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EPSTEIN-BARR VIRUS LATENCY-ASSOCIATED PROTEINS REGULATE MULTIPLE PATHWAYS TO PROMOTE B CELL PROLIFERATION AND SURVIVAL

A Dissertation in

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by

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Abstract

Epstein-Barr virus (EBV) is a DNA tumor virus associated with persistent latent infection of B lymphocytes. Upon initial infection in B cells, EBV expresses a repertoire of 9 latency-associated proteins (EBNA1, -2, -3A, -3B, -3C, -LP, LMP1, -2A, and -2B) to modulate host cellular pathways, promoting survival and proliferation of EBV-infected B cells. Lifelong infection is achieved by expressing a series of latency programs in which latency genes are progressively downregulated to avoid immune surveillance and ensure persistent infection. EBV contributes to both lymphoid and epithelial cell malignancies, and is notably linked to the development of Burkitt lymphoma (BL). This dissertation investigates contributions of EBV latency-associated proteins to the pathogenesis of EBV infection. Our studies focused on the Epstein-Barr virus nuclear antigen 3A (EBNA3A), and in particular, the mechanisms by which EBNA3A drives proliferation of a unique and aggressive subset of BL tumors that exhibit a W promoter-restricted form of latency (Wp-R). Using shRNA-mediated knock-down, we determined that EBNA3A is essential for growth of Wp-R, as EBNA3A knock-down results in abrupt G0/G1 cell cycle arrest followed by induction of apoptosis. Increased expression of the cell cycle inhibitor $p21^{WAF1/CIP1}$ was detected, correlating with the onset of cell cycle arrest. EBNA3A-mediated repression of p21 was required to maintain hyperphosphorylation of Rb and promote cell cycle progression. Additionally, EBNA3A repressed p53-dependent transcriptional activation of p21. Whereas typical BL (Latency I) displays mutant p53, Wp-R BL contains wild-type p53. EBNA3A counteracted the wild-type status of p53 in Wp-R and inhibited induction of p53-responsive gene products. EBNA3A did not alter p53

transcription, but rather protein expression of p53 was elevated following EBNA3A knock-down. EBNA3A likely regulates p53 protein stability via interactions with HDM2. Both EBNA3A and the related family member, EBNA3C, were required for proliferation of lymphoblastoid cell lines (LCLs), but we found EBNA3C was dispensable for proliferation of Wp-R and did not affect p21 levels. Additionally, EBNA3A, but not EBNA3C, activated expression of the oncogenic microRNA, microRNA-155 (miR-155), which may contribute to p21 repression. Here we identify a unique role for EBNA3A in repressing transcriptional activation of p53 target genes, including p21, to promote cell cycle progression.

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List of Abbreviations

4HT	4-hydroxytamoxifen
AGO	argonaute protein
AhR	Aryl hydrocarbon receptor
AIDS	Acquired immunodeficiency syndrome
Akt	RAC-alpha serine/threonine protein kinase
Ang	Angiotensin
APC/C	anaphase promoting complex/cyclosome
ASPP	apoptosis-stimulating protein of p53
ASK1	apoptosis signal-regulating kinase 1
ATR	Angiotensin receptor
BARTs	*Bam*HI A fragment rightward transcripts
BCL-2	B cell lymphoma protein 2
BCL6	B cell lymphoma protein 6
BCR	B cell receptor
BHRF1	*Bam*HI H rightward fragment
BIC	B cell integration cluster
Bim	BCL-2-interacting mediator of cell death
BL	Burkitt lymphoma
BMP	bone morphogenic protein
CAK	CDK activating kinase
CAS	cellular apoptosis susceptibility protein
CD	cluster of differentiation
Cdc25	cell division cycle phosphatase

CDK	cyclin-dependent kinase
Chk	cell cycle checkpoint serine/threonine protein kinase
CKI	cyclin-dependent kinase inhibitor
CLL	chronic lymphocytic leukemia
Cp	*Bam*HI C promoter
CtBP	C-terminal binding protein
CTL	cytotoxic T lymphocyte
DLBCL	diffuse large B cell lymphoma
DMSO	dimethyl sulfoxide
DOX	doxycycline
DP	dimerization partner
DUB	deubiquitinating enzyme
EBER	EBV-encoded small RNA
eBL	endemic Burkitt lymphoma
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated protein kinase
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	germinal center
GFP	green fluorescent protein
HAT	histone acetyltransferase
HAUSP	herpesvirus-associated ubiquitin-specific protease
HDAC	histone deacetylase

HDM2	human double -minute 2
HZF	hematopoietic zinc finger
HIV	human immunodeficiency virus
HL	Hodgkin lymphoma
HPV	human papillomavirus
IFN	interferon
Ig	immunoglobulin
IM	infectious mononucleosis
KSHV	Kaposi sarcoma-associated herpesvirus
LANA	KSHV latency-associated nuclear antigen
LCL	lymphoblastoid cell line
LMP	latent membrane protein
LNA	locked nucleic acid
M phase	mitosis
MDV	Marek disease virus
MHC	major histocompatibility complex
miRNA	microRNA
mRNA	messenger RNA
NF-κB	nuclear factor kappa-light-chain enhancer of activated B cells
NLS	nuclear localization signal
NO	nitric oxide
NPC	nasopharyngeal carcinoma
NSCLC	non-small-cell lung cancer
OHL	oral hairy leukoplakia
oncomiR	oncogenic microRNA

ORF	open reading frame
OriP	EBV latency-associated origin of plasmid replication
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PFTα	pifithrin alpha
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
Pol	polymerase
PP2A	protein phosphatase 2A
pri-miRNA	primary transcript microRNA
PKA	protein kinase A
PKB	protein kinase B
PTLD	post transplant lymphoproliferative disease
PUMA	p53-upregulated mediator of apoptosis
Qp	*Bam*HI Q promoter
Ras	Rat sarcoma oncogene
Rb	retinoblastoma protein
RBP-Jκ	recombination signal binding protein Jκ
REF	rat embryo fibroblast cells
RISC	RNA-induced silencing complex
S phase	DNA synthesis phase
sBL	sporadic Burkitt lymphoma
SCID	severe combined immunodeficiency

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	small hairpin RNA
SOCS1	suppressor of cytokine signaling 1
TAP1	antigen peptide transporter protein 1
TCDD	2,3,7,8-tetrachloro-dibenzo-p-dioxin
TGFβ	transforming growth factor β
TR	terminal repeats
UTR	untranslated region
Wp	*Bam*HI W promoter
Wp-R	W promoter-restricted

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Dedication

This dissertation is dedicated to my family, who have always supported and encouraged me (and drove a moving truck across the country for me). I could not have done any of this without my mom and dad, Martha and Mike, and my sister and brother-in-law, Sarah and Erich. Thank you mom especially for leading by example and teaching me to do what makes you happy, regardless of what others think. I also dedicate this to my grandmother D'Asaro, who I know would have been so proud to see this thesis. I love you all.

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And to Roxy and Liam, who were there through it all.

"All you need is love, and a cat."

Chapter 1: Introduction

Introduction

Epstein-Barr virus (EBV) is a DNA tumor virus associated with persistent latent infection of B cells. Upon initial infection EBV expresses a set of 9 latency-associated proteins, including the EBV-nuclear antigen-3 family of proteins (EBNA-3s). Expression of EBV latency-associated proteins is linked to the development of a range of diseases, including lymphoma. The overall aim of this project was to evaluate the contribution of EBNA3A to the pathogenesis of EBV infection, specifically the mechanisms by which EBNA3A contributes to the development of a unique subset of Burkitt lymphoma (BL) tumors displaying a W promoter-restricted form of latency (Wp-R). Compared to typical BL, Wp-R exhibits accelerated tumorigenicity in mouse models, and increased resistance to apoptosis in cell culture. The expanded set of latent protein production in Wp-R tumors could contribute to their increased oncogenic potential. We determined previously that EBNA3A is required for the proliferation of Wp-R cells. EBNA3A knock-down, using an EBNA3A-targeting shRNA, results in G1 cell cycle arrest that is quickly followed by cell death. In addition, we observed a shift in retinoblastoma (Rb) protein from a predominantly hyperphosphorylated (inactive) state to a hypophosphorylated (active) state. These findings suggested that EBNA3A positively regulates cyclin/CDK complexes thereby permitting Rb phosphorylation. Indeed, we found that following knock-down of EBNA3A, protein levels of the cyclin-dependent kinase inhibitor, p21, are increased. Experiments detailed in this thesis work sought to elucidate mechanisms by which EBNA3A represses expression of p21 and contributes to cell cycle progression.

Objective 1: Determine the contribution of EBNA3A to miR-155 regulation in B cells

Rationale:

Elevated levels of microRNA-155 (miR-155) have been detected in multiple types of cancer, including lymphomas, and it is currently one of the miRNAs most commonly implicated in the development of cancer. EBV infection of resting primary B cells is a potent inducer of miR-155 expression, which may contribute to the activated phenotype of EBV-infected B cells. miR-155 is highly expressed in EBV-infected B cells displaying Latency III, but not in cells displaying Latency I, suggesting latency-associated proteins expressed during Latency III play a role in miR-155 activation. Recently it was determined that miR-155 is essential for EBV-mediated immortalization of B cells and proliferation of lymphoblastoid cell lines (LCLs). Inhibition of miR-155 activity in this system results in abrupt G1 cell cycle arrest and increased apoptotic cell death. Previously, we demonstrated that knock-down of the EBV latent protein EBNA3A also results in loss of proliferation and enhanced apoptotic cell death in both Wp-R BL and LCLs. The similar phenotypes observed following knock-down of EBNA3A and inhibition of miR-155 led us to hypothesize that EBNA3A regulates expression of miR-155, and by this mechanism, promotes cell cycle progression.

Findings:

We concluded that EBNA3A positively regulates miR-155 expression in both Wp-R cells and LCLs. Following shRNA-mediated knock-down of EBNA3A, levels of miR-155 were decreased relative to vector control-transfected cells. We also

determined that expression of EBNA3A is sufficient to induce miR-155 levels, however it is likely that a cooperative effort among multiple EBV latency-associated proteins is necessary to stimulate maximal miR-155 expression. Our results indicated that the related EBNA3 family member, EBNA3C, does not contribute to miR-155 regulation. Whereas EBNA3A contributes to activation of miR-155 in both Wp-R and LCLs, we determined that this function is required only for proliferation of LCLs. Inhibition of miR-155 activity in Wp-R did not significantly alter proliferation of these cells, whereas in LCLs, miR-155 inhibition resulted in rapid cessation of growth. As opposed to that in LCLs, the lower basal levels of miR-155 in Wp-R may not be sufficient to confer a proliferative advantage in these cells. In LCLs, inhibition of miR-155 resulted in increased p21 expression, implicating a potential pathway by which EBNA3A can regulate p21 levels in LCLs.

Objective 2: Identify the mechanism by which EBNA3A represses protein production of the cell cycle inhibitor p21$^{WAF1/CIP1}$

Rationale:

Previous experiments in Wp-R demonstrated that EBNA3A promotes G1/S cell cycle progression by repressing expression of p21, thereby permitting Rb hyperphosphorylation. However, the mechanisms by which EBNA3A mediates repression of p21 were unclear. Regulation of p21 protein levels could occur via alterations to protein stability and degradation, or at the level of transcription of the p21 gene. Since EBNA3A functions as a transcription factor and regulates expression of multiple cellular genes, we hypothesized that EBNA3A may repress

transcription of p21. Additionally, we sought to determine whether EBNA3A-mediated repression of p21 was due to a p53-dependent or -independent pathway. Mutated p53 has been observed in EBV-associated BL, however we previously determined that the Wp-R subset of BL displays wild-type p53. In Wp-R, EBNA3A could contribute to repression of p53 expression or activity, thereby repressing p53 target genes such as p21.

Findings:

The accumulation of p21 we observed in cells transfected with EBNA3A shRNA suggested that this cell cycle inhibitor contributed to the cell cycle arrest that follows EBNA3A knock-down. To test whether this protein mediates loss of proliferation, we used p21-targeting antisense oligonucleotides to prevent the accumulation of p21 following EBNA3A knock-down and evaluated whether this could rescue cell growth. Indeed, p21 knock-down restored proliferation in cells transfected with EBNA3A shRNA.

We also detected increased p21 mRNA levels following EBNA3A knock-down, and further, levels of p21 primary transcript and promoter activity were similarly elevated, suggesting a transcriptional mechanism of repression. To determine whether increased p21 levels following EBNA3A knock-down were dependent on p53, we inhibited p53 activity with the chemical inhibitor, pifithrin-α (PFTα). In cells transfected with EBNA3A shRNA, PFTα treatment rescued cell growth and prevented p21 accumulation. In addition, PFTα treatment promoted hyperphosphorylation of Rb, consistent with reduced levels of p21, an inhibitor of Rb phosphorylation. We

concluded that p21 induction and subsequent cell cycle arrest following EBNA3A knock-down is due, at least in part, to a p53-dependent mechanism.

In reporter assays, EBNA3A did not regulate transcription of the p53 gene, indicating that EBNA3A represses p53 activity rather than expression. Indeed, using a p53-responsive luciferase reporter, we determined that EBNA3A affects p53 activity, likely by regulating p53 protein stability. Protein levels of the p53 negative regulator, HDM2, are decreased following EBNA3A knock-down.

Summary and overall significance:

Both EBNA3A and -3C are required for the immortalization and proliferation of LCLs. This requirement was previously thought to stem from their shared contributions to repress cell cycle inhibitors, p14 and p16. However EBNA3A cannot compensate for EBNA3C, and vice versa, suggesting the two likely have additional distinct functions. We identified two unique functions of EBNA3A: regulation of the oncogenic microRNA miR-155, and repression of the cell cycle inhibitor p21. EBNA3A-mediated repression of p21 seems to be particularly critical in Wp-R, in which p16 is irreversibly silenced, thus possibly negating the requirement for EBNA3C to sustain proliferation. Indeed, we observed that EBNA3C is not required for the proliferation of Wp-R. In LCLs, a cellular context in which p16, p14, and p21 are expressed, both EBNA3A and EBNA3C are critical for proliferation.

We also determined that EBNA3A repression of p21 is p53-dependent. EBNA3A-mediated targeting of p53 may have consequences for the accumulation of multiple p53-regulated gene products, including the pro-apoptotic protein Bim.

Although typical BL displays mutant p53, Wp-R BL and LCLs contain wild-type p53. In typical BL, which does not express EBNA3A, the mutated status of p53 serves to repress expression of downstream effectors such as p21. Our data suggests a model in which expression of EBNA3A counteracts wild-type p53 in Wp-R and LCLs to promote cell cycle progression. We cannot discount the possibility that EBNA3A may also contribute to p21 repression independently of p53, possibly via regulation of miR-155.

Chapter 2: Literature Review

2.1 Epstein-Barr virus

The discovery and biology of EBV

In the early 1960s, concerted efforts of Epstein, Barr, and Burkitt led to the identification of EBV as the first human tumor virus [1–3]. Development of jaw tumors in sub-Saharan African children was linked to the presence of previously unidentified herpesvirus particles. EBV was determined to be a member of the γ-herpesvirus sub-family in the genus *Lymphocryptoviridae*. γ-herpesviruses are lymphotropic, and EBV displays predominantly B cell tropism. EBV contains a linear double-stranded DNA genome of 172 kilobase pairs in length, which is maintained as an episome within nuclei of infected cells [4,5]. The genome encodes roughly 90 genes, in addition to multiple miRNAs and non-coding RNAs [6–8].

The EBV life cycle

EBV infection typically occurs during childhood via salivary transmission, and presents as an asymptomatic infection. However when initial infection is delayed until adolescence, individuals may develop infectious mononucleosis, a relatively benign illness characterized by malaise and splenomegaly [9,10]. By adulthood, approximately 90% of people are infected with EBV. Primary EBV infection occurs in the oropharynx following exposure to infected saliva, and EBV can be detected in oropharyngeal excretions from both healthy and immunocompromised hosts [11–15]. Although exact

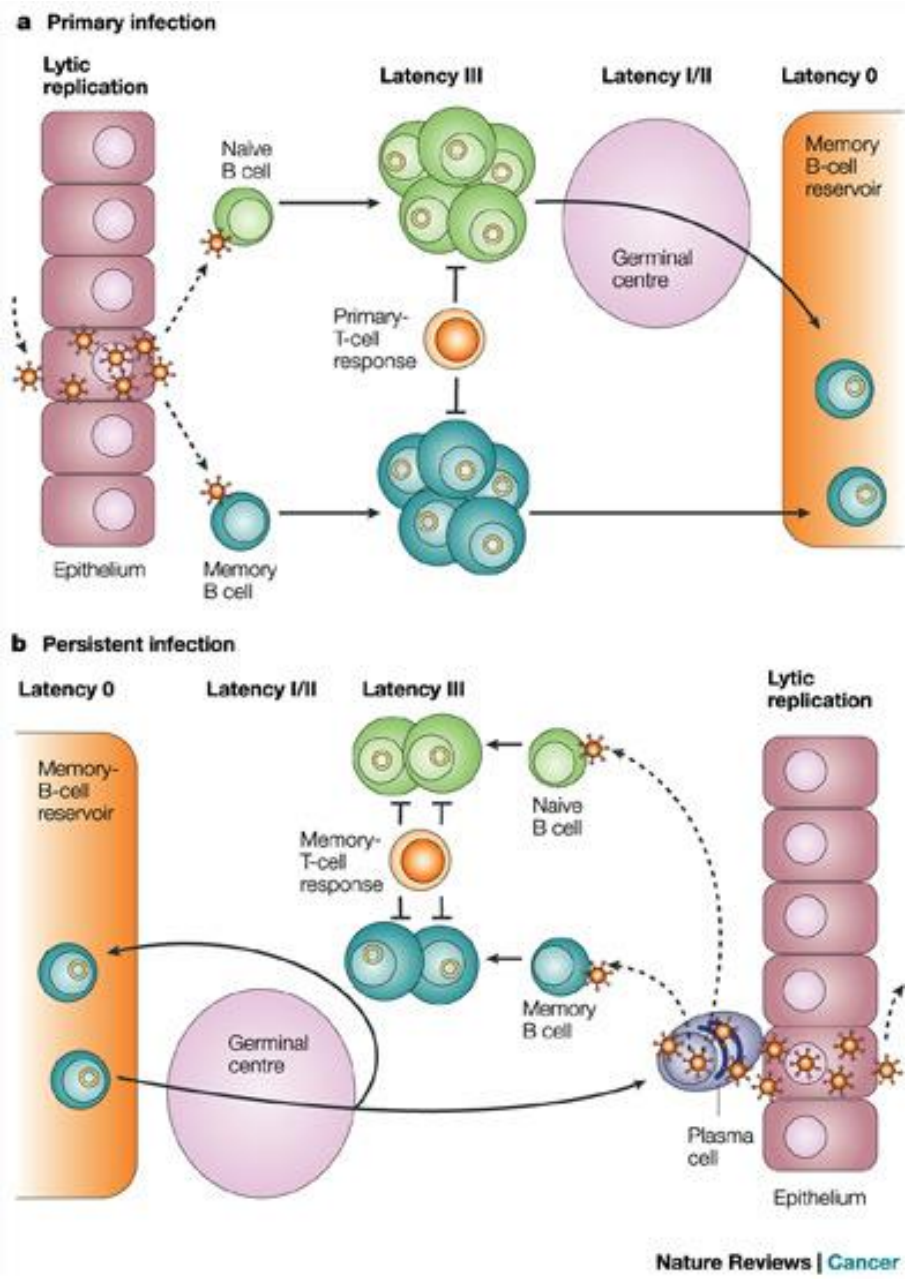
mechanisms by which EBV crosses epithelial barriers to enter the B cell compartment are unclear, replication in the oral epithelium likely facilitates viral amplification and dissemination. Infection of tonsillar B cells is observed following acute infection [16,17]. EBV establishes latency within B cells, and promotes expansion of these cells, thereby increasing the reservoir of EBV within the host (**Fig. 2.1**) Latency-associated proteins stimulate B cell proliferation, however these antigens also activate a robust EBV-specific cytotoxic T lymphocyte (CTL) response in the immunocompetent host. To avoid immune surveillance and ensure persistence, EBV gradually limits its latent protein expression through a series of latency programs [18,19]. Periodically, lytic cycle reactivation in latently infected B cells leads to release of infectious virions [20]. In this way, EBV establishes a carrier state in healthy hosts in which persistent, latent infection is intermittently punctuated by productive viral replication, facilitating transmission between individuals.

