death.

2. Restoration of the p53 pathway as a treatment for KSHV lymphomas *in vivo* (I, II)

2.1 MDM2 inhibition by Nutlin-3 eradicates subcutaneous PEL tumors in mice

Since our *in vitro* studies demonstrated the potential of restored p53 in specific PEL cell killing, we aimed to confirm this observation *in vivo* (I and II). We thus explored the effect of MDM2 inhibition by Nutlin-3 in a human subcutenaeous (s.c.) mouse model for PEL (I). Balb/c nude female mice were xenografted with 6×10^6 BC-3 cells in matrigel, and their tumors were allowed to grow until palpable. To treat these tumor-bearing mice, we administered Nutlin-3 by an i.p. route, at 20 mg/kg of body weight, every second day for the course of two weeks (I). Animals treated with Nutlin-3 showed a dramatic regression in tumor growth, in contrast to mice receiving vehicle control (Fig. 7 in I). After three doses of Nutlin-3, we observed significant tumor shrinkage (Fig. 7 in I), and after seven doses, 40% of the mice showed complete tumor remission (I). We followed these mice for over 90 days and observed no tumor recurrence (I). However, after treatment cessation, those tumors that had displayed a delayed response to Nutlin-3 resumed proliferation. The size of these tumors did, however, remain smaller than in the control mice (I). Furthermore, additional Nutlin-3 treatment of those tumors with partial responses led to their regression without signs of recurrence during the follow-up period (I). This suggests that p53 restoration by MDM2 antagonists may offer an effective treatment modality for KSHV-associated lymphomas (Fig. 5).

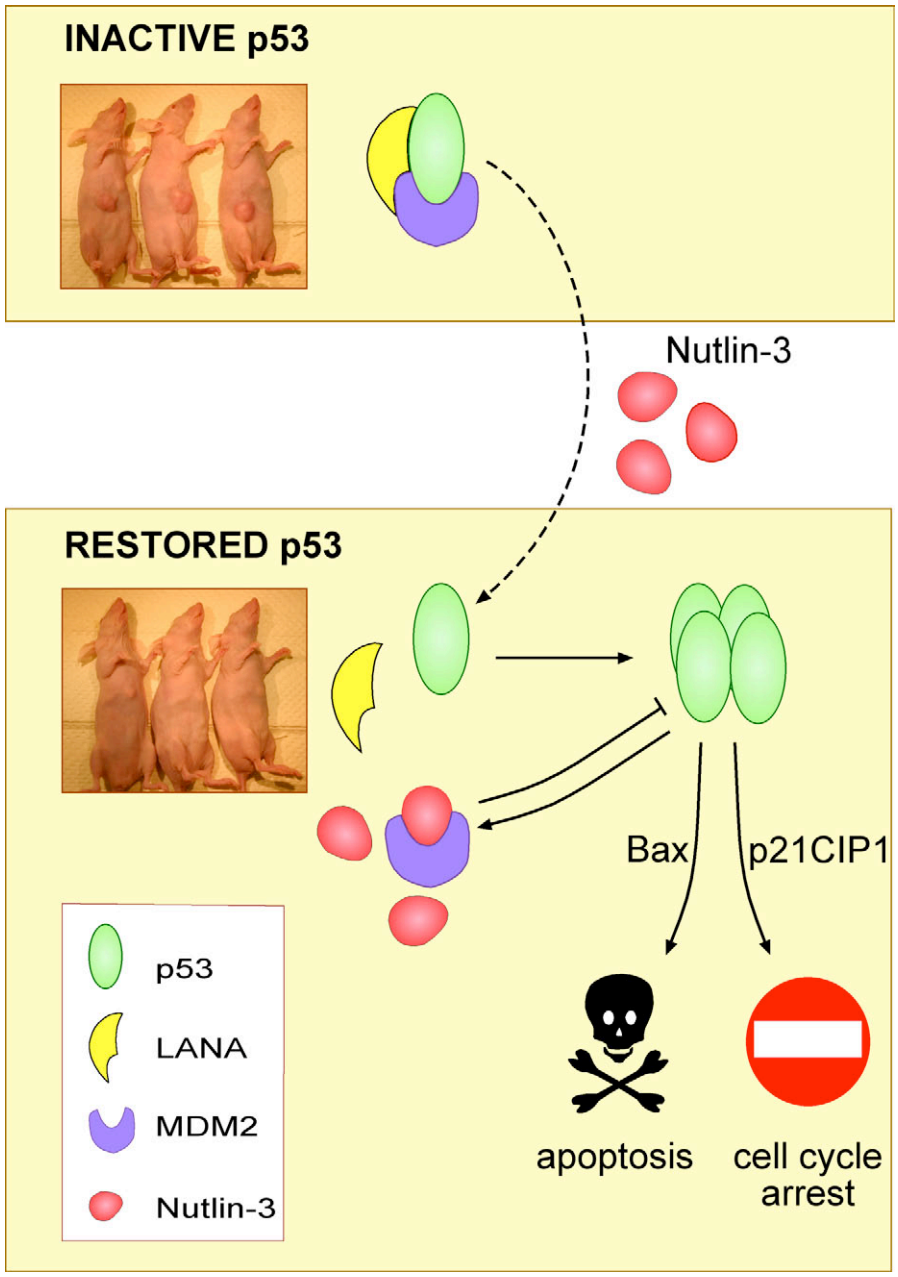

Figure 5. Restoration of p53 function by an MDM-2 antagonist as a therapeutic modality in primary effusion lymphomas. In PEL cells, LANA binds p53 and MDM2, which represses the p53 function and allows tumor development in mice. Treatment with Nutlin-3 releases p53 from the negative regulation of LANA. Inhibition of MDM2 binding to p53 leads to p53 stabilization, activation, and accumulation of transcriptionally active tetramers. The subsequent activation of the p53 pathway and p53-target genes induces cell cycle arrest and apoptosis, thus leading to tumor eradication.

2.2 Anti-tumor activity of Nutlin-3 occurs in intraperitoneal PEL tumors in mice

Investigation of biological processes such as tumor growth and response to specific therapies benefits from preclinical animal models in which quantitative and non-invasive detection of tumor cells can be perfomed. As the results described above were based on s.c. PEL tumors in mice, we next chose an animal model to better recapitulate features of the human disease, and to allow more quantitative follow-up of the

treatment (II). To achieve this, we injected KSHV-positive BC-3 PEL cells (1×10^7) carrying an NF-κB-regulated luciferase reporter gene (BC-3/NF-κB-luc (Keller *et al.*, 2006)) into the body cavity of severely immuno-compromised NOD/SCID mice (II). We monitored tumor formation by whole-body imaging using the IVIS imaging system (II). We started the i.p. Nutlin-3 (40 mg/kg) administration every second day after tumor establishment (average luciferase signal intensity 1×10^5 to 4×10^5 photons), for two weeks. We evaluated tumor response to Nutlin-3 by *in vivo* bioluminescence imaging in real time in live animals (II). We observed that mice within a control group (n=12) that received vehicle (DMSO) showed a continuous increase in the bioluminescence signal associated with tumor growth (Fig. 2A-B in II). In contrast, 87% of Nutlin-3-treated mice (n = 15) showed a reduction in tumor mass. Within this group, after 14 days of treatment, 60% of the mice achieved total tumor regression (Fig. 2A-B in II). One mouse had a residual tumor despite Nutlin-3 treatment and showed a weight gain six days after treatment cessation, indicating resumed cell proliferation (Fig. S2A in II). However, re-treatment with six doses of Nutlin-3 (20 mg/kg) for two weeks resulted in complete tumor eradication (Fig. S2B in II).

Next, to examine the involvement of p53 activation in the Nutlin-3 response of the IP tumors (II), we collected tumors from ascites of Nutlin-3- or DMSO- treated mice 24 hours after the fourth dose. When these tumor cells were examined by immunofluorescence using antibodies against p53 and the p53 target p21CIP1 (Fig. S2C in II), increased p53 and p21CIP1 signal was evident in Nutlin-3-treated tumors, whereas DMSO-treated tumors showed the basal level of p53 and no p21CIP1 induction (Fig. S2C in II). Further examination by TUNEL assay of these tumor cells for apoptosis revealed nearly 20% of apoptotic cells in the Nutlin-3-treated tumor and only 2% in the DMSO-treated tumor (Fig. S2D in II). To conclude, p53 accumulation and induction of apoptosis upon Nutlin-3 treatment led to PEL tumor regression.

Radiosensitive tissues such as small-intestine epithelium and thymus are extremely susceptible to p53-induced apoptosis (Lowe *et al.*, 1993; Potten *et al.*, 1997). Moreover, restoration of p53 function by a genetic approach in the absence of MDM2 triggers massive apoptosis in radiosensitive mouse organs, followed by death of the animals within five days (Ringshausen *et al.*, 2006). This prompted us to study

whether pharmacological activation of p53 will also be toxic to healthy tissues (II). To assess this in our system, we studied the effect of p53 restoration in radiosensitive (small intestine, spleen) and radioresistant (heart, lungs, kidney) organs of NOD/SCID mice treated with eight doses of Nutlin-3 (20 mg/kg) for two weeks (II). The organs were examined by TUNEL immunofluorescence assay, revealing that MDM2 inhibition caused neither apoptosis nor atrophy (Fig. S3 in II). These results are also supported by others' reports that Nutlin-3 is well tolerated by mice and that accumulation of p53 after MDM2 inhibition does not cause profound pathological changes in normal tissues (Vassilev *et al.*, 2004; Tovar *et al.*, 2006).

Taken together, our data provide, to our knowledge, the first *in vivo* experimental evidence of p53 restoration in mice and demonstrate in the mouse models remarkable anti-tumor activity against KSHV lymphomas. This warrants further evaluation in preclinical and clinical studies and may provide new measures to treat aggressive KSHV lymphomas.

2.3 The p53 pathway can be efficiently restored in clinical PEL tumors *ex vivo*

Because an important prerequisite of the novel cancer therapeutic agents is their ability to inhibit the survival and growth of primary tumor cells isolated from humans, we tested whether the p53 pathway can be restored in primary tumors isolated from patients diagnosed with PEL (II). To this end, we used ascitic fluids containing large lymphomatous cells, with all their morphological features consistent with a PEL diagnosis, isolated from HIV-positive patients (II). Primary PEL tumors were treated *ex vivo* with 7 μM Nutlin-3 or vehicle control for 24 or 48 hours, with cell death assessed by trypan blue exclusion (Fig. 1A in II). The data revealed that Nutlin-3 treatment resulted in a two- to six-fold higher, time-dependent induction of cell death than in the vehicle-treated control (Fig. 1A in II). To study whether Nutlin-3 activity in PEL tumors coincides with an increased expression of p53 and its targets MDM2 and p21CIP1, we performed Western blotting analysis that detected an increase in expression of p53 and of its targets in Nutlin-3-treated PEL tumors (Fig. 1B in II). As Nutlin-3 has been reported to trigger apoptosis in primary tumor samples from hematological malignancies (Kojima *et al.*, 2005; Stuhmer *et al.*, 2005; Coll-Mulet *et al.*, 2006; Secchiero *et al.*, 2006), we examined its apoptotic potential also in clinical PEL tumors. Immunoblotting analysis revealed elevated levels of active caspase-3 in PEL tumors upon Nutlin-3 treatment, suggesting efficient execution of apoptosis (Fig.

1B in II). Taken together, these results suggest that the downstream p53-dependent pathway is intact also in primary PEL tumors.

Compared to our control BC-3 cells, the apoptotic response of one clinical PEL tumor (#993) was delayed (Fig. 1A in II). Given our finding that LANA is in a complex with p53 and MDM2 (I), and that it most likely contributes to the specificity of Nutlin-3-induced cell death in the KSHV-infected cells (I, and (Koopal *et al.*, 2007)), we examined LANA expression level in the #993 tumor (II). Indeed, immunoblotting analysis revealed reduced LANA levels in this tumor when compared to the other tumor (#974) and to BC-3 cells (Fig. 1B in II). This prompted us to search for any correlation between levels of LANA and response to Nutlin-3 treatment (II). To this end, we subjected U2OS cells stably expressing LANA or GFP from lentiviral constructs to Nutlin-3 (7 μM) treatment and determined cell viability by the trypan blue exclusion (Fig. S1 in II). We found that the Nutlin-3-induced cytotoxic effect in U2OS cells expressing LANA was almost three-fold higher than in the control cells expressing GFP (Fig. S1 in II). Based on these results, we suggest that LANA expression may promote sensitivity of the cells to the cytotoxic activity of Nutlin-3. However, further studies are required to confirm this observation in a broader panel of PEL cells. A possible *in vivo* validation of the correlation between LANA levels and Nutlin-3 cytotoxicity could be carried out in LANA transgenic animals with B-cell tumors (Fakhari *et al.*, 2006) that resemble the B-cell population of KSHV-associated human lymphomas (Jenner *et al.*, 2003; de Sanjose *et al.*, 2004).

2.4. Viral lytic replication impairs the apoptotic potential of Nutlin-3 *in vivo*

One of the major challenges for effective therapy against cancer in general is to overcome tumor resistance. Our *in vivo* data (see 2.1. and 2.2.) demonstrate that the overall response rate to Nutlin-3 treatment was excellent. However, four mice responded poorly to Nutlin-3 (Fig. 3A in II). In order to study the mechanism behind their poor response, we collected and further analyzed their ascites. Immunofluorescence analysis of tumor cells from one non-responder mouse revealed impaired stabilization of p53 and no activation of its target p21CIP1, unlike the response in the responder mouse (Fig. 3C in II). To examine whether absence of p53 pathway activation in tumor cells from the non-responder mouse leads to a failure of these cells to undergo apoptosis, we performed TUNEL and anti-active caspase-3 immunostaining (Fig. 3D in II). No positive staining was detectable in tumor cells of

the non-responder mouse, sharply contrasting with cells of the responder mouse (Fig. 3D in II). These results suggest that in the tumors of the non-responder mice, the apoptotic response was compromised.