Figure 2.1: Putative in vivo interactions between Epstein-Barr virus and host cells

(A) Primary infection. Incoming virus establishes a primary focus of lytic replication in the oropharynx, after which the virus spreads throughout the lymphoid tissues as a latent (Latency III) growth-transforming infection of B cells. Many of these proliferating cells are removed by the emerging latent-antigen-specific primary T-cell response, but some escape by downregulating antigen expression. This establishes a stable reservoir of resting memory B cells, in which viral antigen expression is mostly suppressed (Latency 0). Different views of these events are shown. One view is that naive B cells are the main targets of new EBV infections *in vivo*. In this scenario, viral transformation drives naive cells into memory by mimicking the physiological process of antigen-driven memory-cell development in lymphoid tissues, a process involving somatic immunoglobulin gene hypermutation during transit through a germinal center. However, this is difficult to reconcile with the finding that EBV-infected B cells in tonsils from patients with infectious mononucleosis (IM) localize to extrafollicular areas — not to germinal centers — and show no evidence of ongoing hypermutation within expanding clones. An alternative view therefore envisages infection of pre-existing memory cells as a direct route into memory; this is consistent with the above observations on IM tonsils, but still leaves unexplained the apparent disappearance of the infected naive cell population. **(B)** Persistent infection. The reservoir of EBV-infected memory B cells becomes subject to the physiological controls governing memory-B-cell migration and differentiation. Occasionally, these EBV-infected cells

might be recruited into germinal center reactions, entailing the activation of different latency programs, after which they might either re-enter the reservoir as memory cells or commit to plasma-cell differentiation, possibly moving to mucosal sites in the oropharynx and, in the process, activating the viral lytic cycle.

Figure 2.1: Putative in vivo interactions between Epstein-Barr virus and host cells



2.2 EBV latency

Latency programs

Establishment of lifelong, latent infection is a hallmark characteristic of all herpesviruses. Although the majority of EBV gene products are associated with lytic replication, expression of a set of 9 latency proteins is particularly important, as virtually all known EBV-associated diseases are characterized by latent infection. Following B cell infection and establishment of latency, EBV expresses all 9 latency-associated proteins that cooperate to promote cellular proliferation and survival in the program Latency III [22]. Over time, latent gene expression is progressively downregulated through various latency programs until true latency is reached, in which no genes are expressed (Latency 0) [23,24] (**Table 2.1**). Currently, 4 naturally occurring latency programs (and 1 unique program associated specifically with disease) have been described, each characterized by the usage of different EBV promoters to drive expression of distinct patterns of latency: *BamHI* Q promoter (Qp), *BamHI* C promoter (Cp), and *BamHI* W promoter (Wp). There are six EBV nuclear antigens (EBNAs), EBNA-1, -2, -3A, -3B, -3C, and -leader protein (LP), and three latent membrane proteins (LMP), LMP-1, -2A, and -2B. In addition, the non-coding RNAs, *BamHI* A fragment rightward transcripts (BARTs) and EBV-encoded small RNAs (EBERs), are also detected in latency[21] (**Fig. 2.2**).

Table 2.1: EBV latency programs and their expression in B lymphocytes and EBV-associated malignancies

http://www.nature.com/onc/journal/v23/n11/fig_tab/1207372t1.html#figure-title

Table 2.1: EBV latency programs and their expression in B lymphocytes and EBV-associated malignancies

Latency program	*Expressed viral genes*	*Normal B cell*	*Disease*
Latency 0	None	Memory B cells	None
Latency I	LMP-2A/EBNA-1	Memory B cells[a]	Burkitt's lymphoma[b]
Latency II	EBNA-1, LMP-1, LMP-2A, -2B	Germinal center centroblasts	Hodgkin's disease, Peripheral T-cell lymphoma, nasal T/NK cell lymphoma, nasopharyngeal carcinoma, lymphoepithelioma (stomach, thymus)
Latency III	EBNA-1, -2, -3, -4, -5, -6, LMP-1, LMP-2A, -2B	Immunoblasts	Infectious mononucleosis, AIDS-related immunoblastic B-cell lymphoma, post-transplant lymphoproliferative disorder (PTLD)

[a]LMP-2A mRNA may be the only viral transcript detected in circulating memory cells. [b]EBNA-2 is usually not expressed in BL cells while some tumors were shown to express EBNA-3, -4 and -6

Figure 2.2: The Epstein-Barr virus genome

(A) Diagram showing the location and transcription of the EBV latent genes on the double-stranded viral DNA episome. The origin of plasmid replication (OriP) is shown in orange. The large green solid arrows represent exons encoding each of the latent proteins, and the arrows indicate the direction in which the genes encoding these proteins are transcribed. The latent proteins include the six nuclear antigens (EBNAs 1, 2, 3A, 3B and 3C, and EBNA-LP) and the three latent membrane proteins (LMPs 1, 2A and 2B). EBNA-LP is made from RNAs containing a variable number of repetitive exons. LMP2A and LMP2B RNAs are composed of multiple exons, which are located on either side of the terminal repeat (TR) region, which is formed during the circularization of the linear DNA to produce the viral episome. The blue arrows at the top represent the highly transcribed non-polyadenylated RNAs EBER1 and EBER2, and BARF0/1 non-coding RNAs from the *Bam*HIA region are shown by black arrows. The red arrow represents the EBNA1 transcript originating from Qp in Latency I/II. Latency III is characterized by a long primary transcript originating from Cp or Wp, which is then differentially spliced to generate the individual EBNA mRNAs (indicated by the green arrow). **(B)** Location of open reading frames for the EBV latent proteins on the *Bam*HI restriction-endonuclease map of the prototype B95.8 genome. Pink boxes located at the linear termini indicate TR region, and the red box represents OriP. *Figure adapted from original by Andrew Brooks*.

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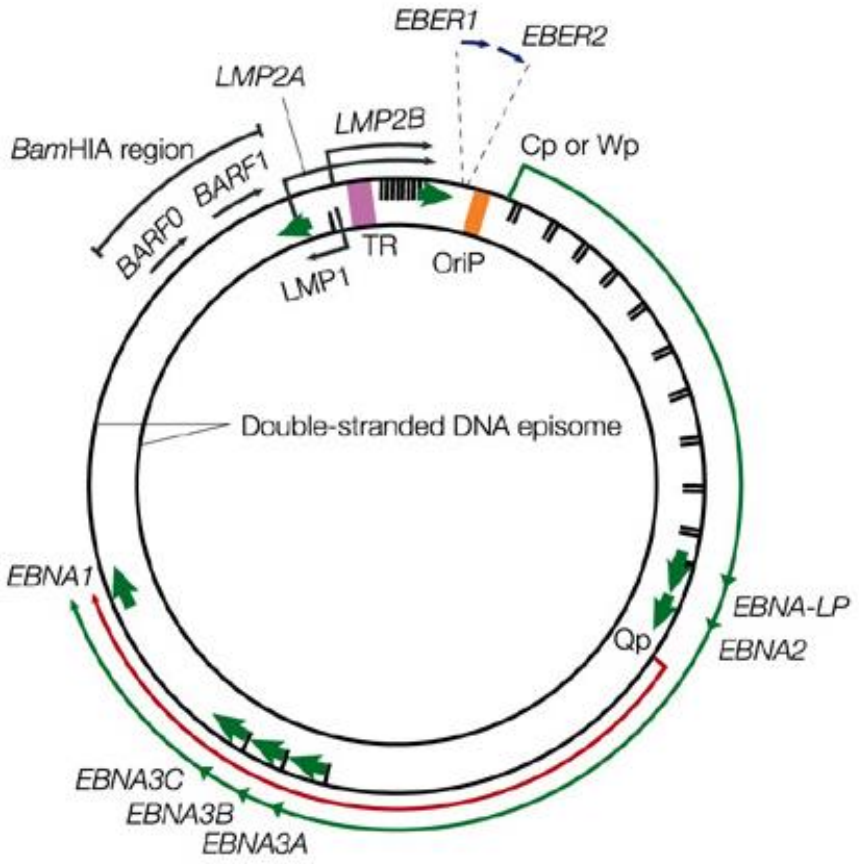
http://www.nature.com/nrc/journal/v4/n10/fig_tab/nrc1452_F1.html

Figure 2.2: The Epstein-Barr virus genome

A.



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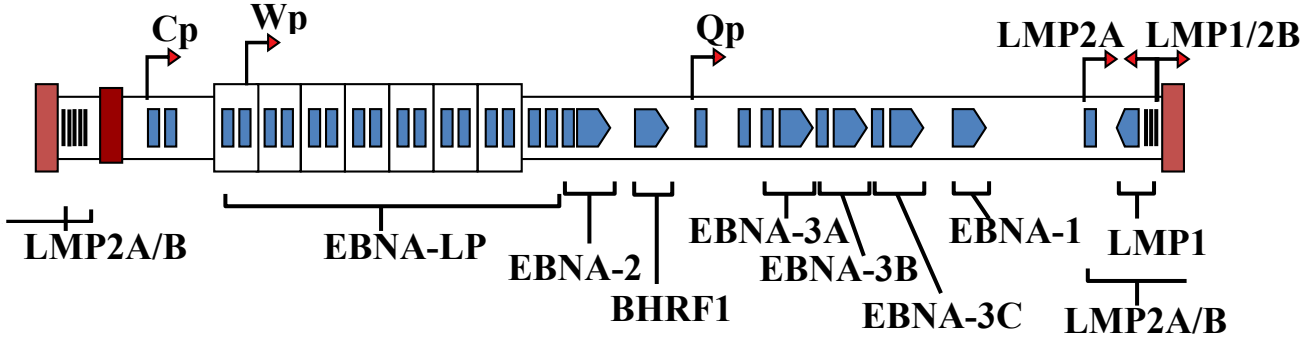


Latency III

Latency III, in which all 9 latency-associated proteins are detected, is the initial program entered by EBV infected B cells. These proteins function to activate B cells and expand the reservoir of EBV within the host [22]. Upon entering the host cell nucleus, the viral genome circularizes by about 12 h post infection [26]. B cell-specific transcription factors then bind and activate Wp, the constitutive B cell promoter, resulting in expression of EBNA2 and EBNA-LP, spliced from a primary mRNA transcript that additionally yields the other EBNAs [27–30]. EBNA2 and EBNA-LP cooperate to transactivate LMP promoters and also activate Cp, which becomes the dominant promoter driving expression of EBV latent genes [31–33]. The EBNA3 proteins compete with EBNA2 and EBNA-LP at the Cp enhancer to regulate their own transcription [34,35]. Of the 9 latency-associated proteins expressed during Latency III, EBNA1, -2, -3A, -3C, -LP, and LMP1 are required for the immortalization of B cells and generation of lymphoblastoid cell lines [36–41]. Latency III is the program most often associated with infectious mononucleosis, post-transplant lymphoproliferative disease (PTLD), and AIDS-related lymphomas [42].

Latency II

In Latency II, gene expression is limited to EBNA1, driven from Qp, and the LMPs, as well as non-coding RNAs [24]. Hodgkin lymphoma (HL) is the only EBV-associated disease consistently harboring Latency II [43]. EBV infection is linked to approximately 40% of HL cases, with tumors expressing monoclonal EBV genomes. Nasopharyngeal carcinoma (NPC), gastric carcinoma (GC), and NK/T cell

lymphomas also display Latency II, however, in these instances expression of LMP1 is variable [44].

Latency I

In Latency I only Qp-driven EBNA1 as well as non-coding RNAs are detected. This restricted latency program is important for immune evasion. EBNA1 contains an internal glycine-alanine repeat region that interferes with proper antigen processing and major histocompatibility complex (MHC) class I presentation to EBNA1-specific CTLs [45,46]. Endemic BL is classically associated with this latency state [23].

Latency 0

Latency 0 represents true latency and is characterized by the absence of any latent gene expression, however the non-coding RNAs, BARTs and EBERs, are still detected in this latency program. Memory B cells display Latency 0, however cycling between Latency 0 and Latency I is crucial for episomal maintenance of the genome [47]. Currently, no human malignancies or diseases have been linked to Latency 0.

Wp-R latency

Wp-R latency, first observed in an atypical subset of BL tumors, drives viral protein production solely from the Latency III-associated promoter, Wp, rather than the Latency I promoter classically linked to BL, Qp. These tumors contain a unique EBV genome with a deletion in the region encoding EBNA2 and a segment of EBNA-LP, resulting in a distinct pattern of latent gene expression that includes EBNA1,

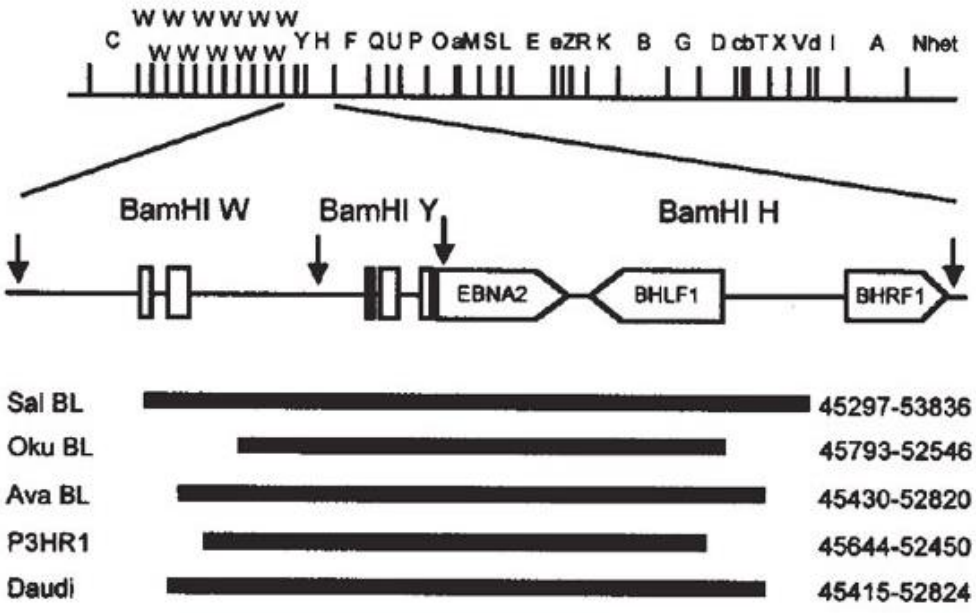
EBNA3s, and truncated EBNA-LP, but no detectable EBNA2 or LMPs [23,48]. The loss of EBNA2 likely explains the lack of LMP expression because EBNA2 mediates transactivation of LMP promoters, [49–52]. Additionally, BHRF1, a viral homolog of BCL2 typically expressed during lytic cycle, is detected in Wp-R as the genomic deletion brings the BHRF1 open reading frame in proximity to Wp, facilitating latency promoter-driven expression [53,54] (**Fig. 2.3**).

Figure 2.3: EBNA2 gene deletion in Wp-R cell lines

Comparison of the EBNA2 gene deletions in Wp-R cell lines, Sal, Oku, Ava, P3HR1, and Daudi cell lines. Shown at the top is a *Bam*HI restriction map of the EBV B95.8 strain virus genome, with an expanded view of the *Bam*HI W, *Bam*HI Y and *Bam*HI H fragments (arrows indicate restriction sites). Open boxes identify the *Bam*HI W1, W2 and *Bam*HI Y1, Y2 and Y3 exons together with the open reading frames encoding EBNA2 and two lytic cycle products BHLF1 and BHRF1. Shown below are the positions of the deletions (solid black bars) and their coordinates relative to the B95.8 nucleotide sequence.

http://www.nature.com/nm/journal/v8/n10/fig_tab/nm758_F6.html

Figure 2.3: EBNA2 gene deletion in Wp-R cell lines



EBV latent proteins and RNAs

EBNA1

EBNA1 is the only protein expressed during all latency programs, with the exception of Latency 0, and is essential for immortalization of B cells [55]. Through its DNA binding domain, EBNA1 functions to tether the viral episome to host chromosomes, thereby ensuring genome maintenance in dividing cells [56–60]. EBNA1 can be variable in size due to the presence of glycine-alanine repeats. These repeats impede proteasome-mediated degradation of EBNA1, thus reducing antigen processing and MHC presentation. Limited mRNA translation of EBNA1 also serves to hinder immune recognition [45,46]. EBNA1 contributes to activation of Cp and also regulates its own expression from Qp [61–63].