2.4.1 KSHV viral reactivation attenuates the p53-dependent apoptotic response to MDM2 inhibition

Because these results suggest that LANA expression sensitizes cells to the cytotoxic effect of Nutlin-3, we first examined whether LANA expression levels differ between responder and non-responder mice (Fig. 3B in II). However, Western blotting analysis failed to reveal any substantial reduction in LANA in the non-responders (Fig. 3B in II).

As rapid progression and dissemination of KS lesions have been linked to the extent of viral reactivation and viral loads in the peripheral blood (Whitby *et al.*, 1995; Brambilla *et al.*, 1996; Campbell *et al.*, 2000; Pellet *et al.*, 2002; Quinlivan *et al.*, 2002; Campbell *et al.*, 2003; Engels *et al.*, 2003; Song *et al.*, 2004; Guttman-Yassky *et al.*, 2007; Laney *et al.*, 2007; Mancuso *et al.*, 2008), we decided to assess the viral replication phase in the PEL tumors from ascites of responder and non-responder mice (Fig. 3E in II). Our quantitative real-time PCR (qRT-PCR) analysis for expression of the KSHV lytic transcripts ORF50 (immediate early lytic gene) and K8.1 (late lytic gene) showed that expression of both lytic transcripts was markedly higher in non-responder mice than in responders (Fig. 3E in II). This clearly correlates with the response of these mice to the Nutlin-3 treatment and to their tumor burden (Fig. 3A in II). Collectively, these results suggest that viral lytic reactivation may affect the outcome of small-molecule-mediated p53 restoration *in vivo*.

2.5 Chemical and physiological induction of viral reactivation recapitulates resistance to MDM2 inhibition *in vitro*

Inspired by the unexpected finding of elevated lytic gene expression in the tumors, we tried to recapitulate the attenuation of the Nutlin-3 response by viral reactivation *in vitro* (II). Since KSHV-infected cells can be induced to enter the productive replication phase by a variety of chemicals (Yu *et al.*, 1999; Miller *et al.*, 2007), we induced lytic replication in PEL cells (BC-3 and JSC-1) with TPA. Following TPA-induced viral reactivation, the cells were treated with Nutlin-3 and analyzed by Western blotting (Fig. 4A in II), revealing a four-fold lowering of the stabilization of p53 levels in TPA-

and Nutlin-3-treated BC-3 and JSC-1 cells, in contrast to Nutlin-3-treated uninduced PEL cells. Importantly, this decrease in p53 levels was not due to an adverse TPA effect, because TPA- and Nutlin-3-treated KSHV-negative lymphoblastoid cells IHE showed no p53 attenuation (Fig. S4 in II). Furthermore, as determined by trypan blue exclusion, induction of lytic replication in both BC-3 and JSC-1 cells led to a significantly impaired apoptotic response to Nutlin-3 treatment (Fig. 4C in II).

We next studied the role of lytic replication in the impaired apoptotic response to Nutlin-3 treatment in a more physiological setting. Hypoxic conditions have induced KSHV lytic replication (Davis *et al.*, 2001; Haque *et al.*, 2003; Cai *et al.*, 2006; Haque *et al.*, 2006). Moreover, as PEL lymphomas arise in a low-oxygen environment (Funahashi *et al.*, 1971; Houston, 1981), hypoxia may predispose the tumor cells to induction of lytic replication. We thus studied the effect of hypoxia-induced viral reactivation on the apoptotic response to Nutlin-3 treatment (II). To this end, BC-3 and JSC-1 cells were cultured under hypoxic conditions (1% O_2) for 96 hours, and analyzed for viral reactivation by immunostaining for early lytic marker ORF59. Quantitative immunofluorescence analysis of hypoxia-treated PEL cells revealed enhanced spontaneous lytic replication, as compared to PEL cells cultured in a normoxic (21% O_2) environment (Fig. 5A in II). To examine whether hypoxia-induced viral reactivation affects p53 expression, we performed immunoblotting analysis of Nutlin-3- or vehicle-treated JSC-1 cells grown under hypoxic or normoxic conditions. Under low oxygen conditions, both the basal level of p53 and its stabilization in response to Nutlin-3 treatment were lower than in normoxia-treated cells (Fig. 5E in II).

To investigate whether the impaired apoptotic response to Nutlin-3 treatment depends on hypoxia-induced viral reactivation, we cultured BC-3 and JSC-1 cells under hypoxic conditions for 18 hours, followed by Nutlin-3 treatment for an additional 48 hours. A trypan blue exclusion viability assay showed a dramatic decrease in Nutlin-3-induced cell death in the hypoxia-reactivated PEL cells (Fig. 5B in II). Next, we investigated whether inhibition of viral reactivation with anti-herpes agent GCV (Kedes and Ganem, 1997), a specific inhibitor of KSHV replication, could restore the apoptotic potential of Nutlin-3 in hypoxia-reactivated PEL cells. We therefore incubated hypoxia-treated JSC-1 cells with GCV or vehicle control for 48 hours, followed by co-incubation with Nutlin-3 for the next 72 hours. Intriguingly, inhibition

of late lytic gene expression by GCV (Fig. 5C in II) resulted in robust restoration of Nutlin-3-induced cell death, as determined by the trypan blue exclusion method (Fig. 5 D in II). These results demonstrate that in PEL cells, KSHV viral reactivation compromises Nutlin-3-induced cytotoxicity. However, this undesirable outcome can be prevented by inhibition of lytic replication.

Cancer-cell resistance to therapeutic agents can be multifaceted, and understanding its causes is fundamental to improving already-existing therapies and to developing new, more efficient treatment strategies. Our data uncover the role of viral lytic replication in the therapies based on p53 restoration (II). Anti-virals for KSHV-associated diseases in humans are essential in patients with advanced AIDS; GCV treatment causes a rapid and dramatic decline in the prevalence of new KS tumors (Glesby *et al.*, 1996; Mocroft *et al.*, 1996; Martin *et al.*, 1999). The search for new treatments against KSHV-associated malignancies has become intertwined with the identification of genes and their products that directly contribute to the malignancy. Recently, a novel role for Ser/Thr Pim kinases in KSHV pathobiology has been reported, demonstrating that Pim-1 and Pim-3 are required for viral lytic reactivation at very early stages (Cheng *et al.*, 2009); as these two kinases thus may contribute also to PEL tumor development, it may be feasible to selectively inhibit these kinases to avoid any resistance of PEL tumors to specific treatments. However, before a potential Pim kinase inhibitor can be considered, one needs to evaluate its cytotoxicity and its ability to specifically inhibit kinase activity and lytic replication. A leading inhibitor for Pim kinases should thus first be tested for suppression of early stages of viral lytic reactivation in PEL cells *in vitro*, and then in the i.p. PEL mouse model to assess whether even higher therapeutic efficacies are possible in combination with the MDM2 inhibitor.

2.6 Decrease in p53 protein level in lytic PEL cells is proteasome-mediated

As the ubiquitin-proteasome pathway is an important system to control the half-life of various proteins including transcription factors such as p53 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997; Muratani and Tansey, 2003), we studied whether the decrease in the level of p53 in lytically reactivated PEL cells (see 2.5 and Fig. 5E in II) results from its degradation by the ubiquitin-proteasome system. We thus preincubated JSC-1 cells in hypoxia and then monitored changes in their p53 protein upon treatment with the 26S proteasome inhibitor MG132 or DMSO. Western blotting revealed that these hypoxia-treated JSC-1 cells displayed decreased turnover of p53 in the presence of

MG132, suggesting that reduction in p53 protein levels in lytically reactivated JSC-1 cells (see 2.5 and II) is proteasome-mediated (Fig. 5E in II).

To discover which E3 ubiquitin ligase(s) will specifically mark p53 for enhanced proteasomal degradation in PEL cells upon KSHV viral reactivation, we examined whether MDM2 could mediate this degradation. To this end, we monitored changes in MDM2 levels in hypoxia- or normoxia-treated JSC-1 cells upon Nutlin-3 treatment. Western blotting showed that Nutlin-3 raised the levels of MDM2 protein in normoxia-treated cells, but no increase in MDM2 was detectable in hypoxic cells undergoing lytic replication (Fig. 5E in II). Thus, although MDM2 predominantly controls the amount of p53 protein during the latent infection, after the switch to lytic replication, another E3 ligase is most likely involved in the ubiquitin-mediated proteasomal p53 degradation.

Several KSHV proteins have been identified in the regulation of p53 stability. The BZLF1 protein of EBV, which functions as an adaptor component of the ECS ubiquitin ligase complex, enhances the ubiquitin-mediated degradation of p53 (Sato *et al.*, 2009a; Sato *et al.*, 2009b). Interestingly, KSHV encodes a homolog of BZLF1, a lytic protein K-bZIP, which belongs to the basic region-leucine zipper (bZIP) family of transcription factors (Lin *et al.*, 1999). Recently, K-bZIP has been demonstrated to possess activity of the SUMO E3 ligase, with specificity towards SUMO2/3 (Chang *et al.*, 2009) and to interact with and inhibit p53-driven transcription in PEL cells (Park *et al.*, 2000). Further studies are required to clarify whether K-bZIP targets p53 for proteasomal degradation during the lytic replication phase. An alternative mechanism of p53 degradation may involve proteins that themselves lack E3 ligase activity, such as immunomodulatory KSHV lytic proteins (vIRFs). These vIRFs could theoretically form multi-protein complexes with ubiquitin ligase activity components. Accordingly, vIRF-4 has recently been shown to interact with MDM2 and to facilitate the proteasome-mediated degradation of p53 (Lee *et al.*, 2009a), whereas vIRF-1 has been reported to interact with p53 and inhibit its phosphorylation and acetylation (Lin *et al.*, 2001; Seo *et al.*, 2001; Shin *et al.*, 2006). As these events may lead to ubiquitination and degradation of p53, the role of vIRF-1 and of vIRF-4 in p53 degradation upon lytic replication requires examination.

3. The switch from latency to lytic replication - a critical step in the KSHV pathogenesis (III, IV; unpublished data)

Latency provides a unique opportunity for KSHV to escape host immune surveillance and to establish persistent infection (Coscoy, 2007). However, to maintain viral reservoirs and to spread to other sites and hosts, KSHV must reactivate from latency and enter the lytic growth phase to generate more viral progeny. The switch between the latent phase and lytic replication is therefore a critical step determining the outcome of viral infection and influencing the pathogenesis of KSHV-induced malignancies. Viral reactivation can be induced and studied in KSHV-infected B-type lymphocytes in cell culture, but our current knowledge of specific virus-host interactions that repress or activate lytic replication is still limited.

3.1 Nucleophosmin is a novel regulator of the latent-lytic switch

3.1.1 Nucleophosmin is phosphorylated by v-cyclin-CDK6 *in vivo* in PEL cells

As transient expression of v-cyclin causes NPM redistribution from nucleolus to nucleoplasm in transfected U2OS osteosarcoma cells (Kurki *et al.*, 2004), we studied the localization of NPM in naturally KSHV-infected PEL cells (IV). As immunofluorescence analysis revealed NPM in both nucleoplasm and nucleolus in PEL cells (data not shown in IV), we investigated whether v-cyclin and NPM could have a functional relationship *in vivo* in a biologically relevant KSHV model.

To this end, we needed to develop a biochemical approach to identify and further characterize the functional relationship between v-cyclin and its *in vivo* cellular interaction partners in PEL cells. We summarize here the development and validation of the methodology described in detail in Study III. As the results were not within the scope of this thesis, they are not discussed in detail.

As a first step to study v-cyclin interaction partners *in vivo* we generated a new affinity-purified polyclonal antibody against v-cyclin protein (III). This antibody we tested for its ability to specifically and efficiently recognize full-length v-cyclin protein in native and denatured conditions by immunoblotting and immunoprecipitation. This antibody served to identify v-cyclin substrates in all subsequent steps: (1) by gel filtration chromatography, and fraction analysis by immunoblotting, (2) immunoprecipitation of v-cyclin from v-cyclin-containing fractions, and (3) determination of v-cyclin kinase activity towards the co-precipitated endogenous

proteins (III). By this approach, we were able to confirm *in vivo* in PEL cells an earlier observation that p27KIP1 is a substrate for the v-cyclin-CDK6 complex *in vitro* (Ellis *et al.*, 1999; Mann *et al.*, 1999)(III).

Given the applicability of this methodology (III and Jarviluoma *et al.*, 2006), we went on to investigate whether NPM forms a complex with v-cyclin and becomes phosphorylated by v-cyclin-CDK6 in PEL cells (IV). To accomplish this, PEL cells were fractionated by gel filtration chromatography, and the fractions containing NPM and v-cyclin identified. These fractions were subjected to immunoprecipitation and a subsequent *in vitro* kinase assay towards the coprecipitated proteins. This revealed an approximately 37-kDa band at the position of NPM on the SDS-PAGE gel, which was phosphorylated by the v-cyclin-associated CDK6 kinase (Fig. S1A-C in IV). This strongly suggests that NPM may be a novel v-cyclin target in PEL cells *in vivo*.

NPM phosphorylation by v-cyclin–CDK6 *in vitro* occurs mostly on Thr199 (Cuomo *et al.*, 2008). We confirmed this finding in our experimental *in vitro* system by transfecting U2OS cells with expression vectors for v-cyclin or a vector control together with wt NPM (eGFP-NPM), or its phosphorylation site mutant T4A (Thr199Ala, Thr214Ala, Thr234Ala, Thr237Ala) and mutant T3A in which all but Thr199 is replaced by Ala (Fig. S1D in IV). We analyzed phosphorylation of NPM by immunoblotting with phospho-NPM antibody (pNPM Thr199). This analysis revealed that phosphorylation of NPM was detectable only in cells co-transfected with v-cyclin and with the eGFP-NPM retaining an intact Thr199 site (Fig. S1D in IV).