The role of EBNA1 in tumorigenesis is still controversial, even though it has been detected in all forms of EBV-associated malignancy. In mouse models, EBNA1 was proposed to cooperate with Myc in the development of lymphoma [64], however recent studies failed to corroborate these findings [65].

EBNA2

Following infection, EBNA2 is among the first proteins expressed [66]. It was determined to be required for B cell immortalization after virus harvested from the Wp-R cell line, P3HR1, was found to be non-immortalizing until EBNA2 was complemented in trans [41,67]. EBNA2 is an important transactivator of both viral and cellular genes through its association with recombination signal binding protein-

Jκ (RBP-Jκ/CBF1) [68]. While RBP-Jκ can mediate transcriptional repression when associated with a co-repressor complex, its interactions with intracellular Notch, activated following Notch receptor engagement, functions to activate transcription [69]. EBNA2 mimics constitutively active Notch, binding RBP-Jκ to mask its repressor domain and recruit transcriptional machinery [70] (**Fig. 2.4**). Microarray data implicated EBNA2 in the regulation of over 500 genes, including B cell activation markers, CD23 and CD21, as well as the Myc oncogene [70–75]. It is likely EBNA2 can regulate gene expression via interactions with additional transcription factors, such as Spi-1/PU.1, CBF2, and AUF1 [76–78].

EBNA-LP

EBNA-LP is encoded by the leader sequence of EBNA2 [79]. Similar to EBNA1 and -2, EBNA-LP is essential to immortalize B cells [80]. It was demonstrated that mutations in EBNA-LP, such as truncations observed in Wp-R, lead to reduced transformation efficiency [37]. EBNA-LP cooperates with EBNA2 to activate viral promoters, however EBNA-LP does not play a role in EBNA2-mediated transcriptional activation of cellular genes, indicating its ability to coactivate is promoter specific [32,33,81].

LMP1

LMP1 is critical to the B cell-immortalizing ability of EBV, and activates numerous pro-growth and survival signaling pathways. LMP1 can transform rodent fibroblasts *in vitro*, as well as contribute to lymphoma development in transgenic

mouse models [82,83]. LMP1 signaling increases cell surface expression of activation markers, CD23, CD39, CD44 [84]. Notably, LMP1 mimics a constitutively active CD40 receptor, stimulating NF-κB signaling and B cell activation [85,86].

Activities of LMP1 also have effects on cell cycle regulation. In both rat and human fibroblasts, LMP1 prevents Ras-induced senescence via repression of the cell cycle inhibitor p16 [87]. LMP1 promotes CRM1-dependent nuclear export of Ets2, a transcription factor necessary for p16 expression. Furthermore, LMP1 inhibits the cell cycle inhibitory activity of p16 by promoting nuclear export of E2F4/5, essential downstream mediators of p16-induced growth arrest [88]. Additionally, expression of LMP1 can induce Cdk2 and CyclinD1 [89]. Signaling activated by LMP1 interferes with p53-dependent apoptosis by modulation MAPK/JNK phosphorylation and induction of an anti-apoptotic mediator, A20 [90,91]. LMP1 also contributes to p53 regulation by promoting expression of a negative regulator of p53, HDM2 (Mdm2 in mice), thereby enhancing p53 degradation [92].

LMP2

The LMP2 gene encodes both LMP2A and LMP2B. These proteins are not essential for the immortalization of B cells; however LMP2A can provide signals that support proliferation and survival, such as activation of the Ras-PI3K-Akt pathway [93–98]. The amino terminus of LMP2A contains a tyrosine-based activation motif which plays a role in blocking B cell receptor-mediated signal transduction, including calcium mobilization and activation of EBV lytic replication [99]. Activity of LMP2A can be regulated by LMP2B [100].

EBERs and BARTs

The EBERs (EBER1 and EBER2) are small non-polyadenylated non-coding RNAs abundantly expressed in all EBV latency programs. The exact functions of the EBERs are still relatively unclear; however, it was established they are non-essential for B cell transformation [101]. One crucial role for the EBERs is to inhibit type I interferon (IFN) response [102]. Furthermore, EBERs support tumorigenicity of BL by inducing IL-10 and inhibiting apoptosis [103,104].

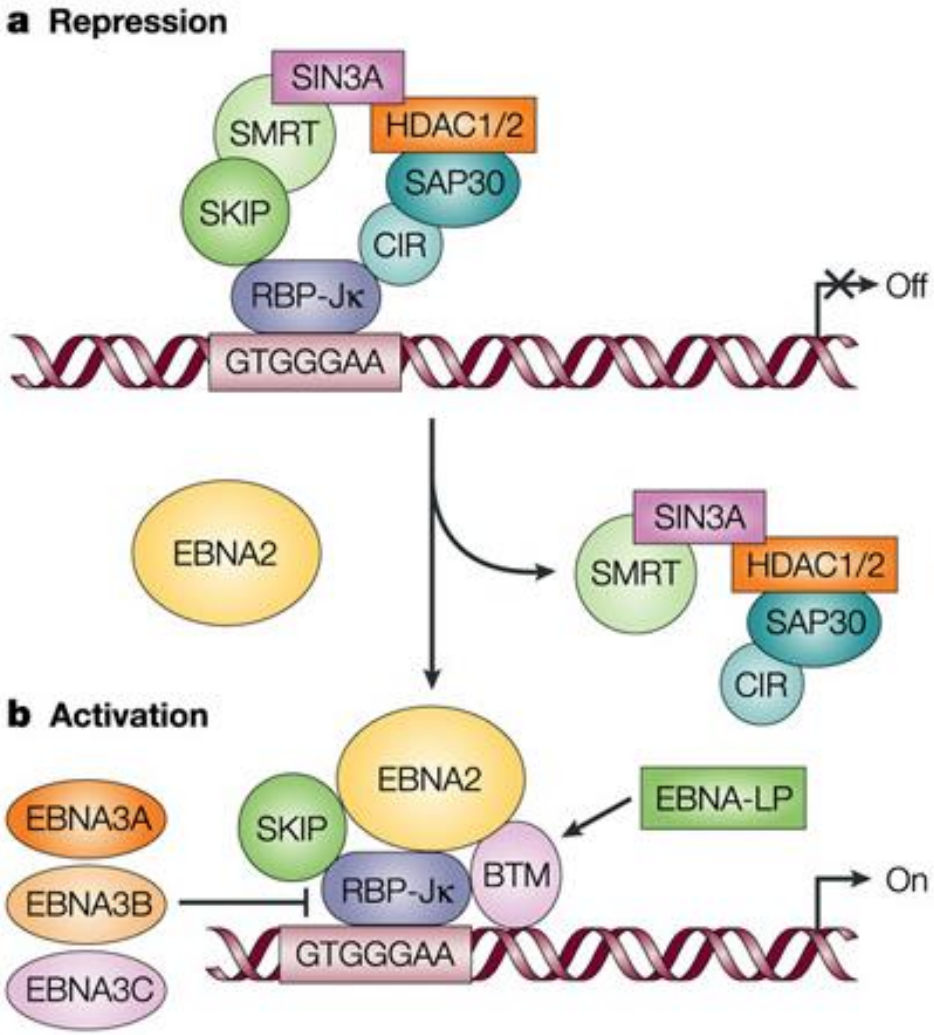
The BART family of non-coding RNAs are expressed at low levels in all forms of EBV latency [21]. BART miRNAs have been detected in both healthy individuals and EBV-associated malignancies which may suggest a role in viral persistence [105–107]. While exact BART functions are unknown, they were shown to inhibit apoptosis [108,109].

Figure 2.4: Regulation of EBNA2 and EBNA3-mediated transcription through interactions with RBP-Jκ

(A) EBNA2 functions as a transcriptional activator by interacting with RBP-Jκ and relieving the transcriptional repression mediated by a large multiprotein complex consisting of SMRT, SIN3A, histone deacetylase 1 (HDAC1) and HDAC2. SKIP (Ski interacting protein) is another RBP-Jκ-interacting protein that also associates with the SMRT–HDAC corepressor complex. EBNA2 abolishes RBP-Jκ-mediated repression by competing for the SMRT–HDAC corepressor complex through binding to both RBP-Jκ and SKIP. **(B)** In cooperation with EBNA-LP, EBNA2 recruits the basal transcription machinery (TFIIB, TFIIH and p300; not shown) to activate transcription. The EBNA3 family of proteins modulate EBNA2-mediated RBP-Jκ activation by interacting with RBP-Jκ and competing for binding and activation by EBNA2. EBNA3 association with RBP-Jκ results in transcriptional repression.

http://www.nature.com/nrc/journal/v4/n10/fig_tab/nrc1452_F2.html

Figure 2.4: Regulation of EBNA2 and EBNA3-mediated transcription through interactions with RBP-Jκ



2.3 The EBNA3 family of proteins (EBNA3A, 3B, and 3C)

Discovery

EBNA3A was first discovered when a 140-kDa nuclear protein was detected by immunoblot analysis of LCLs[110]. Soon after, two additional nuclear proteins of similar size, EBNA3B and 3C, were observed in immunoblots of LCL lysates incubated with human sera [111–113]. The EBNA3s were confirmed to be nuclear proteins associated with the nuclear matrix and excluded from nucleoli [114].

The EBNA3 proteins are arranged tandemly in the genome and likely formed following gene duplication events. While overall sequence homology between the EBNA3s is low, they share highly conserved functional domains [35]. However the EBNA3s lack any direct DNA binding domains [115]. The EBNA3s, observed in Latency III and Wp-R, are transcribed from either the C or W promoter to generate one primary transcript that is differentially spliced into EBNA3A, 3B, and 3C [28,116].

Functions

EBNA3A and EBNA3C are required for immortalization of B cells, while EBNA3B is not, suggesting some functional divergence among the EBNA3s [40]. Furthermore, EBNA3A and EBNA3C cannot compensate for one another in immortalization assays, again indicating unique roles for the EBNA3s, even though their only currently known functions are shared between 3A and 3C [117,118].

Binding to RBP-Jκ

The EBNA3s all interact with RBP-Jκ via a conserved amino-terminal domain and in this way can mediate regulation of gene transcription [119,120]. Unlike EBNA2, binding of the EBNA3s to RBP-Jκ results in transcriptional repression of target genes rather than activation (**Fig. 2.4**). Since their binding sites are in close proximity, EBNA3s could function to displace EBNA2 from RBP-Jκ and inhibit EBNA2-mediated gene transactivation [119]. The EBNA3s contribute to viral gene regulation, including their own expression, via the RBP-Jκ -binding domain, as RBP-Jκ-binding sites have been identified in both the C and LMP promoters. RBP-Jκ-binding sites have also been detected in numerous cellular genes, including Myc, p21, and p53 [73,121,122]. The interaction of the EBNA3s with RBP-Jκ is a critical component of EBNA3-mediated transcriptional regulation. Additionally, EBNA3A and EBNA3C interaction with RBP-Jκ is required for immortalization of LCLs. In complementation assays, EBNA3A and 3C with RBP-Jκ-binding domain mutants also fail to maintain growth of LCLs [123,124].

Binding to CtBP

In addition to RBP-Jκ, EBNA3A and 3C are known to interact with one other cellular protein, C-terminal binding protein (CtBP). CtBP was first identified to interact with the carboxy-terminus of adenovirus E1A, and is critical for the immortalizing function of E1A[125]. CtBP also associates with polycomb group (PcG) repressive complexes, thus contributing to histone and chromatin modification to mediate transcriptional repression [126]. Most CtBP binding partners, including EBNA3C, associate with CtBP via a Pro-Leu-Asp-Leu-Ser (PLDLS) motif [127]. In contrast,

EBNA3A binds CtBP through two cooperative non-canonical sites, ALDLS and VLDLS, and mutation in either site abolishes binding [127]. While interactions between EBNA3A/3C and CtBP are important for EBNA3-mediated gene regulation, recombinant viruses expressing EBNA3A or EBNA3C with CtBP-binding domain mutations were still immortalizing, although proliferation was reduced (Cheeyancheri *et al*., Manuscript in Preparation). EBNA3 binding to CtBP likely promotes, but is not essential for, cellular proliferation.

Repression of cell cycle inhibitors, p14^{ARF} and p16^{INK4a}

EBNA3A and EBNA3C cooperate to repress expression of the cell cycle inhibitors p14 and p16 by interacting with CtBP [128,129]. In LCLs that conditionally express EBNA3A and EBNA3C, loss of EBNA3C results in robust increases in p14 and p16 protein levels, but only modest changes are observed in the absence of EBNA3A [129]. Even so, following loss of EBNA3A, LCLs rapidly cease proliferating. When EBNA3A is knocked-down, transfection of shRNA specific for either p14 or p16 does not restore growth, and co-transfection of both p14 and p16 shRNAs results in only a partial rescue. However expression of human papilloma virus (HPV) E6 and E7, which inactivate p53 and Rb respectively, completely rescues LCL proliferation [129]. Cooperation between EBNA3A and EBNA3C, in association with CtBP, is essential to repress p14 and p16, but these data suggest this may not be the only role for EBNA3A in maintaining proliferation of LCLs. Rather, another elusive function of EBNA3A remained, likely connected to p53 or Rb activity.

Repression of the pro-apoptotic protein, Bim

Another example of cooperation between EBNA3A and EBNA3C is in the repression of BCL-2-interacting mediator of cell death (Bim). BCL-2 family members contribute to regulating the balance of survival and apoptosis in lymphocyte homeostasis. Bim mediates activation of BCL-2 family death effector proteins to initiate apoptotic signaling cascades, a process which is critical for autoantigen-driven deletion during lymphocyte development [130–132]. In a murine model of BL (the Eμ-*myc* mouse) deletion of Bim accelerates tumorigenesis, implicating Bim as an important mediator in the development of BL [133]. Additionally, EBV-positive BL cells are more resistant to apoptosis compared to EBV-negative BL [134]. Infection of EBV-negative B cells with EBNA3A or 3C knock-out viruses demonstrated that these EBNA proteins are primarily responsible for Bim repression [135]. Because RBP-Jκ and CtBP are the only proteins known to associate with the EBNA3s, Bim repression is likely mediated by one of these interactions. Indeed, recent data from our lab suggest that the RBP-Jκ-binding region of EBNA3C is essential to repress Bim expression in LCLs (Cheeyancheri *et al*. Manuscript in Preparation). However, other recent studies link Bim repression to the PcG repressive complexes 1 and 2, which are often associated with CtBP [136]. Increased histone methylation (H3K27), in addition to decreased histone acetylation (H3K and H4K), of the Bim locus is important for EBNA3A/3C-mediated Bim repression. This pattern of epigenetic regulation is observed in both BL cell lines and LCLs, as well as in EBV-positive BL biopsies [137]. Further studies are necessary to elucidate the mechanism of EBNA3A/3C-mediated repression of Bim.

Unique functions of the EBNA3s

Most known functions of EBNA3A and EBNA3C are redundant, whereas very little is known about EBNA3B, apart from its non-essential role in the immortalization of B cells and association with RBP-Jκ [35,138]. EBNA3A and EBNA3C act cooperatively on some promoters to regulate gene expression, however it is unclear whether EBNA3A and 3C interact with each other [139–141]. Since EBNA3A and EBNA3C cannot compensate for one another in either the immortalization process or continued proliferation of LCLs, it is reasonable to assume that they possess unique functions.

EBNA3A binds to the aryl hydrocarbon receptor (AhR), mediating AhR nuclear translocation [142]. AhR, a transcription factor induced following exposure to environmental pollutants, promotes proliferation in the absence of its ligand (2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)) by activating Ras signaling. Additionally, AhR induces c-Myc expression and can overcome G1/S cell cycle check points [143]. In contrast, following ligand binding, AhR mediates cell cycle arrest through interactions with Rb and activation of p21 and p27 [143–145]. It is currently unknown if and how EBNA3A exploits this interaction with AhR to promote B cell proliferation, however expression of EBNA3A does counteract the growth inhibitory effects of TCDD in LCLs [142]. EBNA3A could facilitate ligand-dependent activation of AhR.

In overexpression systems, EBNA3C directly binds Rb and promotes its degradation [146], and enhances kinase activity of the S/G2 cyclin, cyclin A [147], both of which should promote cell cycle progression. There is some controversy about the role of EBNA3C in cyclin D1 regulation. One report demonstrated stabilization of

cyclin D1 in the presence of EBNA3C [148], however we and others failed to corroborate this role for EBNA3C, and in fact cyclin D1 is not detectable in Wp-R [149,150].

In studies designed to examine the contribution of EBNA3B to the pathogenesis of EBV, humanized mice infected with EBNA3B knock-out virus subsequently developed aggressive B cell lymphomas marked by very little T cell infiltration [151]. These EBNA3B-negative tumors secrete reduced levels of CXCL10, a chemokine important for tumor immune control. Repression of CXCL10 in EBNA3B-negative cells likely allows these tumors to escape CTL recognition and subsequent lysis [151]. In a clinical study, after bone marrow transplant a patient developed highly aggressive lymphoma that was unresponsive to donor-derived EBV-specific CTL immunotherapy [152]. Tumors were subsequently found to harbor a mutant EBNA3B lacking critical epitopes necessary for CTL recognition. Thus, although non-essential for virus transformation, EBNA3B could have important functions for survival of the virus within the host.