To demonstrate that NPM phosphorylation in KSHV-infected cells was indeed v-cyclin-dependent, we silenced v-cyclin expression in SLK endothelial cells stably infected with the recombinant rKSHV.219 (Vieira and O'Hearn, 2004), using retrovirus-mediated RNA interference (RNAi) (IV). Immunoblotting analysis showed an approximately five-fold lower NPM phosphorylation on Thr199 in cells with stably down-regulated v-cyclin (sh-v-cyclin-expressing cells) than for the control (sh-scr) cells (Fig. 1A in IV). RNAi against v-cyclin triggers a concomitant depletion of v-FLIP (Godfrey *et al.*, 2005), because v-FLIP translation is carried out from a bicistronic transcript (comprised the *ORF72*/v-cyclin and *ORF71*/v-FLIP genes) through an internal ribosome entry site located in the v-cyclin coding region (Talbot *et al.*, 1999; Bieleski and Talbot, 2001; Grundhoff and Ganem, 2001). We therefore

needed to eliminate the possibility that inhibition of NPM phosphorylation in sh-v-cyclin-expressing cells is due to the loss of v-FLIP. To accomplish this, rKSHV.219 SLK cells expressing sh-v-cyclin were reconstituted for v-FLIP by use of retrovirus-mediated gene transfer. The RT-qPCR analysis revealed that expression of sh-v-cyclin reduced the levels of both v-cyclin and v-FLIP transcripts (Fig. S3A in IV). Based on our immunoblotting result, this reduction was accompanied by a substantial decrease in NPM Thr199 phosphorylation in the sh-v-cyclin cells expressing either the empty control vector (pBMN) or v-FLIP (v-FLIP-pBMN) (Fig. S3B in IV). These data suggest that for NPM phosphorylation on Thr199, v-cyclin but not v-FLIP is required.

As CDK6 has been identified as a predominant kinase partner for v-cyclin (Chang *et al.*, 1996; Godden-Kent *et al.*, 1997; Li *et al.*, 1997; Platt *et al.*, 2000), we further delineated whether CDK6 is required for v-cyclin-mediated phosphorylation of NPM. To accomplish this, we silenced CDK6 in rKSHV.219-infected EA.hy926 endothelial cells, and, in addition, CDK4 or CDK6 in BC-3 cells by lentivirus-mediated RNAi, as detailed in Methods. Immunoblotting analysis showed markedly attenuated NPM phosphorylation on Thr199 in both endothelial and BC-3 cells silenced for CDK6 as compared to the CDK4-silenced and non-silenced controls (Fig. 1B-D in IV). This indicates that in KSHV-infected endothelial and PEL cells, the v-cyclin-CDK6 complex mediates the NPM phosphorylation on Thr199.

3.1.2 Phosphorylation of NPM promotes the NPM-LANA association, which is further regulated by acetylation

Transcripts of v-cyclin and LANA are generated from a common promoter (Dittmer *et al.*, 1998; Talbot *et al.*, 1999). As NPM has been reported to interact with LANA and the terminal repeats of the KSHV genome in PEL cells (Si *et al.*, 2006), and we found that NPM interacts with v-cyclin (Fig. S1B in IV), we studied the role of v-cyclin in the formation of the NPM-LANA complex in more detail. We transfected U2OS cells stably expressing LANA or GFP with an Myc-tagged v-cyclin expression vector (Myc-v-cyclin) or with an empty vector as a control. Cell extracts were subjected to immunoprecipitation with anti-LANA or anti-NPM antibodies. Immunoblotting analysis revealed that transient transfection of v-cyclin in U2OS cells stably expressing LANA, but not GFP, resulted in the NPM-LANA complex formation (Fig. 2A in IV).

To investigate whether NPM-LANA interaction in PEL cells is CDK6-dependent, we silenced CDK6 in BC-3 cells by lentiviral RNAi (IV). Cell lysates were subjected to gel filtration chromatography, and NPM/LANA-positive fractions were further immunoprecipitated with anti-LANA antibody. Immunoblotting showed that silencing of CDK6 in BC-3 cells reduced interaction of NPM with LANA (Fig. 2B in IV), indicating that v-cyclin-CDK6 kinase activity is required for the NPM-LANA complex formation (Fig. 6).

Given that Thr199 is the major phosphorylation site on NPM (Fig. S1D in IV), we examined the importance of NPM phosphorylation on Thr199 for the NPM-LANA interaction. We ectopically expressed in U2OS cells stably expressing LANA, v-cyclin, along with the wild-type NPM (eGFP-NPM) or its phosphosite mutants that were harboring Thr to Ala mutations of NPM codons. Western blotting analysis of LANA immunoprecipitates showed that only NPM with an intact phosphorylation site at Thr199 was associated with LANA (Fig. 3B in IV). Thus, NPM phosphorylation on Thr199 by v-cyclin-CDK6 seems to be required for efficient NPM-LANA interaction.

Maintenance of the KSHV latency is associated with deacetylation of the chromatin due to recruitment and activity of HDACs (Miller *et al.*, 2007; Pantry and Medveczky, 2009). As NPM associates with histones (Okuwaki *et al.*, 2001b) and changes chromatin structure by HDAC recruitment (Liu *et al.*, 2007a), we examined whether NPM associates with HDAC1 in PEL cells (IV). We thus reciprocally immunoprecipitated BCBL-1 whole-cell extracts with anti-HDAC1 or anti-NPM antibodies. Immunoblotting analysis revealed NPM in a complex with HDAC1 in latent BCBL-1 cells (Fig. 3C in IV). Furthermore, this complex greatly dissociated in the presence of NaB, a histone deacetylase inhibitor that induces viral reactivation (Fig. 3C in IV). To examine whether LANA also forms a complex with HDAC1, we subjected HDAC1 immunoprecipitates to immunoblotting with anti-LANA antibody, detecting the complex in latent BCBL-1 cells and demonstrating that it was NaB-resistant (Fig. 3D in IV). We therefore examined whether NPM could affect the integrity of the LANA-HDAC1 complex. To this end, we silenced NPM in PEL cells by use of lentivirus-mediated RNAi. Cell extracts were subjected to immunoprecipitation with anti-HDAC1 antibody, and immunoblotting analysis revealed that NPM silencing abolished the interaction of LANA and HDAC1 (Fig. 3D in IV). This suggests that in latently infected PEL cells, NPM recruits HDAC1 and

allows its interaction with LANA, and possibly with core histones (Fig. 6). In agreement with this, HDACs have been demonstrated to associate with lytic ORF50 promoter and to contribute to its stable repression, thereby inhibiting lytic gene expression (Gwack *et al.*, 2001; Lu *et al.*, 2003).