2.4 Burkitt lymphoma (BL)

In 1958 Denis Burkitt first described the disease that would become known as BL [2]. Epstein, Barr, and Achong then discovered an unknown herpesvirus in BL tumors, subsequently identified as EBV, and determined that all African patients with BL were infected with EBV [3]. Today, BL is the most common non-Hodgkin lymphoma in children and adolescents. It proved difficult to resolve whether EBV was a true cofactor of disease or simply a passenger.

c-Myc translocation and BL mutations

BL cells exhibit a characteristic chromosomal translocation of the proto-oncogene c-Myc to an immunoglobulin (Ig) heavy or light-chain locus [153,154]. The Ig enhancer elements result in constitutive transcription of what is normally regulated Myc expression. Although Myc translocations are not commonly observed in other lymphomas, this genetic change is observed in all forms of BL [155]. Myc deregulation is implicated in the development of many kinds of human tumors [156,157]. Myc tumorigenic function predominantly involves driving cell proliferation and differentiation, although excessive expression of Myc can be a potent inducer of apoptosis [158]. Myc mediates transcriptional activation of genes, such as cyclins and cellular dependent kinases (CDKs), that promote cell cycle [159,160]. When Myc is overexpressed, p14 is upregulated leading to stabilization of p53 and induction of p53-mediated apoptosis [159]. In addition, excess Myc induces genomic instability [161].

The Eμ-*myc* murine model of BL is a transgenic mouse in which production of murine Myc is under control of the Eμ IgH enhancer [162]. These mice develop tumors with similar chromosomal translocations to those observed in BL. Crossing Eμ-*myc* mice with p53-null, MDM2-null, or p19-null (murine p14 functional homolog) leads to accelerated tumor formation, a result suggesting that counteracting Myc-induced apoptosis is a critical step in lymphoma development [163–165]. Like those arising in Eμ-*myc* mice, development of BL tumors requires a 'second hit' such as p53 pathway mutation or EBV infection to counteract Myc-driven apoptosis and support cellular proliferation. p53 mutations are often observed in EBV-associated BL displaying Latency I, as well as in a high percentage of AIDS-associated cases [166–168]. Many

BL tumors also contain mutations in HDM2 and p14 genes [169,170], and frequently the p16 locus is epigenetically silenced [171].

Types of BL

There are three categories of BL: endemic BL (eBL), sporadic BL (sBL), and HIV or AIDS-associated BL. eBL typically presents as tumors of the jaw, and sBL and AIDS-associated BL are often observed in the abdomen [160,172]. These different types of BL also differ in their degree of association with EBV. eBL is highly linked to EBV infection (80-90%), however sBL and AIDS-associated BL contain monoclonal EBV less frequently (~20% and ~40%, respectively) [43,160]. Given the strong correlation with EBV infection as a cofactor of disease, this dissertation will focus on eBL

Tumor etiology and contribution of EBV to eBL

BL tumors arise from germinal center (GC) B cells [173]. The chromosomal translocation of c-Myc is considered the 'first hit' for lymphoma development. A secondary 'hit' prior to lymphomagenesis is likely EBV infection, which can function to promote cell cycle progression and transformation as well as inhibit apoptotic signaling pathways activated by Myc expression. The extremely high prevalence of EBV in BL tumors and the clonal nature of EBV episomes suggests EBV is a cofactor of disease [174]. *In vitro* studies determined that loss of the EBV genome in BL cell lines results in loss of proliferation and cell death [134,175,176]. EBV-positive and EBV-negative clones of the same Akata BL cell origin demonstrated EBV is essential for

malignant phenotypes of BL (growth in serum deprivation, anchorage-independent growth in soft agar, tumorigenicity in nude mice) [176]. Tumorigenicity can be restored in EBV-negative Akata cells by reinfecting with EBV, confirming the virus is required for oncogenesis [134,175].

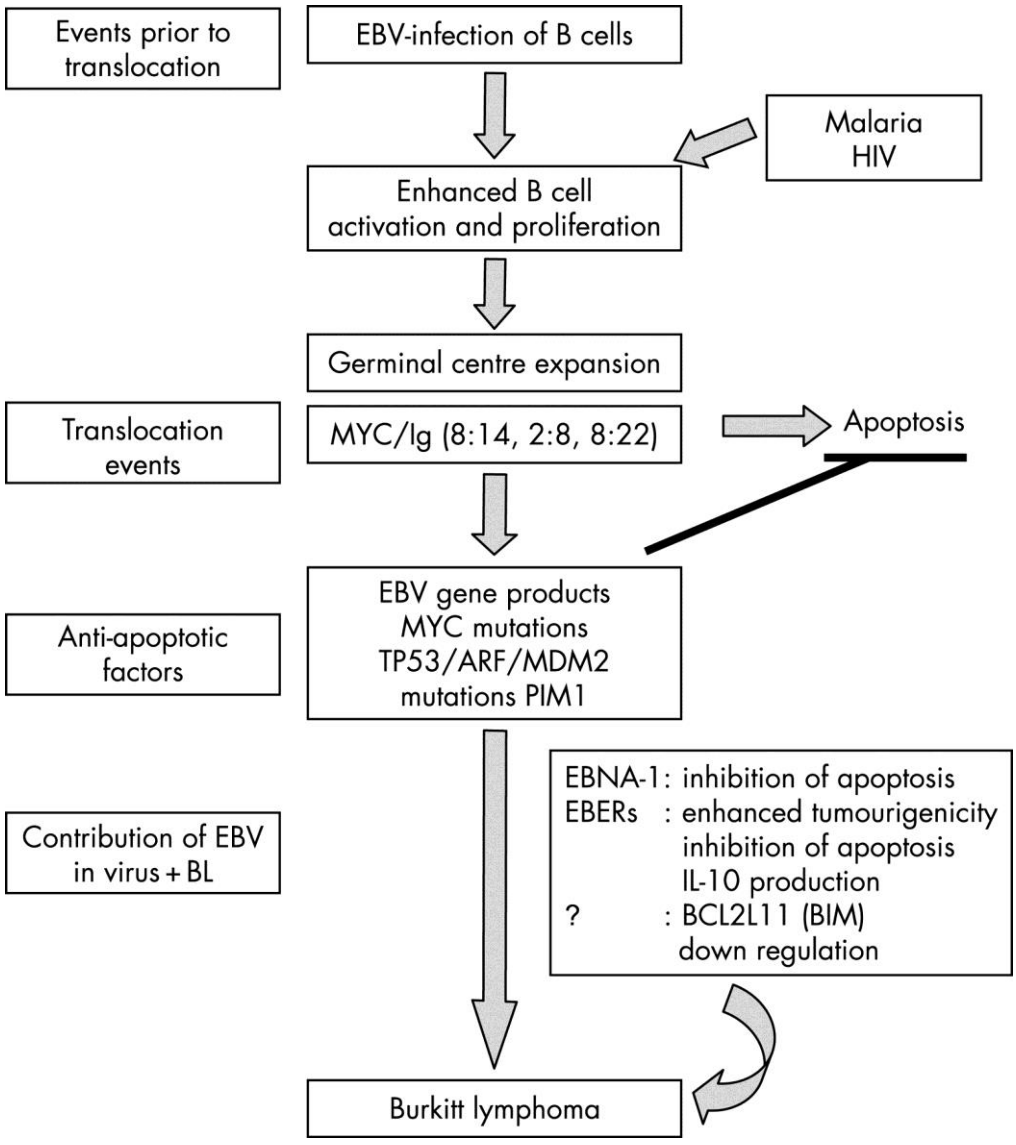
EBV could be contributing to BL development by several possible mechanisms. EBV infection of naive B cells drives them through a GC reaction to establish a pool of infected memory B cells [160]. This process likely creates a favorable environment for Myc translocation because recombination events occur during the germinal center phase of B cell development [55,177]. Additionally, EBV could stimulate proliferation and promote survival of GC B cells, prolonging their life-span and increasing the probability of Myc translocation. Latency-associated proteins also help counteract Myc-induced apoptosis and sustain cellular proliferation [160] (**Fig. 2.5**).

Figure 2.5: Contributions of EBV to the development of BL

Reproduced from Epstein-Barr virus and Burkitt lymphoma, Brady G., MacArthur GJ, and Farrell PJ, 60:1397-1402, Copyright 2007 with permission from BMJ Publishing Group Ltd. [178]

http://jcp.bmj.com/content/60/12/1397/F1.large.jpg

Figure 2.5: Contributions of EBV to the development of BL



Wp-restricted (Wp-R) subset of BL

Approximately 20% of eBL display a distinct pattern of latency characterized by exclusive use of the W promoter. These Wp-R tumors contain a unique EBV genome with a deletion that encompasses the EBNA2 open reading frame and a portion of EBNA-LP. In the absence of EBNA2 expression in Wp-R, the LMP promoters are not transactivated and thus no LMP expression is observed. Protein expression does, however, include EBNA1, the EBNA3s, truncated EBNA-LP, and BHRF1, an anti-apoptotic protein typically only expressed during lytic replication. The Wp-R genomic deletion brings the BHRF1 open reading frame in proximity to the W promoter close enough to facilitate latency-driven BHRF1 production.

These atypical Wp-R tumors resemble Latency I BL in that they contain a wild-type, transformation-competent EBV genome. Unexpectedly, they also carry a second genome, derived from wild-type, harboring the EBNA2 deletion [48,179,180]. The wild-type genome is silenced, but it is likely maintained in cells by the EBNA1 made from the deletion virus. Given the potency of T cell responses against cells making the latent EBV antigens, particularly the EBNA3s, immune surveillance may predominantly drive the restriction of EBV gene expression in BL [181]. *In vitro*, normally EBNA3-specific CTLs do not recognize Wp-R cells, suggesting a defect in antigen processing or presentation [48]. Also, because LMP1 upregulates transporter associated with antigen processing (TAP) and human leukocyte antigen (HLA) class I, LMP1 status in EBV-associated malignancies is critical for effective CTL targeting [182,183]. Accordingly, a scenario to explain *in vivo* selection for BL subsets in which Wp remains active is that deletion of EBNA2, and resulting absence of LMP1, contributes

to reduced antigen presentation, negating the requirement for Qp promoter switching in EBV gene restriction and immune evasion.

Wp-R BL tumors are more resistant to apoptosis than Latency I BL, suggesting that the expanded set of Wp-R latency proteins play a role in the resistance [150,184,185]. Enhanced apoptotic resistance also occurs following infection of EBV-negative BL with virus isolated from Wp-R cells, showing that the Wp-R EBV genome suppresses apoptotic signaling [184]. The truncated EBNA-LP protein, observed exclusively in Wp-R, also is implicated in resistance to apoptosis [186]. Truncated, but not full length, EBNA-LP inhibits apoptosis by interacting with protein phosphatase 2A (PP2A), a regulator of caspase activity [186]. On the other hand, some Wp-R BL cell lines maintain apoptotic resistance despite very low levels of truncated EBNA-LP. Additionally, while our laboratory identified a role for EBNA3C in promoting survival of Wp-R under growth limiting conditions (***Chapter 3***), a key role for BHRF1 has also been demonstrated [54]. BHRF1 is homologous to the anti-apoptotic, cellular protein BCL-2 [187]. The structure of BHRF1 and its interactions with BH3-only domain proteins Bim and p53-upregulated modulator of apoptosis (PUMA) are similar to that of BCL-2 [188]. Studies using Akata-BL (Latency I) and P3HR1 (Wp-R) implicate BHRF1 as the principle mediator of apoptotic resistance [54,189]. However, our studies demonstrated intrinsic differences between Wp-R BL cell lines in their dependence on BHRF1 (***Chapter 3***). Considering the differences between Wp-R BL cell lines, it is likely that BHRF1, truncated EBNA-LP, and EBNA3C all contribute to apoptosis inhibition to varying degrees.

Although 90% of Latency I BL contain mutations in the p53-ARF-HDM2 pathway, wild-type p53 is observed in Wp-R [160,190]. Accordingly, Latency I BL cell lines are less dependent on EBV for survival than Wp-R cell lines [191]. These findings lead us to propose a model in which expression of the EBNA3s in Wp-R overcomes the requirement for p53 inactivation to allow extended cell survival.

2.5 MicroRNAs

Almost 25 years ago, investigators first demonstrated that specific intronic regions of the genome, previously considered non-functional, did indeed have gene regulatory capacity. lin-4, the first described microRNA (miRNA), was shown to be essential for developmental timing of *Caenorhabditis elegans* by regulating expression of the lin-14 protein [192]. miRNAs are a class of small, non-coding RNAs approximately 21-23 nucleotides in length that negatively regulate gene expression at the post-transcriptional level [193]. They repress target mRNAs by preventing translation initiation and elongation [192,194–196], and in some cases mediate mRNA cleavage and decay [197]. In humans, over 1000 miRNAs have been identified and are predicted to regulate activity of more than 60% of all protein-coding genes, leading to profound consequences for cellular homeostasis [198,199]. The miRNA seed sequence (5' nucleotides 2-8) mediates miRNA interaction with the 3' untranslated region (UTR) of target mRNAs [200]. Not only can a single gene be regulated by multiple miRNAs, but a particular miRNA may also have several different mRNA targets. This allows for intricate regulation of biological processes such as cellular differentiation, proliferation, and survival [201–203].

Biogenesis of miRNAs

miRNAs are frequently encoded by intronic sequences of protein-coding genes. Many of these miRNA genes use the same promoter as the protein-coding gene, however some are under control of alternative intronic promoters and often have multiple transcriptional start sites[204,205]. Although RNA polymerase (Pol) III transcribes some viral miRNA genes, most miRNA genes are transcribed by Pol II, yielding a long primary transcript (pri-miRNA) containing a stem-loop structure from which the miRNA is eventually processed [8,206] (**Fig. 2.6**). Following transcription, the RNase III-type endonuclease, Drosha, initiates pri-miRNA processing, cleaving the stem-loop into small "hairpin" RNAs (pre-miRNA) of approximately 70 nucleotides in length [207]. Since Drosha cleavage defines miRNA termini, and thus the miRNA seed sequence and target-specificity, proper and precise recognition is essential for miRNA biogenesis. Drosha forms a microprocessor complex with its essential cofactor, DGCR8, which interacts with pri-miRNA both directly and specifically via two dsRNA-binding domains [208]. DGCR8 functions as a molecular anchor and aids in pri-miRNA recognition [209,210].

Following Drosha cleavage of the pri-miRNA, pre-miRNA hairpins are exported from the nucleus into the cytoplasm where they are recognized and cleaved by Dicer (another RNase III-type endonuclease), yielding a small RNA duplex [211–214]. Dicer contains an N-terminal helicase domain that facilitates recognition of the pre-miRNA and a PAZ domain that binds and anchors to the miRNA termini. The region between PAZ and RNase III domains serves as a 'molecular ruler' to generate small RNAs of discrete size [215–218]. The resulting small RNA duplexes are sorted onto

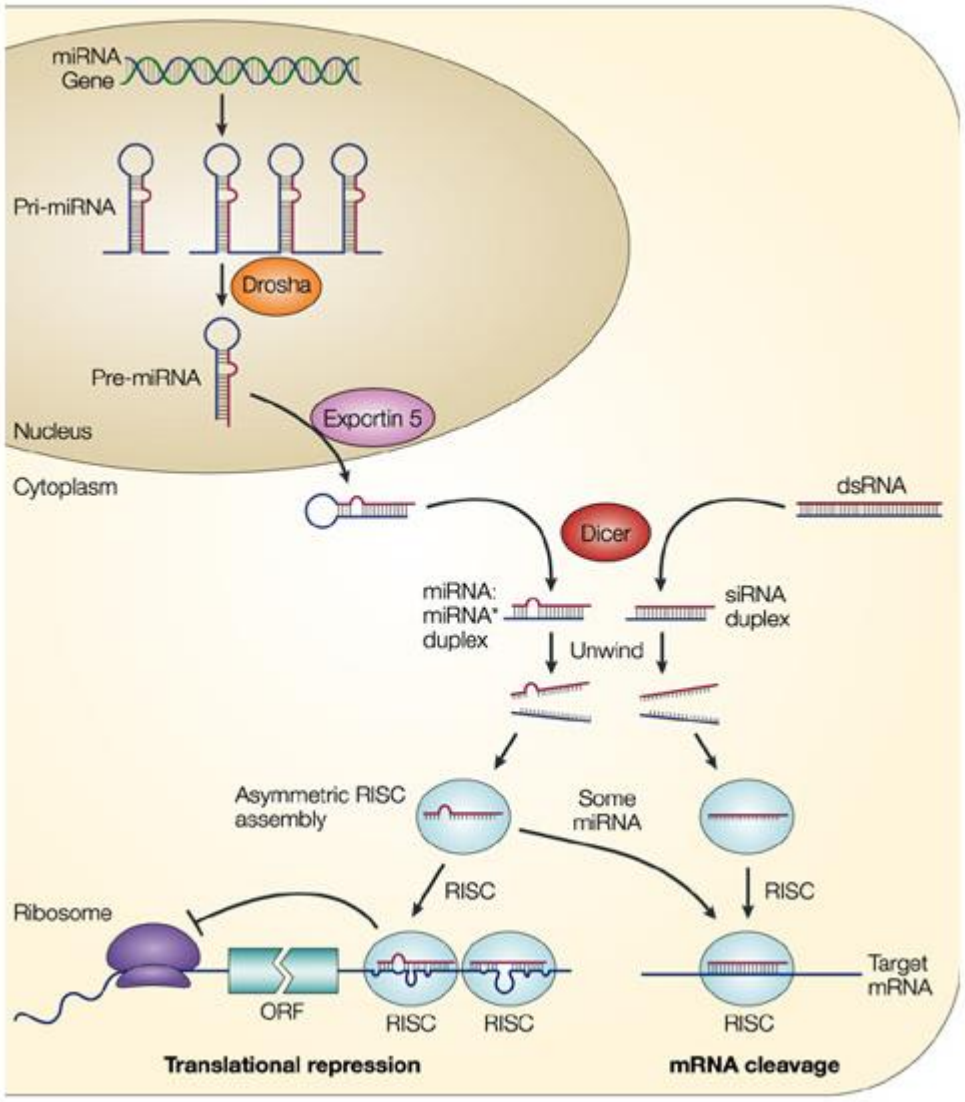
particular argonaute (AGO 1-4) protein scaffolds to form the effector complex, RNA-induced silencing complex (RISC) [219–221]. Dicer, along with its binding partner, PACT/TRBP, were proposed to play a role in RISC assembly and loading of RNA duplexes [222–224], but this idea remains controversial [225–227]. Once the miRNA duplex is loaded onto RISC, one strand of the duplex is cleaved and removed by AGO2 to generate a mature RISC [223,228,229].