Inhibition of the HDAC activity induces lysine acetylation of LANA, its dissociation from the core histones, and transcriptional de-repression of the ORF50 promoter (Lu *et al.*, 2006). Since NPM silencing abolished HDAC1-LANA interaction (IV and Fig. 6), we determined LANA acetylation status upon NPM silencing. We immunoprecipitated NPM-silenced BCBL-1 cell extracts with anti-acetylated lysine (Ac-K) antibody. Western blotting analysis with LANA revealed a remarkable increase both in the intensity and appearance of newly acetylated LANA bands when compared to those of the non-silenced control (Fig. 3E in IV). Thus, depletion of NPM led to enhanced LANA acetylation (Fig. 6).

The p300-mediated acetylation of NPM is required for nucleosomal disassembly and for active chromatin transcription (Swaminathan *et al.*, 2005). We therefore analyze the extent of NPM acetylation in BC-3 cells in the presence or absence of the histone deacetylase inhibitor NaB (IV). Immunoblotting analysis, with anti-Ac-K antibody, of NPM immunoprecipitates showed elevated NPM acetylation upon HDAC inhibition (Fig. 3F in IV). In order to examine whether inhibition of HDAC activity promotes association of NPM with p300, we performed NPM immunoprecipitation from BCBL-1 cells treated with NaB. Western blotting demonstrated that NaB treatment enhanced NPM-p300 interactions (Fig. 3G in IV).

Because protein acetylation modulates the interaction between LANA and NPM, we examined whether NaB could affect this interaction (IV), by treating BCBL-1 cells with NaB or vehicle control, then by subjecting their lysates to immunoprecipitation with anti-LANA antibody. Immunoblotting analysis showed that association of NPM with LANA was abolished in the presence of NaB (Fig. 3H in IV), suggesting that protein acetylation controls the interaction between NPM and LANA (Fig. 6). Lysine acetylation has been reported to impair phosphorylation-dependent interactions of large macromolecular complexes (Choudhary *et al.*, 2009). Our results thus suggest that the initial association of NPM with LANA is regulated by v-cyclin-mediated

phosphorylation of NPM, and is further controlled by lysine acetylation (Fig. 6).

3.1.3 Silencing of NPM leads to viral reactivation

As we had demonstrated that NPM silencing induces an increase in PEL-cell LANA acetylation (Fig. 3E in IV), and acetylation of LANA has been linked to viral reactivation (Lu *et al.*, 2006), we next examined the expression of lytic replication markers in NPM-silenced PEL cells (IV). These NPM-silenced BC-3 and BCBL-1 cells were analyzed by immunofluorescence using antibody against an early lytic marker, ORF59. Silencing of NPM led to an increase in ORF59-positive cells as compared to those in the non-silenced control (Fig. 4A-B in IV). The qRT-PCR analysis of NPM-silenced BCBL-1 cells for lytic genes (ORF50, ORF57, and K8.1) corroborated further these results (Fig. 4C in IV), suggesting that viral reactivation was induced in response to NPM silencing.

Because production of new viral particles is the final event in the lytic replication cascade, we investigated the production and release of infectious virions in response to NPM silencing. We therefore transduced BCBL-1 cells with lentivirus expressing short hairpin RNA (sh-RNA) against either NPM (sh-NPM) or a non-target control (sh-Scr) (IV). Western blot analysis revealed that silencing of NPM dramatically induced expression of the late lytic glycoprotein K8.1, whereas transduction with a scrambled control had no effect (Fig. 4D in IV). Next, we confirmed that the observed increase in K8.1 expression corresponded to the production of infectious KSHV virions. To this end, we used supernatants of BCBL-1 cells stably expressing sh-RNA against NPM to infect naive SLK endothelial cells (IV). Immunofluorescence studies revealed a great number of LANA-positive cells in the infected SLK target population, two days post-infection, confirming that NPM silencing resulted in efficient production and release of infectious viral particles (Fig. 4E-F in IV). In addition, when we used concentrated virus (obtained by ultracentrifugation of supernatants of BCBL-1 cells stably expressing sh-RNA against NPM) to infect SLK cells, the intensity of the LANA signal and the number of nuclear speckles typical for the LANA immunostaining greatly increased (Fig. 4E in IV). Considered together, these data confirm induction of the KSHV full lytic cascade in cells depleted for NPM expression. Based on other reports and our findings in this thesis (3.1.2, 3.1.3, and IV)

we suggest a model of NPM as a critical regulator of the KSHV latent-lytic switch (Fig. 6).