Figure 2.6: miRNA biogenesis and post-transcriptional regulation

The nascent pri-microRNA (pri-miRNA) transcripts are first processed into ~70-nucleotide pre-miRNAs by Drosha inside the nucleus. Pre-miRNAs are transported to the cytoplasm by Exportin 5 and are processed into miRNA:miRNA duplexes by Dicer. Dicer also processes long dsRNA molecules into small interfering RNA (siRNA) duplexes. Only one strand of the miRNA:miRNA duplex or the siRNA duplex is preferentially assembled into the RNA-induced silencing complex (RISC), which subsequently acts on its target by translational repression or mRNA cleavage, depending, at least in part, on the level of complementarity between the small RNA and its target. ORF, open reading frame.

http://www.nature.com/nrg/journal/v5/n7/fig_tab/nrg1379_F2.html

Figure 2.6: MiRNA biogenesis and post-transcriptional regulation



Deregulation of miRNAs in cancer

Aberrant miRNA expression is associated with several diseases such as cancer, with miRNAs implicated as both tumor suppressors and oncogenes. The first evidence of miRNA involvement in cancer came from studies of chronic lymphocytic leukemia (CLL). While examining a chromosomal region frequently deleted in CLL (13q14) for the presence of protein-coding tumor suppressor genes, two tumor suppressor miRNA genes, miR-15a and miR-16-1, were discovered instead [231]. In early studies of miRNA genes, most known miRNAs were mapped to chromosomal loci prone to amplifications or deletions in many different human cancers [232]. Genome-wide profiling demonstrated that miRNA expression signatures can be used to classify specific types of cancer with high accuracy [233,234]. More recently, miRNA expression profiles have been successfully used as prognostic markers predicting cancer outcomes. For example, a unique miRNA signature accurately predicts disease progression in CLL [235]. Several miRNAs also are linked to treatment response predictions. In multiple types of cancer, elevated miR-21 expression not only correlates with aggressive tumor formation and decreased survival, but also with poor response to chemotherapy treatment [236–238].

miRNA expression in cancer is often regulated by genetic alterations in fragile sites [239]. Sequence mutations are less frequently observed, but can contribute to improper miRNA targeting in cancer-related pathways [240,241]. Interestingly, mutations predicted to affect secondary structure of the primary transcript do not impact processing of the mature miRNA, suggesting that such mutations are not as physiologically relevant as expected [242]. Approximately half of all known miRNA loci are associated with CpG islands and thus are subject to epigenetic changes in cancer

[243,244]. Additionally, miRNAs themselves regulate expression of epigenetic machinery components, leading to feedback loops affecting their own expression as well as expression of other cancer-related genes [245,246].

miRNA deregulation also results from alterations in transcription factor function and transcriptional activity of the promoter. For example the miR-34 family, often deleted in cancers, is directly induced by p53 and plays a role in p53-induced apoptosis [247]. Defects in miRNA biogenesis machinery modulate miRNA expression as well. Aberrant Drosha and Dicer activity in tumor cells is linked to irregular miRNA expression profiles [248–251]. Cofactors such as DGCR8 are also implicated in proper regulation of miRNAs, and in fact DGCR8-null mice present with severe miRNA-related developmental defects [252].

2.6 MicroRNA-155

miR-155 is encoded by a primary transcript transcribed from the B cell integration cluster (BIC) on chromosome 21 [253]. Originally identified in chickens, the BIC non-coding RNA is encoded by a locus that in avian leukosis virus-induced lymphomas is a common site for retroviral integration [254,255]. The proto-oncogene BIC collaborates with c-Myc in B cell lymphomagenesis [256]. miR-155, encoded within exon 2 of BIC, is processed from BIC and expressed in mice and humans in addition to chickens[253]. miR-155 is induced by a number of inflammatory stimuli including Toll-like receptor (TLR) ligands, tumor necrosis factor-α (TNF-α), interferon-γ/-β, and B and T cell receptor engagement [257–259].

Tissue expression and functions

miR-155 is detected in all human tissues, but is most highly expressed in lymphoid organs. miR-155 is critical for regulation and differentiation during haematopoiesis [260]. Upon activation of T and B lymphocytes, miR-155 synthesis is induced, and its expression is closely tied to the inflammatory response and adaptive immunity [257,261,262]. miR-155 expression augments IFN-γ signaling, and IL-2 and TNF-α production [263]. Analysis of T cell function in miR-155$^{-/-}$ mice revealed a propensity for Th2, rather than proinflammatory Th1/Th17 differentiation [264]. As a result of impaired Th1 and Th17 pathogen-specific responses, miR-155$^{-/-}$ mice are unable to control *Helicobacter pylori* infection [265]. Defective Th1 and Th17 development was also demonstrated in miR-155$^{-/-}$ mice as these animals were shown to be highly resistant to experimental autoimmune encephalitis (EAE), the pathogenesis of which is mediated by Th1 and Th17 cells [266].

miR-155 also regulates the germinal center response. Germinal centers are formed in both wild-type and miR-155$^{-/-}$ mice; however the number and size are reduced in the null mice [263,267]. In the absence of miR-155 expression there are decreased numbers of antigen-specific IgG1 plasma cells, impaired high-affinity antibody production, and functional defects in memory B cells [267,268]. miR-155 targeting of the transcription factor PU.1 is essential for proper B cell antigen driven maturation and IgG1 class-switched differentiation [268].

Involvement in disease

miR-155 plays a significant role in carcinogenesis and is overexpressed in a high percentage of neoplastic diseases, including cancers of the breast, lung, colon, cervix, pancreas, and B and T cell lymphomas [269,270]. miR-155 transgenic mice, in which miR-155 is overexpressed in B cells, rapidly develop high-grade B cell lymphomas [271]. miR-155 regulates over 100 genes, many of which are tumor suppressors. Targeting of PU.1 and C/EBPβ is of particular importance in haematopoietic and myeloproliferative disorders. PU.1 regulation affects oncogenesis by contributing to differentiation and activation of B cells and macrophages, and is implicated in the development of myeloid leukemia [272,273]. C/EBPβ, a target of miR-155 in the development of acute myeloid leukemia (AML), is a transcription factor that plays a role in haematopoietic cell growth and differentiation [272].

In many human breast cancer cell lines, miR-155 represses expression of suppressor of cytokine signaling (SOCS) 1, thereby augmenting JAK/STAT3 signaling to promote tumor inflammation and growth [274]. miR-155 also stimulates oncogenic metabolism by regulating glycolysis through indirect induction of hexokinase 2, a glycolytic enzyme. Through direct targeting of C/EBPβ, miR-155 suppresses miR-143, which in turn is a negative regulator of hexokinase 2 [275]. miR-155 targets the pro-apoptotic transcription factor, FOXO3a, and also blocks caspase-3 activation, thus serving as a potent suppressor of apoptosis [276,277].

Additionally, miR-155 expression is implicated in the development of cardiovascular disease. Activation of the renin-angiotensin-aldosterone system

(RAAS) and the effector molecules Angiotensin II (Ang II) and Angiotensin II receptor (AT1R) are major contributors to hypertension. miR-155 targeting of Ang II and AT1R inhibits vascular smooth muscle cell proliferation and suppresses Ang II-induced ERK1/2 phosphorylation and activation [278–280]. Furthermore, decreased endothelial nitric oxide (NO) or endothelial nitric oxide synthase (eNOS) is characteristic of endothelial dysfunction, and recently eNOS was identified as a direct target of miR-155. miR-155 is essential for the regulation of endothelium-dependent vasorelaxation[281].

EBV-mediated activation of miR-155

Consistent with the findings that miR-155 contributes to the activated phenotype of B cells, EBV infection of primary B cells significantly induces miR-155 expression [282]. Additionally, miR-155 is much more highly expressed in EBV-infected B cells displaying Latency III than in Latency I, suggesting that EBV proteins produced in Latency III activate miR-155 [283–285]. Both LMP1 and EBNA2 are implicated in induction of miR-155; however, neither of these proteins is sufficient to raise miR-155 production to levels observed in LCLs [282,286,287]. It is likely that multiple EBV latency-associated proteins cooperatively regulate miR-155 expression in B cells.

miR-155 contributes to normal expansion of B cells following antigen stimulation or B cell transformation [255,263,271]. It is the most abundant miRNA in EBV-transformed LCLs and is required for LCL proliferation. Following knock-down of miR-155 in LCLs, cells abruptly arrest in G0/G1 phase of the cell cycle and undergo apoptotic cell death [288]. The exact mechanisms underlying the requirement for miR-

155 in B cell proliferation are not known. It was recently proposed that miR-155 stabilizes EBV latency, and thus loss of miR-155 could induce lytic replication and cell death [289]. However we and others determined that miR-155 knock-down does not always reactivate lytic virus [288]. Notably, in BL cell lines displaying Latency I, in which miR-155 levels are much lower than in Latency III cells, silencing of miR-155 has no significant effects on growth [288]. This finding suggests that increased levels of miR-155 do not confer a proliferative advantage, and these cells may have acquired additional mutations rendering miR-155 dispensable.

Two other herpesvirus family members, Kaposi's sarcoma herpesvirus (KSHV) and Marek's disease virus (MDV), encode functional orthologs of cellular miR-155 [290,291]. Although EBV does not encode a viral miR-155 equivalent, its ability to induce cellular miR-155 suggests that there is a critical and conserved role for miR-155-like miRNAs among the herpesviruses. Both miR155 and the KSHV miR-155 ortholog, miR-K12-11, directly target BACH1, a transcriptional repressor that binds consensus AP-1 promoter elements, suggesting downregulation of BACH1 may be a conserved activity of herpesviruses [290,292]. B cell receptor (BCR) engagement activates the BIC promoter through an Ap-1 binding element [286,293]. Because JunB and FosB activate Ap-1, and LMP1 induces JunB expression, it is possible that LMP1 contributes to initial induction of miR-155, but that other EBV latency genes cooperate to sustain the robust miR-155 levels observed in Latency III LCLs [294]. miR-155-mediated inhibition of BACH1 may facilitate derepression of AP-1 promoter elements, allowing EBV to regulate Ap-1 responsive genes.

2.7 The cell cycle

The eukaryotic cell cycle is a highly conserved, complex process in which numerous regulatory proteins direct the duplication of chromosomal DNA and production of two identical daughter cells in response to mitogenic stimuli. This cycle is characterized by two main phases, DNA synthesis (S phase) and mitosis (M phase), which are separated by two gaps, G1 and G2 (**Fig. 2.7**). Quiescent cells not actively participating in the cell cycle enter G0 phase. The G1/S restriction point is of particular importance to this dissertation project is regulation of, therefore this review will focus primarily on these events.

Cyclins and cyclin-dependent kinases

Regulation of the cell cycle is mediated by the activity of 11 different serine/threonine kinases called cyclin-dependent kinases (CDKs) [295]. CDKs are activated in the cytoplasm by cyclins, which contain a cyclin box domain that binds to and facilitates accessibility of the CDK active site [295,296]. Additionally cyclins target CDKs to the nucleus using a nuclear localization signal that CDKs lack [297]. Cyclin/CDK complexes translocate to the nucleus where full CDK activation is achieved following phosphorylation by CDK activating kinase (CAK) [298,299]. CDK activity is critical for progression through the cell cycle and their inactivation prevents mitosis [300–302]. Inhibitory kinases such as Wee1 phosphorylate residues in the CDK active sites to prevent premature activation [301,303]. The Wee1 kinase phosphorylates cyclin/CDK complexes upon their entry into the nucleus, thereby ensuring complete DNA replication prior to mitosis [303]. When the cell is ready to divide, cdc25

phosphatase dephosphorylates the inhibitory phospho-residue put in place by Wee1 [302,304].

The cyclins were named based on their cyclic expression within the cell cycle [305]. Cyclin expression patterns are crucial for their CDK regulatory function since this means CDKs can only become activated at specific points in the cell cycle. Much of this cyclic expression is achieved by regulated degradation. The anaphase promoting complex/cyclosome (APC/C) is a ubiquitin ligase that selectively recognizes substrates at specific points in the cell cycle to regulate key events in mitosis, initiation of anaphase, and preparation for the next round of DNA replication. Notably, it is a crucial mediator of cyclin proteolysis in mitosis [306–308].

In G1 phase, an important target of the CDKs is Rb. Rb phosphorylation is sequential such that in early G1, cyclin-D/CDK4/6 phosphorylate Rb and in late G1, Rb hyperphosphorylation is mediated by cyclin-E/CDK2 complexes [309–312]. Cyclin-E/CDK2 phosphorylates additional substrates involved with centrosome duplication, DNA replication and repair, and histone modification [313]. Cyclin E activity also regulates pre-replication complex formation during the G1/S transition, making degradation of cyclin E critical in S phase to avoid aberrant replication of DNA [314,315]. Upon entry to S phase, cyclin A/CDK2 activity functions to maintain Rb hyperphosphorylation, which mediates transcriptional activation of genes essential for DNA replication and progression through the cell cycle [316]. Cyclin A/CDK2 phosphorylation also regulates HDM2 and p53 expression and activity during S phase [317,318].

Figure 2.7: Regulation of cell cycle progression

Mammalian cell cycle regulation by cyclin/CDK complexes and CKIs. The cell cycle consists of four distinct phases: G_1, S (DNA replication), G_2, and M (mitosis). Activation of specific cyclin/CDK complexes drives progression through these cell cycle phases. CKIs of the CIP/KIP and the INK4 families interact with and suppress cyclin/CDK activity, thereby blocking cell cycle progression and cell proliferation.

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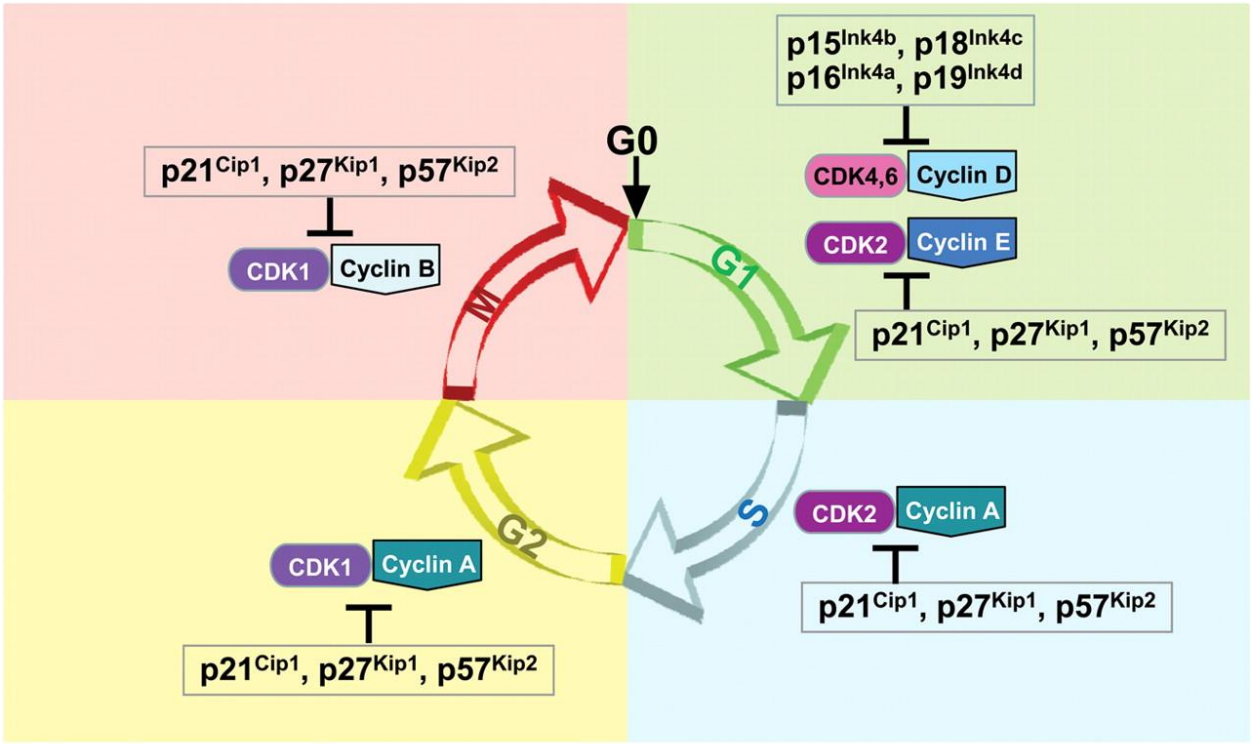
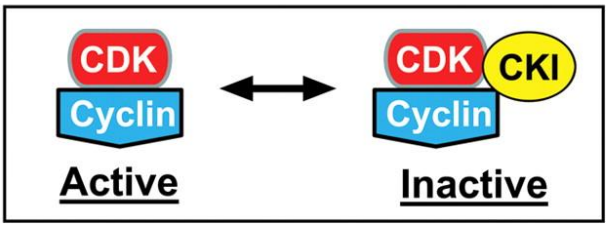
Figure 2.7: Regulation of cell cycle progression





Cip / Kip CKIs	Ink4	
$p21^{Cip1}$	$p15^{Ink4b}$	$p16^{Ink4a}$
$p27^{Kip1}$	$p18^{Ink4c}$	$p19^{Ink4d}$
$p57^{Kip2}$		

Cyclin-dependent kinase inhibitors

The most important class of cyclin/CDK inhibitors are the cyclin-dependent kinase inhibitors (CKIs). There are two predominant families of CKIs: INK4a (p15, p16, p18, p19) and CIP/KIP (p21, p27, p57) (**Fig. 2.7**). Generally, INK4a CKIs are thought to bind primarily to CDK4 and CDK6, while CIP/KIP members inhibit cyclin A/D/E containing complexes [320–324]. INK4a proteins all bind directly to CDKs 4 and 6, initiating a conformational change to inhibit cyclin binding [325]. While structurally and functionally homologous, INK4a family members can be differentially induced. TGFβ signaling activates p15 expression, cell aging induces p16, and p18/p19 are associated with terminal cell differentiation [326–328].

CIP/KIP proteins are capable of inhibiting a broader range of cyclin/CDK complexes. Most proteins in this family are unstructured, allowing them to adopt multiple diverse conformations dependent on the individual target protein encountered [329]. These CKIs bind both the cyclin and CDK subunits and inhibit their activity by physically blocking substrate binding [330,331]. In addition to cyclin/CDK binding, p21 inhibits DNA synthesis by binding to proliferating cell nuclear antigen (PCNA) and blocking its association with DNA polymerase δ [330,332,333]. Furthermore, p21 promotes apoptosis via both p53-dependent and -independent pathways [334].

Growth promoting functions of p21

p21 also facilitates cyclin D/CDK complex assembly and nuclear translocation, suggesting that p21 has complex and diverse roles in cell cycle regulation [335–338]. Subcellular localization and post-translational modification of p21 affects its roles in

cell cycle regulation as well. Cytoplasmic expression of p21 allows for interaction and inhibition of caspases [334,339]. Protein kinase A (PKA)-mediated phosphorylation of p21 impedes its nuclear localization, enabling p21 to inhibit Fas-induced apoptosis by proteolytic activation of caspase-3 [340,341].

Akt/protein kinase B (PKB) is another key mediator of p21 phosphorylation at the T145 residue, functioning to promote cell survival by enhancing p21 stability and cytoplasmic localization [342,343] (**Fig. 2.8**). Following T145 phosphorylation, p21 can no longer bind PCNA and inhibit DNA synthesis [344]. Phosphorylation of this residue also masks a nuclear localization signal (NLS) contributing to relocalization of p21 from the nucleus to the cytoplasm [342,345,346]. Cytoplasmic p21 is exposed to a different set of binding partners, such as apoptosis signal-regulating kinase 1 (ASK1), where it inhibits ASK1 activation and interaction with substrates [347]. p21 phosphorylation can have profound consequences for its function. There are numerous examples of tumors displaying elevated levels of p21 despite maintaining rapid cellular proliferation [348–351]. These cancer cells may exhibit specific p21 phosphorylation to promote growth and counteract apoptosis.

Figure 2.8: The role of p21 in cell survival and death

Under non-stressed conditions, p21 is expressed at low levels and promotes cell cycle progression. Under stress conditions, p21 production is increased through p53-dependent and -independent pathways. Increased p21 interacts with and inhibits cyclin/CDKs activity. In most tumor cells, the cell cycle checkpoint function of p21 is lost due to p53 mutations. Akt phosphorylates and then stabilizes p21 protein for cell survival. p21 inhibits apoptosis by interacting with pro-apoptotic molecules such as caspase-3 and ASK1.

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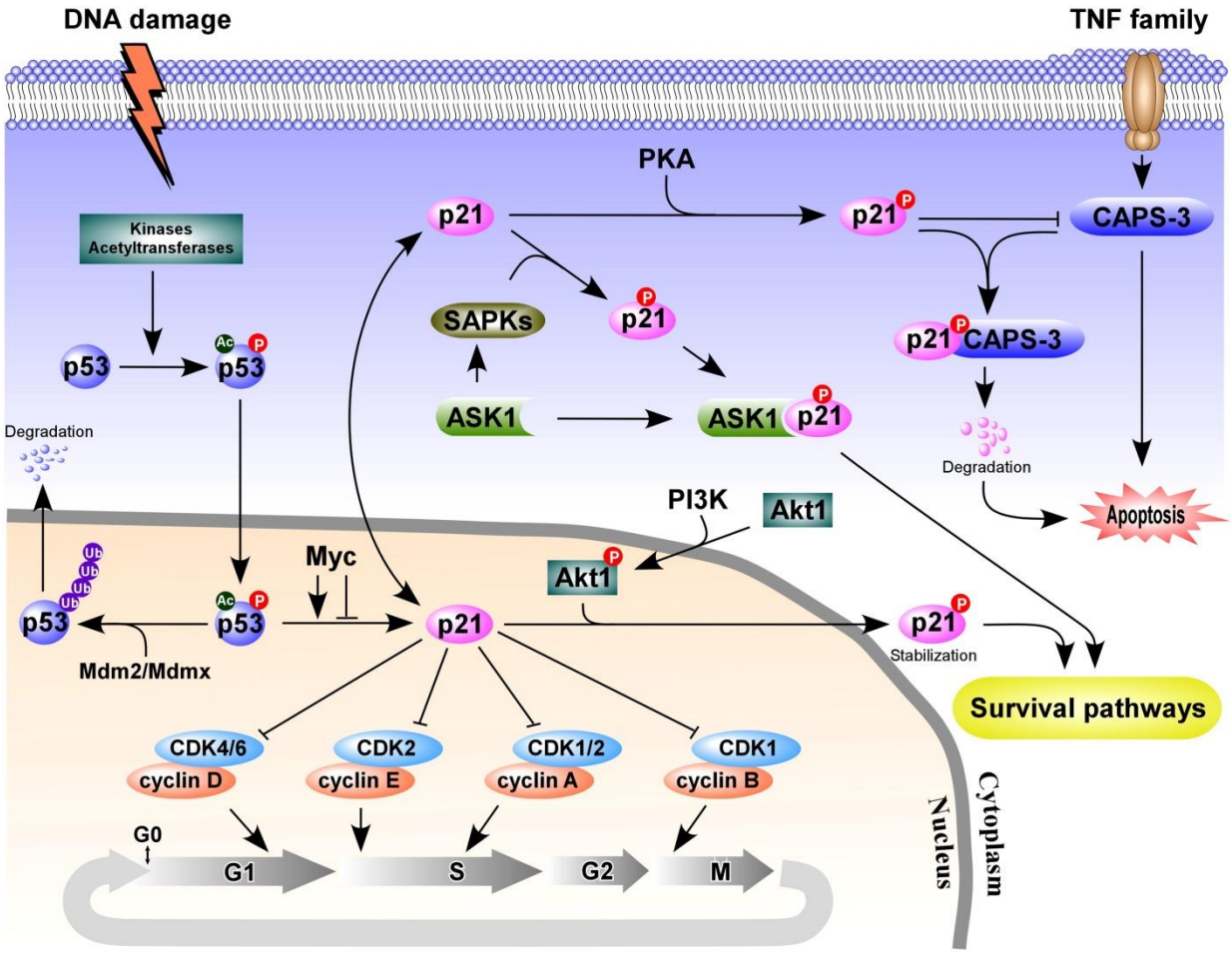
Figure 2.8: The role of p21 in cell survival and death



Regulation of p21 expression

The p21 gene, located on chromosome 6p21.2, encodes 8 alternative transcripts produced from 3 unique transcriptional start sites [320,353,354] (**Fig. 2.9 A**). Most notably, p53 regulates p21 gene expression by directly binding to a p53 response element; however, the p53 homologues, p73 and p63, also transactivate p21 by binding these response elements [355] (**Fig. 2.9 B**). Expression of alternate p21 transcripts vary in their dependence on p53, most likely due to proximity of the transcriptional start site to p53 response elements. Additional transcription factors, such as Sp1/Sp3, Smads, and E-box-binding proteins, can activate p21 in response to various stress stimuli in a p53-independent manner [320,352]. In epithelial cells, Rb can induce p21 expression via Sp1/Sp3 promoter sites [356]. Conversely, c-Myc was shown to be a potent transcriptional repressor of p21 by sequestering Sp1 transcription factor from the p21 promoter [357].

Several miRNAs, including miR-17, miR-20a/b, miR-93, miR-106a/b, have been demonstrated to directly bind the p21 3'-UTR and repress expression of p21 [358–362]. Additionally, many miRNAs target p53 expression thereby indirectly regulating p21 levels [363]. miR-155 promotes lymphomagenesis by directly targeting Smad expression to regulate TGFβ signaling-mediated expression of p21 [364].

Proteasomal degradation plays an essential role in p21 regulation, both in ubiquitin-dependent and -independent pathways [365–367]. While p21 can be ubiquitinated at the N terminus, the 20S proteasome also mediates degradation via binding directly to the C terminus of p21 in the absence of ubiquitin [368–371]. Ras expression stabilizes p21 by promoting cyclin D/p21 heterocomplexes, preventing

proteasomal recognition and degradation of p21 [372]. Although there is some controversy concerning how phosphorylation of p21 affects protein stability, studies have demonstrated phosphorylation can either increase or decrease p21 half-life depending on cellular context, indicating an important and intricate role for phosphorylation in regulation of p21 stability [343,371–374].

Figure 2.9: Human genomic p21 locus

(**A**) Schematic presentation of various known p21 transcripts. The position of the first nucleotide of p21 transcript (GenBank Accession # Z85996) in the genomic DNA is assigned as the start site of the p21 locus. p21 is the classical $p21^{Waf1/Cip1/Sdi1}$ transcript [320]. The alternate transcripts, $p21^{variant-2}$, $p21^{alt-a}$, $p21^{alt-b}$, $p21^{alt-c}$, and $p21^{alt-a'}$, are presented as reported previously [354]. p21B and p21C are also presented as reported previously [353]. The protein coding regions are marked with solid black boxes. Alternative promoters, P1-P3, and the transcripts they generate are bracketed. (**B**) Schematics of the p21 promoters along with various responsive elements, transcription factors, and upstream signals. p53-response elements are numbered and indicated by green boxes.

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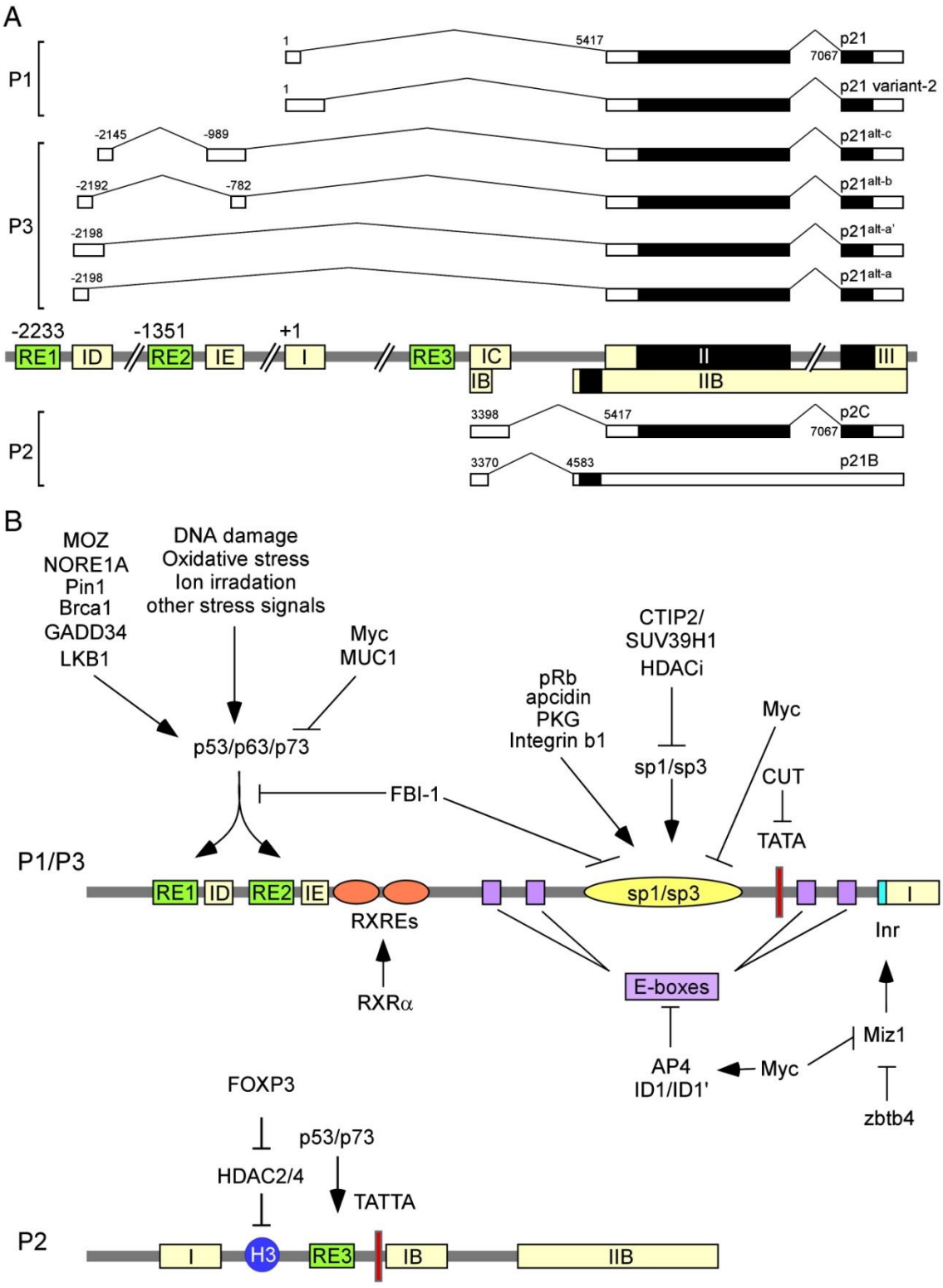
Figure 2.9: Human genomic p21 locus



Retinoblastoma protein (Rb) and E2F

Rb was first characterized as a tumor suppressor protein after identification in a malignant retinal tumor known as retinoblastoma. The Rb gene is often inactivated in human tumors by mutation, deletion, or altered expression of upstream regulators [375,376]. Rb is a major inducer of G1 cell cycle arrest and functions by binding to and suppressing E2F-mediated transcription [377–379]. Phosphorylation of Rb regulates its ability to act as a tumor suppressor and potently inhibit E2F. Rb hyperphosphorylation, mediated by cyclin D,E/CDK complexes in G1, results in inactivation of Rb and dissociation from E2F, while hypophosphorylated Rb is active and maintains E2F binding capability [316,380–382] (**Fig. 2.10**).

E2Fs 1-3 are activators of transcription and target genes that promote the G1/S transition, DNA synthesis, and DNA repair [383–388]. E2Fs form heterodimers with a dimerization partner (DP) that facilitate binding to promoter sites [386]. Cyclins A and E are two examples of Rb-regulated E2F target genes that further serve to hyperphosphorylate Rb and perpetuate cell cycle progression [389,390].

E2F is additionally regulated by cyclin A/CDK2-mediated phosphorylation during S/G2 phase. This regulation serves to inhibit E2F activity, ensuring E2F only transactivates genes promoting DNA synthesis at appropriate stages of the cell cycle [391]. Upon initiation of the DNA damage response, E2F7 and E2F8 function to repress E2F1-3 activator proteins and block cell replication [392,393]. Alternatively, overexpression of E2F1 is a potent inducer of apoptosis [394–396]. Not only do E2F proteins serve as crucial mediators of cell cycle regulation, but there is significant cross-talk between cell cycle and apoptotic regulatory proteins.

Figure 2.10: Regulation of G1/S transition by Rb and E2F

In G1 active cyclin/CDK complexes trigger the hyperphosphorylation of Rb. This releases the transcription factor E2F thereby permitting E2F-mediated transactivation of genes involved in cell cycle progression and DNA synthesis.

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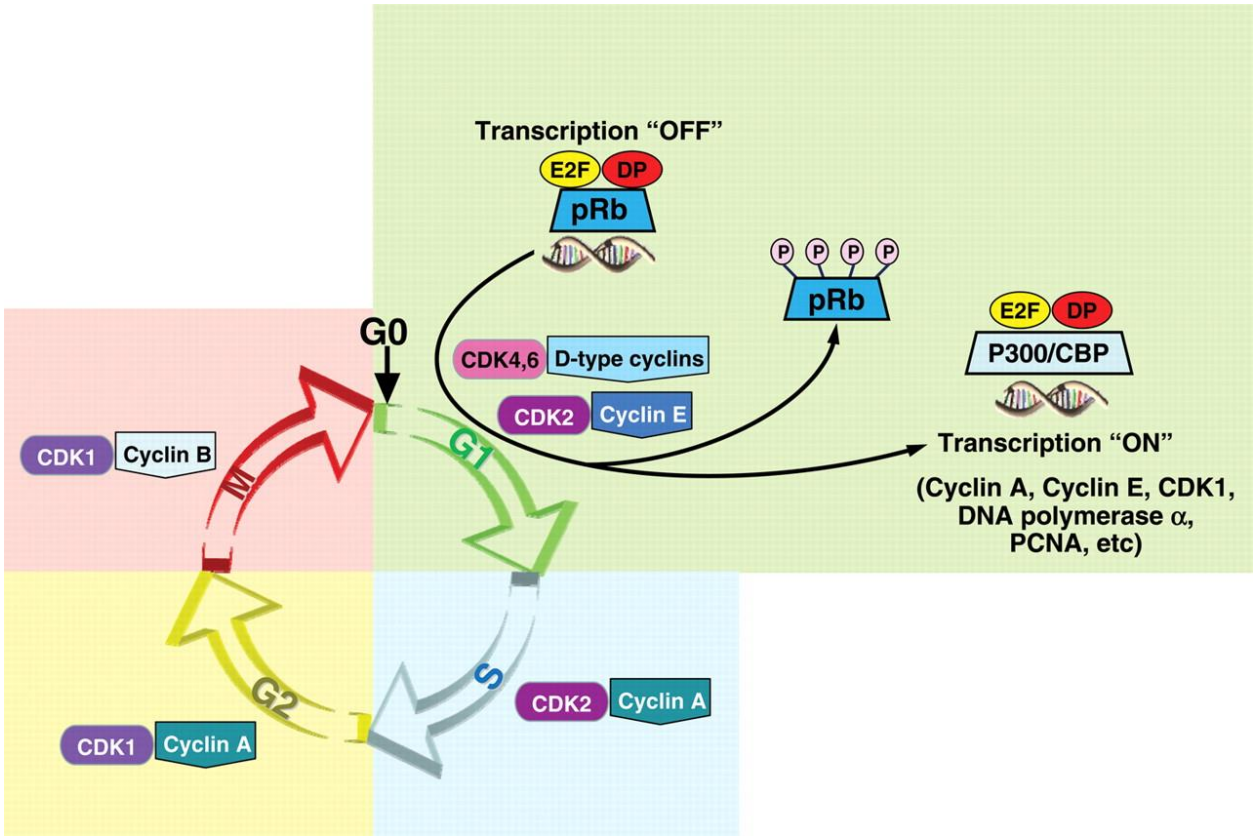
Figure 2.10: Regulation of G1/S transition by Rb and E2F



p53-ARF-HDM2 pathway

Called the ‘guardian of the genome’ [397], the p53 protein is at the center of a complex transcriptional network involved in anti-proliferative cellular responses including cell cycle inhibition, senescence, apoptosis, and modulation of autophagy [398–404]. Activation of p53 in response to cellular stress is followed by stabilization of p53, sequence-specific DNA binding, and transactivation of target genes [405]. p53 stabilization is primarily mediated by interactions with HDM2, a negative regulator of p53 that initiates ubiquitin-mediated degradation [406] (**Fig. 2.11**). HDM2 activity maintains p53 at basal levels during normal cellular conditions [407]. Since p53 regulates its own expression by inducing HDM2, mutant p53 often displays increased protein stability compared to wild-type as it can no longer activate HDM2 [408]. p53-HDM2 interactions can be interrupted by post-translational modification of p53 as well as alterations in HDM2 expression levels [409]. $p14^{ARF}$ (p19 in mouse) is an important negative regulator of HDM2, thus functioning to stabilize p53 [410]. p14 mediates degradation of HDM2, however it can additionally inhibit HDM2 activity by direct binding and sequestering of HDM2 [411–413] (**Fig. 2.11**).