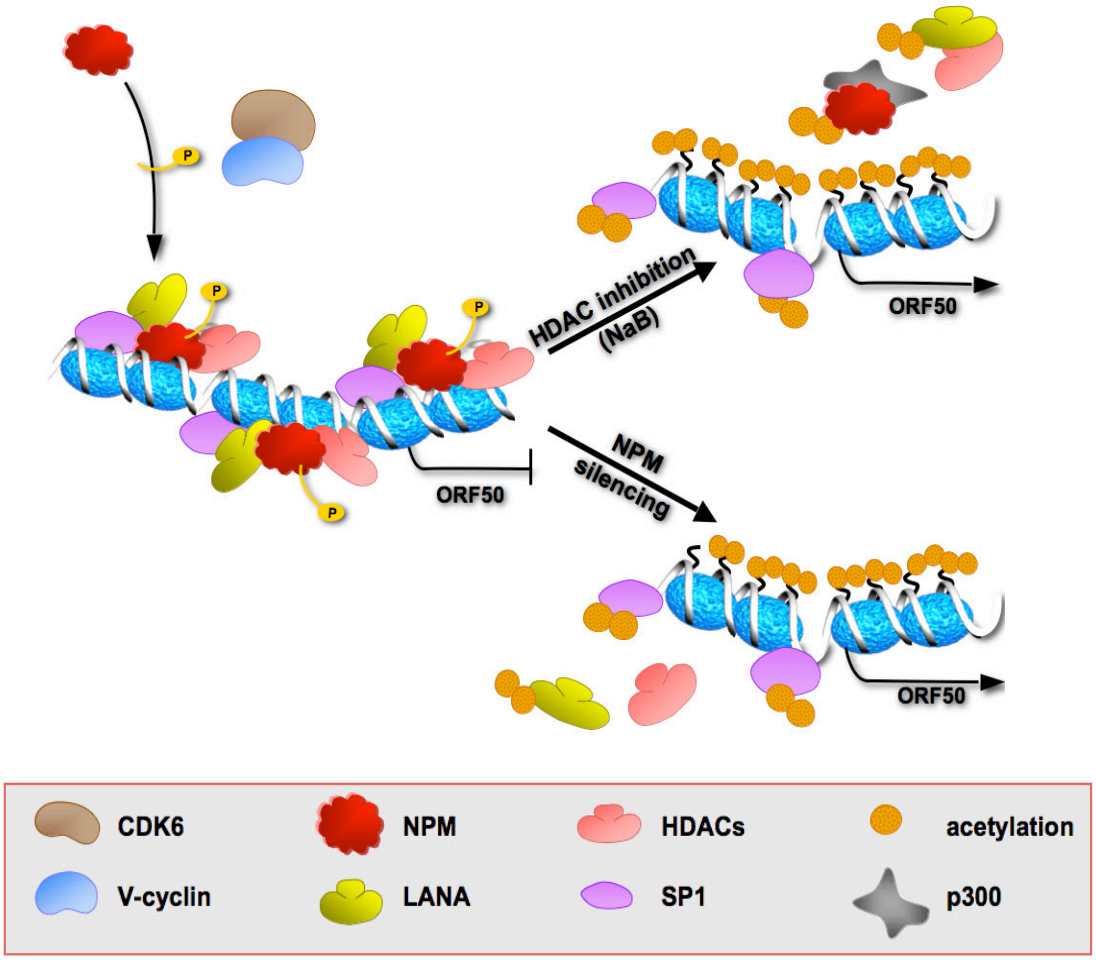

Figure 6. NPM as a novel regulator of the KSHV latent-lytic switch. This schematic model depicts the role of NPM and acetylation in the regulation of KSHV replication. In latency, NPM is phosphorylated by v-cyclin-CDK6, and this phosphorylation is necessary for NPM association wih LANA. The NPM-LANA complex is bound to chromatin and recruits HDACs to the immediate/early lytic promoters. This is essential to maintain the transcriptional repression of viral lytic genes. Following treatment with an HDAC inhibitor, NaB, NPM and LANA are acetylated, and the LANA-NPM complex dissociates, leading to viral reactivation. Similarly, silencing of NPM leads to an increase in the LANA acetylation and its dissociation from Sp1 and core histones, which facilitates viral reactivation. Both NPM depletion and NaB treatment are associated with an increase in the acetylation of LANA and Sp1. This may lead to derepression of the ORF50 promoter through decreased LANA occupancy at the ORF50 promoter regulated by interactions with Sp1 and with core histones or may lead to dissociation of LANA from the RBP-Jk sites. HDACs: histone deacetylases (Adapted and modified from SABiosciences, 2009).

3.2. Phosphorylation of NPM on Thr199 correlates with KSHV latency

Since v-cyclin promoted NPM-LANA interaction, we examined whether there existed any correlation between levels of v-cyclin, pNPM Thr199, and spontaneous viral reactivation in PEL cells (IV). Western blotting analysis, performed on total PEL cell extracts, revealed a correlation between phosphorylated NPM and v-cyclin expression levels (Fig. 5A in IV). To investigate whether NPM phosphorylation indeed

contributes to the interaction between NPM and LANA, we performed LANA immunoprecipitation from extracts of JSC-1 and BC-3 cells. We found a reduced amount of NPM complexed with LANA in JSC-1 cells, which express a low level of pNPM Thr199, in contrast to BC-3 cells expressing a high level of pNPM Thr199 (Fig. 5B in IV). To examine whether NPM phosphorylation correlates with extent of viral reactivation, we performed a qRT-PCR analysis for lytic transcripts ORF50 and ORF57. Interestingly, the level of endogenous pNPM Thr199 inversely correlated with the spontaneous expression of both lytic markers (Fig. 5C in IV). The cells with lower pNPM Thr199 levels had higher levels of spontaneous expression of the lytic markers than did cells with elevated pNPM Thr199 (Fig. 5C in IV). These results thus suggest that the phosphorylation of NPM on Thr199 may play a key role in regulating of viral latency.

Finally, we should consider the clinical implications of the results obtained. Despite major improvements in the treatment of KSHV-associated malignancies, overall survival of patients remains poor, making new prognostic markers and therapeutic strategies urgently needed. Apart from the potential diagnostic value of measuring KSHV viral loads by routine PCR assays, or by serological methods mainly used to differentiate KS from its mimics, the lack of any reliable prognostic factor able to predict patient outcome complicates potential therapies. Because we could detect pNPM Thr199 both in PEL cells (Fig. 5A in IV) and in biopsies from KS patients (Fig. 6 in IV), and the phosphorylation of NPM on Thr199 correlated with extent of viral reactivation (Fig. 5C-D in IV), one could suggest pNPM Thr199 as a prognostic factor for progression of KSHV-associated diseases. This phosphorylation could therefore be analyzed in early (patch/plaque) and late (nodular) tumor stages of the AIDS-related and endemic KS clinical forms, in relation to tumor aggressiveness and viral loads in these lesions. Although further studies need to validate pNPM Thr199 as a prognostic factor, our results provide a proof of principle for the investigation of more accurate markers for the recognition of patients at higher risk.

3.3 NPM phosphorylation is required for efficient establishment of latency (unpublished data)

The relationship between viral latency and extent of NPM phosphorylation on Thr199 inspired us to investigate whether the Thr199 phosphorylation on NPM plays a role in the establishment of latency upon *de novo* KSHV infection.

To this end, we silenced by lentivirus-mediated RNAi, CDK6 in EA.hy926, and CDK6 or CDK4 in SLK endothelial cells. Cells were then infected with rKSHV.219, and the number of LANA-positive cells was determined by immunofluorescence with anti-LANA antibodies as a measure of latent infection starting at four days after infection, and analyzed on every other day until day ten. As early as day four, the KSHV-infected, CDK6-silenced (sh-CDK6) Ea.HY926 and SLK cells showed about 30% fewer LANA-positive cells than did the non-silenced (sh-Scr) or CDK4-silenced (sh-CDK4) control cells (Fig. 7A). In line with previous studies (Grundhoff and Ganem, 2004), we observed a decline in the number of LANA-positive cells in all cell lines analyzed (Fig. 7A). These results suggest that inhibition of NPM phosphorylation in CDK6-silenced cells affects the establishment of latency, but not the rate of KSHV episome loss. Interestingly, immunoblot analysis revealed that the level of NPM Thr199 in the non-silenced control cells increased from day six to day eight post-infection and remained elevated until day ten (Fig. 7C-D). This increase correlated with an increase in the protein levels of CDK6 (Fig. 7C-D), which may suggest that KSHV infection induces CDK6 stabilization in the course of infection. Comparable results also came from NPM-silenced cells (data not shown), which further supports the role of NPM phosphorylation in the establishment of KSHV latency.

We also addressed the effect of CDK6 silencing on KSHV genome copy number in the latently infected rKSHV.219-EA.hy926 and rKSHV.219-SLK cells at four days post-infection (p.i.). To validate the method for analyzing genome copy numbers, we performed a qRT-PCR analysis using DNA derived from BCBL-1 cells. The average number of episomes in this cell line was approximately 67 copies (data not shown), and is in accordance with previous results (Lallemand *et al.*, 2000; Cai *et al.*, 2005). The qRT-PCR analysis of the KSHV-infected CDK-silenced endothelial cells demonstrated that silencing of the expression of CDK6, but not of CDK4, led to a striking reduction in the KSHV copy numbers by 80% in EA.hy926 and 53% in SLK cells at day four after infection (Fig. 7B).