The p53 transcription factor has two DNA-binding domains that bind DNA in a sequence-specific manner [414]. These domains are considered 'hot spots' for mutation, and indeed the majority of p53 mutations identified in cancer occur in these regions [415]. DNA-binding domain mutations will either alter the sequence such that p53 no longer recognizes target genes, or mutations can induce conformational shifts in the protein, masking the binding domain [416]. Another important p53 domain often mutated in human cancers is the tetramerization domain. p53 is only transcriptionally

active when the protein is tetrameric, as this conformation allows for high affinity DNA binding [416–418].

Activation of the p53 pathway generally occurs in response to cellular stress or DNA damage [419]. DNA damage activates ATM/ATR signaling which induces Chk1 and Chk2 kinases responsible for phosphorylating and stabilizing p53, leading to G1 arrest [420,421]. p53-mediated cell cycle arrest in G1 is primarily due to transactivation of p21 which inhibits G1/S transition [422]. Additionally, in response to DNA damage p53 activates 14-3-3σ, a protein that prevents nuclear localization of G2/M-promoting cyclin/CDK complexes [423]. p53 activation also initiates apoptotic signaling cascades. Proapoptotic proteins, Bax, PUMA, and NOXA, are transcriptionally regulated by p53. These proteins inhibit activity of antiapoptotic BCL-2 family members to ensure caspase activation and induction of apoptosis [424–426].

Figure 2.11: The p53 pathway

(**A**) p53 exists in non-stressed cells at a very low concentration. Under stress conditions, the p53 protein accumulates in the cell, binds in its tetrameric form to p53-response elements and induces the transcription of various genes that are involved in cell-cycle control, apoptosis, DNA repair, differentiation and senescence. The loss of p53 tumor-suppressor activity by mutation/deletion or inhibition of p53 permits aberrant proliferation of damaged cells. This uncontrolled proliferation can lead to tumor development. (**B**) p53 and HDM2 form an auto-regulatory feedback loop. p53 stimulates the expression of HDM2 and in turn HDM2 inhibits p53 via blocking p53 transcriptional activity and mediating degradation. DNA damage can induce p53 phosphorylation, preventing its association with HDM2. The ARF protein ($p14^{ARF}$) prevents the HDM2-mediated degradation of p53.

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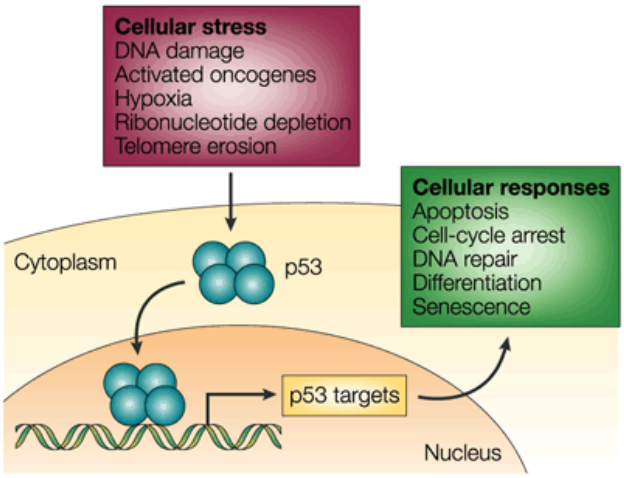
Figure 2.11: The p53 pathway

A.



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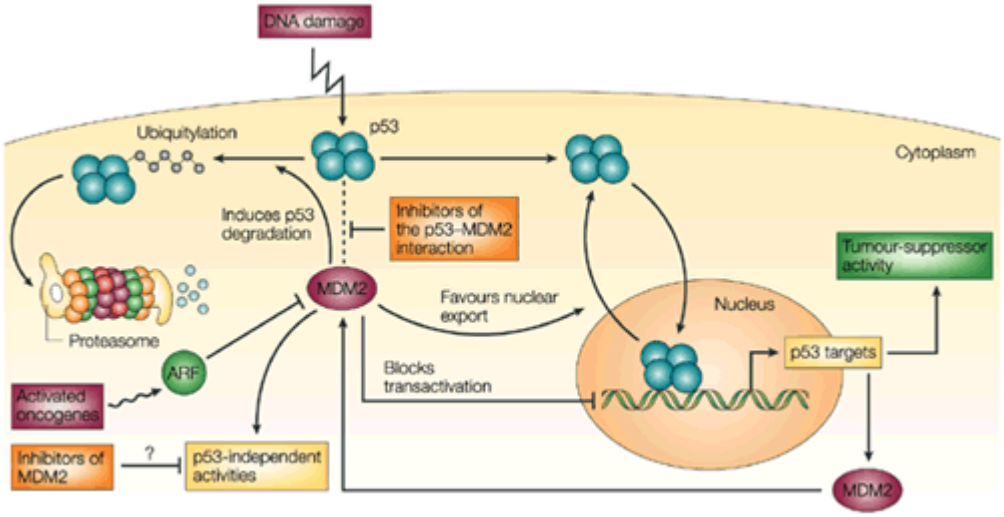


Chapter 3: Contribution of EBV latency-associated proteins to the pathogenesis of Wp-R BL

3.1 Introduction

Translocation of the Myc oncogene to a highly transcriptionally active immunoglobulin locus is a critical lymphomagenic event in the pathogenesis of BL [174]. While Myc functions to promote proliferation and tumorigenesis, it is also a potent inducer of apoptotic cell death [158]. In mouse models of Myc-induced lymphomagenesis, a crucial requirement for the progression to malignancy is the inhibition of apoptotic signaling pathways activated by Myc [133,163,428–430]. While an exact mechanism for the contribution of EBV to BL development is unclear, these data suggested EBV could function as an infectious cofactor by countering apoptotic pathways. Indeed several studies demonstrated EBV-positive cell lines display increased resistance to apoptotic stimuli compared to EBV-negative lines [134,175].

The unique subset of BL, Wp-R, contains an EBV genome in which the region encompassing EBNA2 and a segment of EBNA-LP are deleted, resulting in a distinct pattern of latency that includes a broader range of protein expression than Latency I BL [48]. Wp-R tumors exhibit increased apoptotic resistance compared to Latency I BL [184]. Additionally, compared to Latency I BL, Wp-R cells were found to be more tumorigenic in xenograft assays [135,150]. The high incidence of Wp-R tumors, approximately 20% of BL cases, could indicate biological selection for this unique EBV genome as it may enhance the potential of EBV to act as a cofactor in BL. We hypothesized latency-associated proteins expressed in Wp-R promote proliferation and survival, favoring tumor development.

It is not uncommon for pathogens such as oncogenic viruses to manipulate cellular pathways and processes in order to facilitate their replication and survival. Following its discovery in BL, EBV was the first human virus suggested to contribute to tumor development [431]. Since then, many studies have investigated mechanisms by which additional DNA tumor viruses target cell cycle regulatory machinery. Both adenovirus and HPV encode proteins that can bind to and inhibit tumor suppressors such as p53 and Rb [432–435]. KSHV, a γ-herpesvirus related to EBV, encodes a latency-associated nuclear antigen (LANA) that binds and suppresses p53 transcriptional activity [436], and furthermore directly targets the Rb-E2F pathway [437]. KSHV also encodes a viral homolog of the cellular protein, cyclin D [438].

In contrast, much less is known about strategies used by EBV to promote proliferation. EBNA3A and 3C are both essential for immortalization of B cells [40] and in LCLs cooperate to repress the cell cycle inhibitors p14 and p16 [129]. No conclusive evidence has identified a role for EBV latency-associated proteins in direct regulation of p53 or Rb activity. Although some reports have proposed a role for EBNA3C in the inhibition and degradation of p53 [439,440], these studies were performed using Flag-tagged EBNA3C and EBNA3C overexpression vectors. Results from our laboratory have failed to corroborate these findings in physiologically relevant B cell lines with endogenous levels of EBNA3C.

Furthermore, there is a correlation between p53 status and EBV latent protein expression. Latency I BL tumors contain mutant p53 while in Wp-R BL and LCLs, which express an expanded repertoire of latency-associated proteins, wild-type p53 is most frequently observed [135], suggesting proteins expressed in Latency III and Wp-

R may function to suppress the p53 response. Proteins expressed in Wp-R, but not Latency I BL, include the EBNA3 family (-3A, -3B, and -3C) and BHRF1, a viral homolog of BCL2 predominantly expressed during lytic replication [53]. To assess how these proteins contribute to Wp-R tumorigenesis, we investigated the requirement for EBNA3A, EBNA3C, and BHRF1 in the proliferation and survival of Wp-R BL.

3.2 Results

EBNA3A promotes G1/S cell cycle progression in Wp-R

To investigate the role of EBNA3A in Wp-R, we generated two distinct shRNAs (3AshRNA-1490 and -601) to specifically target EBNA3A in our Wp-R cell lines, Sal and Oku (**Fig. 3.1 C**). These shRNAs are expressed from an *oriP*-GFP vector, which contains a neomycin-resistance gene, and *oriP*, the EBV origin of latent DNA replication, ensuring episomal maintenance of the plasmid in EBNA1 expressing cells. We transfected B cells using the Amaxa Nucleofection system (Lonza) which resulted in transfection efficiencies of approximately 75-85%. EBNA3A knock-down was accompanied by a loss of proliferation over time in both Sal and Oku compared to empty vector (Vec-Ctrl) or non-specific shRNA (Ctrl-shRNA) control transfected cells (**Fig. 3.1 A,B**). In addition to cessation of growth, we observed increased cell death and initiation of apoptosis, as evidenced by Poly-ADP-ribose polymerase (PARP) and Lamin B cleavage. Both of these proteins are known substrates of caspases [441–443] (**Fig. 3.2**). Propidium iodide staining of transfected Wp-R cells indicated EBNA3A facilitates entry into S phase, as a higher percentage of EBNA3A shRNA-transfected cells were detected in G0/G1 compared to controls (**Fig. 3.3**). We hypothesized EBNA3A functions to promote passage through the G1/S cell cycle restriction point.

During our studies with Sal and Oku cells, we observed that Oku are particularly sensitive to nucleofection, resulting in decreased viability post transfection. Since this introduced difficulties in our ability to collect enough cells for

adequate downstream analysis, we focused our experiments investigating EBNA3A to the Sal cell line.

EBNA3A knock-down results in accumulation of p21 protein and hypophosphorylation of Rb

To determine the mechanism by which EBNA3A promotes G1/S transition, we assessed whether EBNA3A could regulate key mediators of the cell cycle. While previous work identified a role for EBNA3A in repression of $p14^{ARF}$ and $p16^{INK4a}$ [129], no significant changes in $p14^{ARF}$ expression were observed in Wp-R following EBNA3A knock-down, and $p16^{INK4a}$ was not expressed in the Wp-R lines, likely due to epigenetic silencing. Instead, we observed increased protein expression of the cell cycle inhibitor $p21^{WAF1/CIP1}$ in cells transfected with EBNA3A shRNA compared to controls (**Fig. 3.4 A**). p21 is a potent pan cyclin-dependent kinase inhibitor capable of repressing both cyclin D and cyclin E-containing complexes and mediating G1 cell cycle arrest [444–446].

Since the cyclin/CDK complex activity targeted by p21 functions to hyperphosphorylate Rb [380,381], we reasoned the Rb phosphorylation state might be altered following EBNA3A knock-down. When Rb is hyperphosphorylated it can no longer bind to and inhibit E2F-mediated transcription of DNA synthesis machinery and S-phase cyclins [383,384]. We observed the appearance of a prominent hypophosphorylated species of Rb, accompanied by a corresponding decrease in hyperphosphorylated Rb, following EBNA3A knock-down (**Fig. 3.4 B**). The shift in

Rb phosphorylation was concomitant with accumulation of p21 and cessation of growth.

Since the EBV lytic cycle transactivator Zta can induce expression of p21 [447], we wanted to ensure the observed changes in p21 protein expression were due to EBNA3A expression rather than unforeseen upregulation of Zta. Zta levels were undetectable in the presence or absence of EBNA3A, indicating not only p21 regulation is a function specific to EBNA3A but also the cell death and loss of proliferation observed following EBNA3A knock-down is not due to activation of lytic replication (**Fig. 3.5**).

Figure 3.1: EBNA3A knock-down results in loss of proliferation of Wp-R cell lines

Sal (**A**) and Oku (**B**) cells were transfected with 5 μg of the indicated DNA. 2 days post transfection, cells were seeded at $3.5X10^5$/mL in 10 mL of growth medium containing G418. Live cells were counted at specified time points using Trypan blue exclusion, and fed and re-seeded at $3.5X10^5$/mL every 2 days. (**C**) Efficacy of shRNA-mediated knock-down of EBNA3A expression was previously verified in both Sal and Oku cell lines. Shown here is a representative immunoblot demonstrating EBNA3A knock-down with 2 shRNAs: 3AshRNA-1490 and -601. Sal cells were harvested at day 4 post transfection and lysates run on a 7% SDS-PAGE prior to transfer to PVDF for immunoblotting.

Figure 3.1: EBNA-3A knock-down results in loss of proliferation of Wp-R cell lines

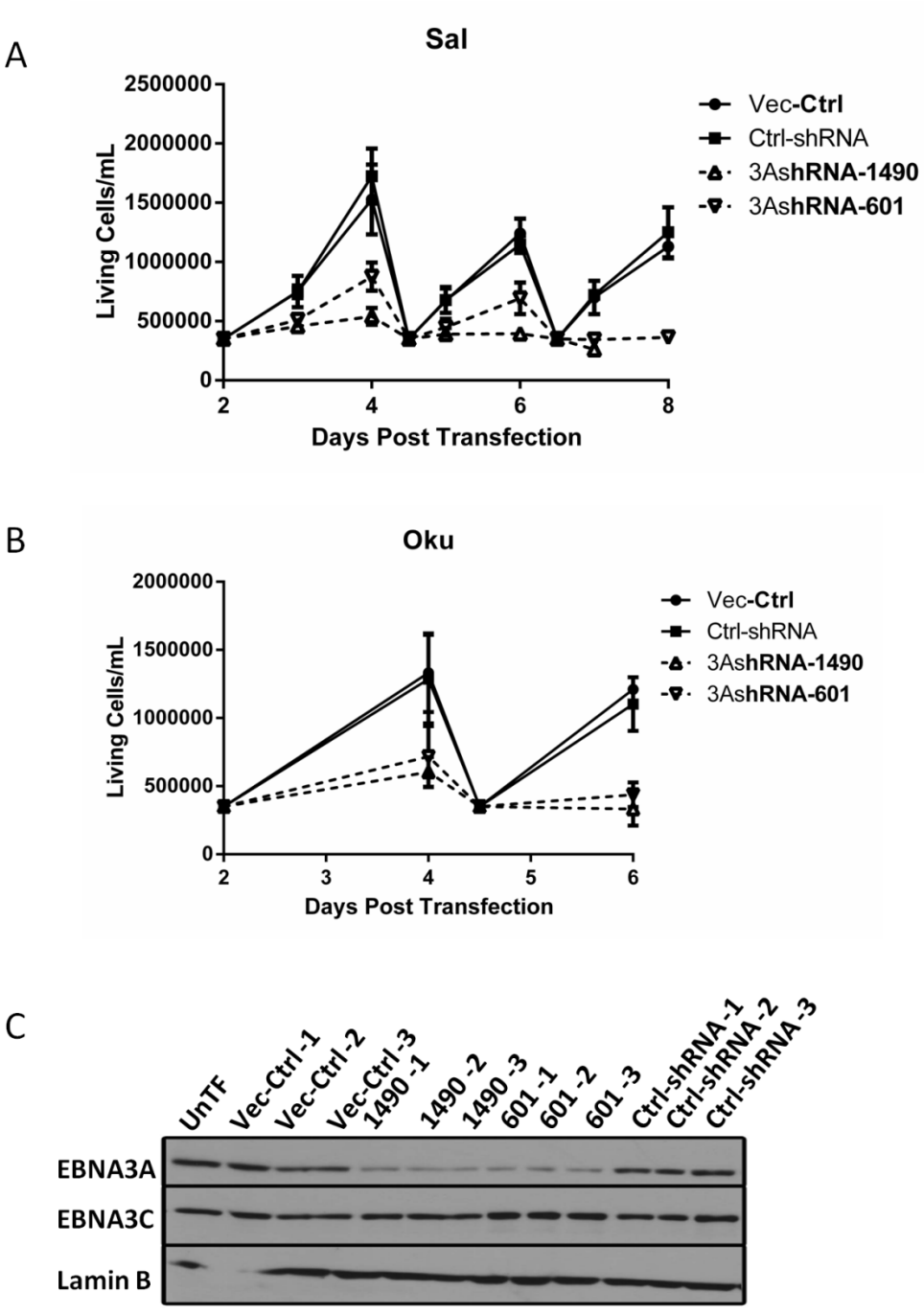


Figure 3.2: Loss of EBNA3A results in induction of apoptosis

Transfected Sal cells were collected at indicated time points post transfection for immunoblot analysis. Cleavage products, observed migrating at a lower molecular weight, were detected in lysates from EBNA3A shRNA-transfected cells indicating caspase activity and apoptotic signaling cascades.