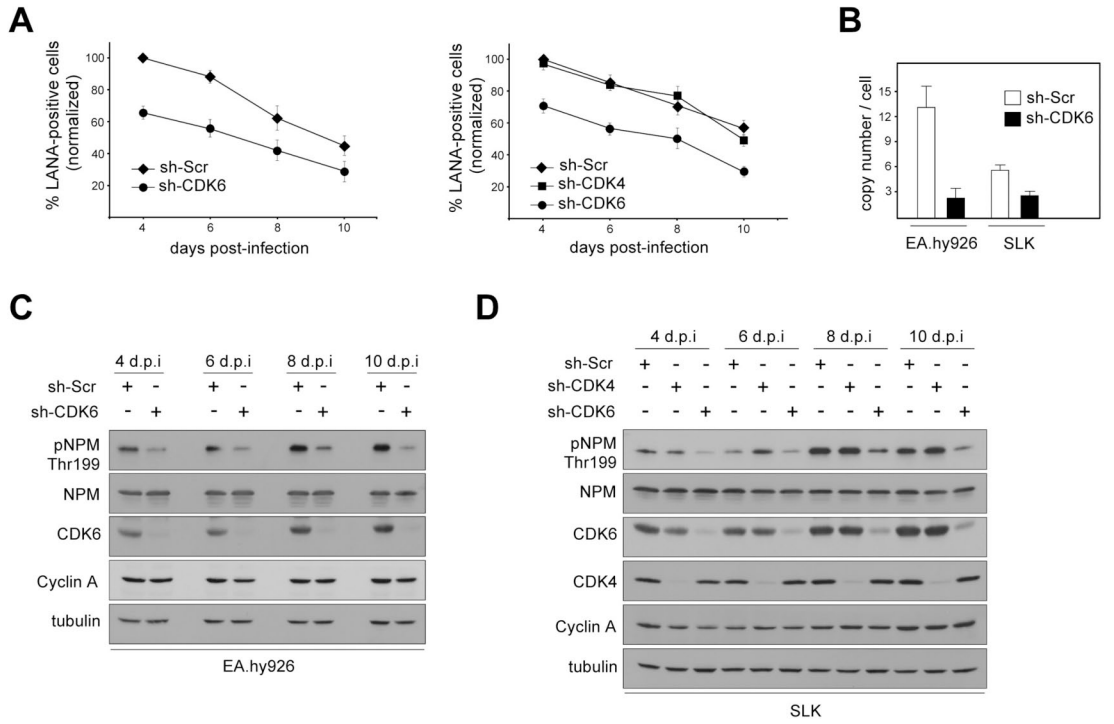

Figure 7. CDK6 silencing reduces the efficiency of establishment of KSHV latency in endothelial cells. (A) EA.hy926 cells expressing control sh-Scr or sh-CDK6 (left panel) and SLK cells expressing control sh-Scr, sh-CDK6, or sh-CDK4 (right panel) were infected with rKSHV.219 and analyzed for LANA expression by immunofluorescence at indicated time-points. Infection efficiency in EA.hy926 and SLK cells expressing control sh-RNA was set at 100%. The graph shows normalized percentage of LANA-positive cells relative to infection level in sh-Scr expressing cells. (B) The sh-Scr and sh-CDK6-expressing cells used in A were assayed for the abundance of KSHV genome copies by qRT-PCR. Each value represents the mean of two independent experiments ± SD. Western blot analysis of a time-course of rKSHV.219-infected EA.hy926 (C) and SLK (D) cells used in panel A. Whole cell extracts were subjected to SDS-PAGE, followed by immunoblotting and analysis for pNPM (Thr199), total NPM, CDK6, CDK4, and Cyclin A. Tubulin served as a loading control.

The ability of KSHV to establish and maintain latency in the host enables this virus to successfully persist in cells of endothelial and lymphoid lineages, from which it can later reactivate and thus complicate disease outcome or response to therapies. We showed that phosphorylation of NPM on Thr199 is a prerequisite for NPM-LANA interactions which allow recruitment of HDACs and maintenace of the latent infection (IV). Identification of cellular factors that mediate the establishment of latency may provide potential targets for the improvement of anti-viral strategies: preventing or inhibiting this process and the dramatic consequences that arise from viral reactivation from latency. Concerning clinical use, these approaches could include development of specific CDK-6 inhibitors, NPM antagonists, or RNA aptamers (Qi *et al.*, 2008; Jian *et al.*, 2009) targeting latency in KSHV-induced cancers.

CONCLUSIONS AND FUTURE PROSPECTS

Approximately 15% of human cancers globally are related to viral infection. KSHV is consistently associated with KS, the most widespread cancer in patients with AIDS and a primary cause of cancer deaths in Africa. In addition, KSHV causes devastating lymphoproliferative disorders such as PEL and MCD.

This thesis work evaluates the functional consequences of p53 restoration by MDM2 inhibition in KSHV-induced lymphomas. The ability of the MDM2 antagonist Nutlin-3 to effectively restore p53 function and eradicate PEL tumor cells was demonstratable both *in vitro* and *in vivo*. In mice, the notable anti-tumor effect in PEL tumors in combination with minimal side-effects suggests that restoration of p53 is a valuable, novel treatment modality for KSHV-induced malignancies. Although the strategy of targeting the p53-MDM2 interaction appears to be a promising to combat PEL malignancies, clinical trials are necessary to completely evaluate the efficacy and risks of MDM2 inhibition in these lymphomas. Restoration of p53 function has led to impressive anti-neoplastic activities in several models of common human cancers. In addition, inhibition of p53-MDM2 interaction has been suggested as a therapy for malignancies associated with extensive angiogenesis. Thus, pharmacological inhibition of MDM2 calls for evaluation of its applicability also in appropriate preclinical KS models. In fact, restoration of p53 could be tested either in mice bearing s.c. xenografts of KSHV-transformed endothelial cells or in a recently reported mouse model in which KS-like tumors form upon introduction of a recombinant GFP-expressing KSHV into murine bone marrow endothelial-lineage cells (Mutlu *et al.*, 2007).

The future of MDM2 inhibition as a treatment against KSHV-associated cancers will largely depend on successful development of targeted combinatorial therapies. The data presented here implicate viral reactivation as a factor, which can hamper the apoptotic response of p53 restoration *in vivo.* This further underscores the importance of identifying critical factors involved in the latent-lytic switch of KSHV. This study identified NPM as a novel cellular regulator of KSHV replication, meaning that NPM or inhibition of its Thr199 phosphorylation may thus represent attractive, novel targets for intervention. Knowledge achieved thus far suggests the potential for a combination treatment in which MDM2 inhibition would be combined with drugs that inhibit viral

reactivation even at very early stages. Promising drug candidates against viral reactivation would include inhibitors for Pim-1 and -3 kinases, CDK6 kinase, or NPM.

Another important focus for targeted therapies in the future against KSHV-induced malignancies will be introduction of reliable prognostic markers to assess which patients are likely to be at risk for a poor response to a given treatment, for instance due to increased viral reactivation in their tumors. In this regard, the data in this thesis imply that pNPM Thr199 levels could be further explored as a prognostic marker for a more aggressive disease or poor outcome for therapies in patients with KSHV-associated malignancies.

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