Figure 3.2: Loss of EBNA3A results in induction of apoptosis

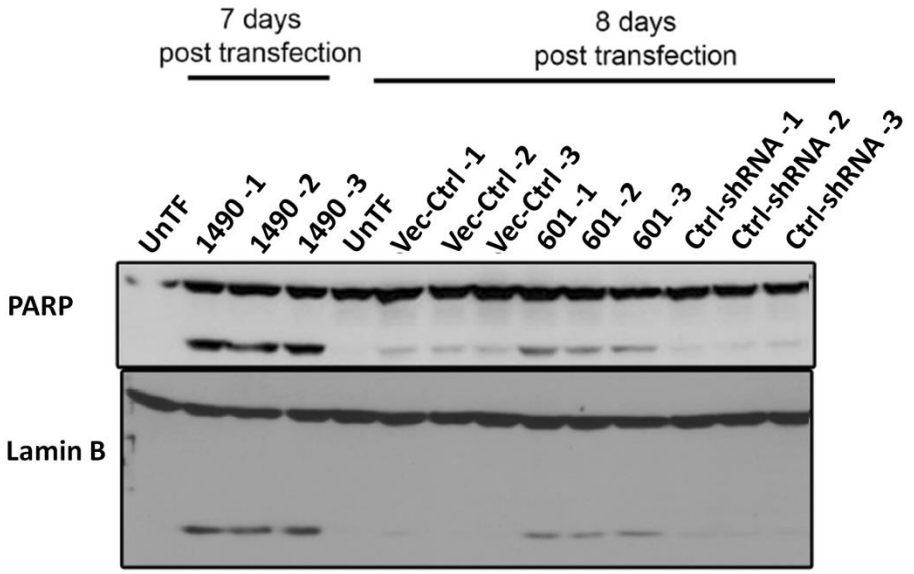


Figure 3.3: Wp-R cells transfected with EBNA3A shRNA arrest in G0/G1 phase of the cell cycle

(**A**) Cell cycle profiles of GFP-positive Sal cells at 3 days post transfection. Vec-Ctrl or 3AshRNA-1490-transfected were analyzed by flow cytometry after staining with propidium iodide. (**B**) Compiled data from three independent experiments, performed in duplicate, indicating percentage of cells contained in each cell cycle phase. A higher proportion of cells in G0/G1 were observed in cells expressing EBNA3A shRNA compared to vector control.

Cell cycle analysis was performed by Melissa Tursiella with assistance from Nate Sheaffer in the Pennsylvania State University Flow Cytometry Core.

Figure 3.3: Wp-R cells transfected with EBNA3A shRNA arrest in G0/G1 phase of the cell cycle

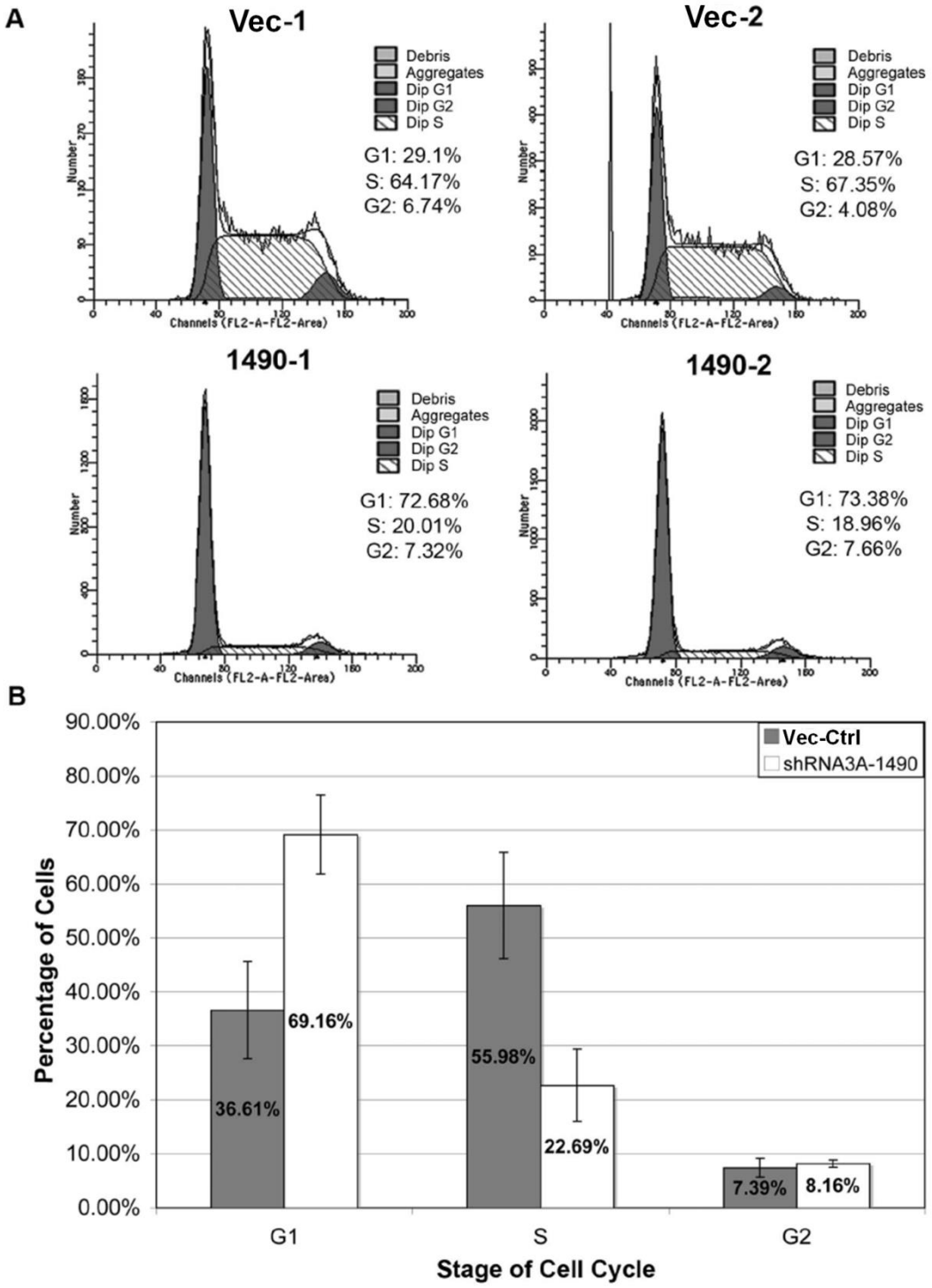


Figure 3.4: EBNA3A represses p21 and promotes Rb hyperphosphorylation

(**A**) Sal cells were transfected with indicated shRNA and harvested at 2 and 4 days post transfection. p21 levels were assayed by immunoblot. GAPDH served as loading control. (**B**) Transfected Sal cells were collected at 4 days post transfection and analyzed for Rb expression. Levels of total Rb and pRb, using a phospho-specific antibody, were measured by immunoblot. Lamin B served as loading control.

Representative immunoblots published in:

Tursiella ML, **Bowman ER**, Wanzeck KC, Throm RE, Liao J, Zhu J, et al. (2014) Epstein-Barr Virus Nuclear Antigen 3A Promotes Cellular Proliferation by Repression of the Cyclin-Dependent Kinase Inhibitor p21WAF1/CIP1. PLoS Pathog 10(10): e1004415. doi:10.1371/journal.ppat.1004415

Figure 3.4: EBNA3A represses p21 and promotes Rb hyperphosphorylation

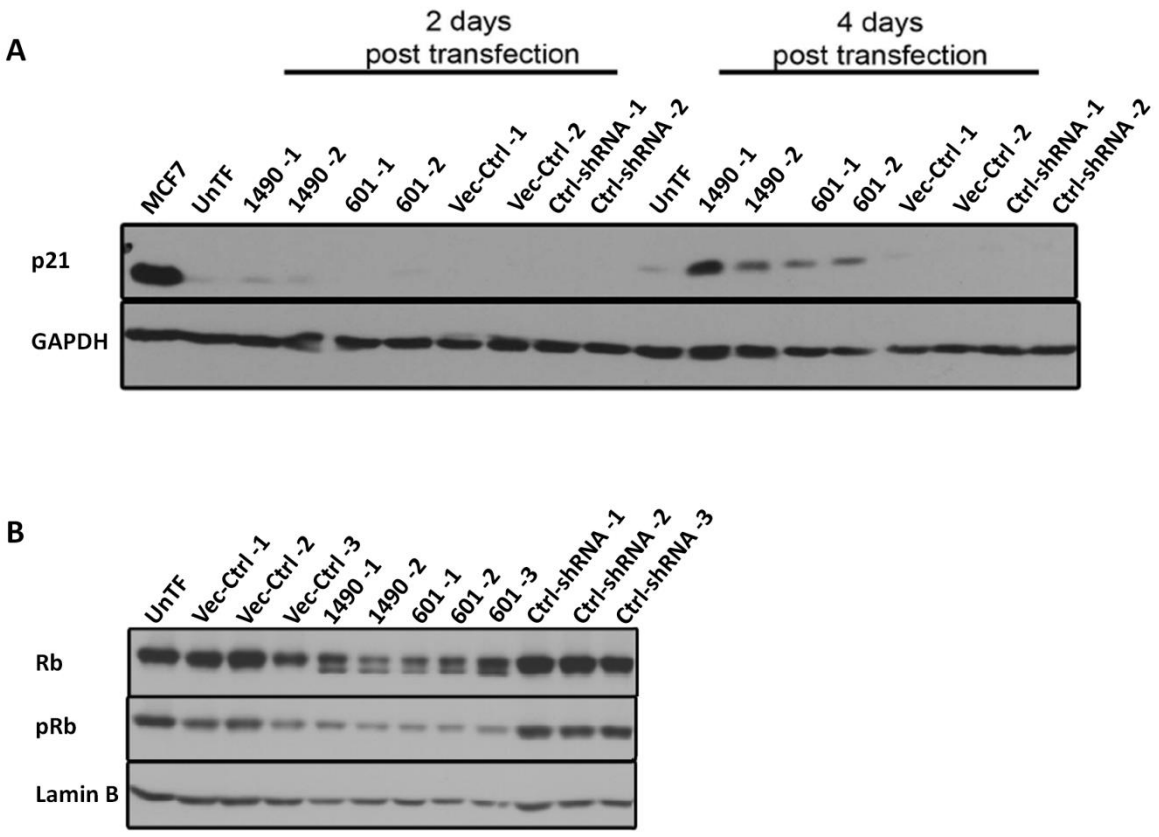
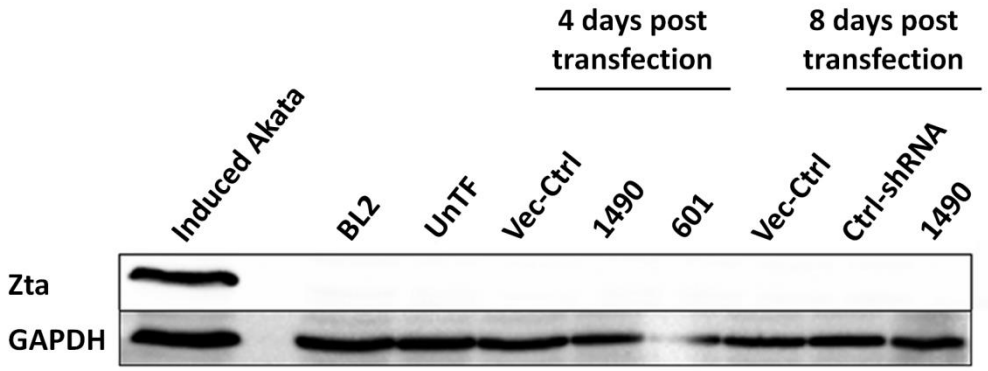


Figure 3.5: EBNA3A knock-down does not induce Zta expression and reactivate lytic cycle replication

Sal cells were transfected and harvested at indicated time points of two independent experiments. As a positive control for Zta expression, lytic replication was induced in EBV-positive Akata cells.

Figure 3.5: EBNA3A knock-down does not induce Zta expression and reactivate lytic cycle replication



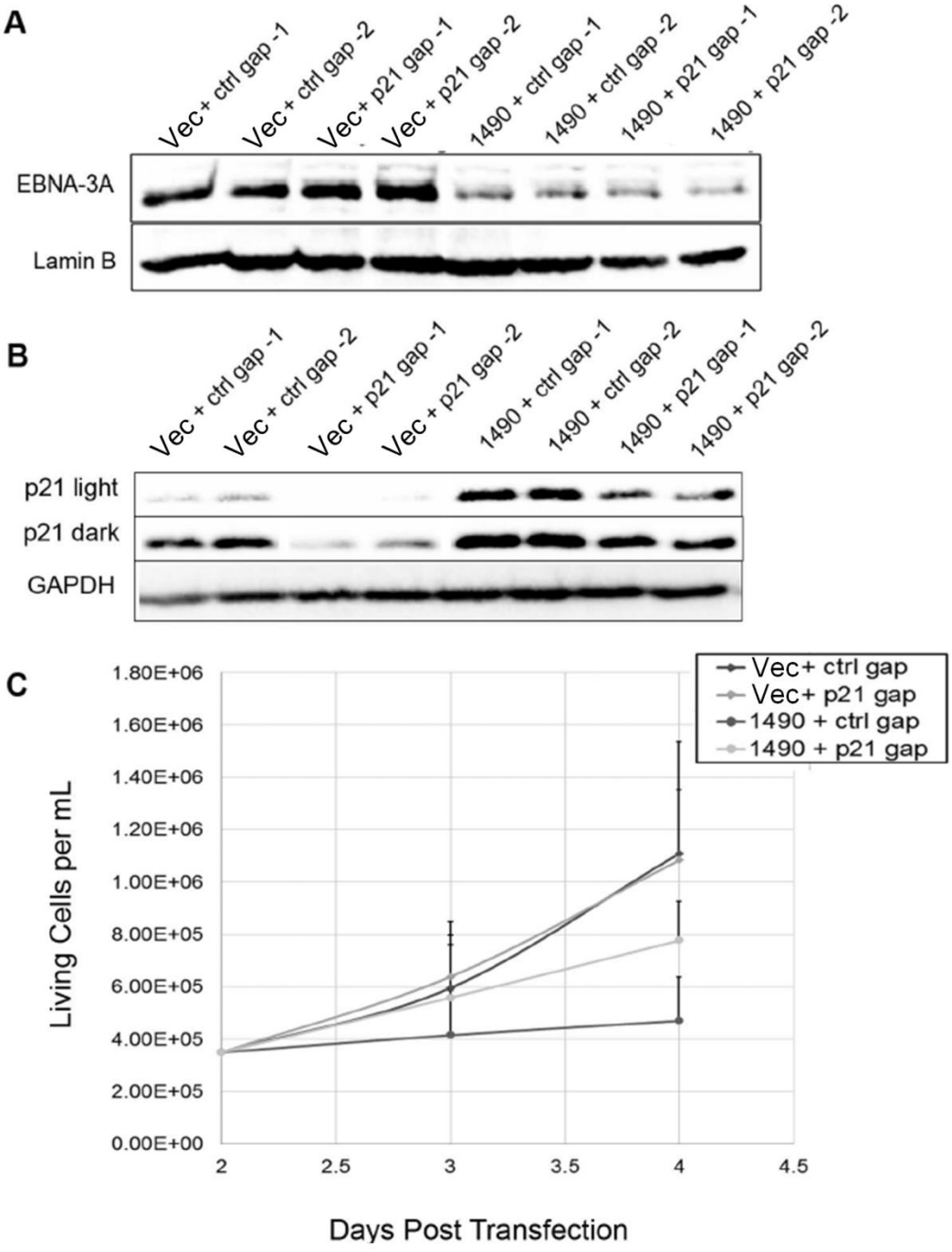
p21 mediates loss of proliferation following EBNA3A knock-down

The observation that p21 levels are increased following EBNA3A knock-down suggested to us that this cell cycle inhibitor is what mediates the G0/G1 cell cycle arrest. To confirm this protein is responsible for loss of proliferation, we used p21-targeting antisense oligonucleotides (AOS) to prevent the accumulation of p21 following EBNA3A knock-down and determined whether this could rescue cell growth. These specific AOS, called gapmers (Exiqon), contain phosphorothioate linkages flanked by locked-nucleic acid modifications that confer higher resistance to nuclease activity and the ability to bind target sequences with high affinity and stability [448]. We introduced control or p21-targeting gapmers into Sal via the process of gymnosis, during which cells internalize the oligo without the use of transfection reagent. Gapmers were added to the cell media two days prior to transfection with the EBNA3A-targeting shRNA. We determined that addition of a p21 gapmer knocked-down expression of p21 in cells compared to a control non-targeting gapmer (**Fig. 3.6 A,B**). Cells co-transfected with the EBNA3A shRNA and p21 gapmer displayed increased proliferative capacity compared to cells co-transfected with EBNA3A shRNA and control gapmer (**Fig. 3.6 C**). Growth was not restored to the level of control cells, potentially due to the inability of gapmers to reduce p21 protein expression to control levels (**Fig. 3.6 B,C**). These data suggest EBNA3A-mediated repression of p21 is functionally relevant to the continued proliferation of Wp-R. This repression, at least in part, promotes cell cycle progression in Wp-R.

Figure 3.6: p21 accumulation mediates growth arrest following EBNA3A knock-down

Sal cells were transfected with p21-targeting and control gapmers via the process of gymnosis. Cells were treated for 2 days prior to nucleofection with 100nM final concentration of gapmer. 4 days following nucleofection, cells were harvested and subjected to immunoblot analysis. (**A**) EBNA3A expression was analyzed to ensure expression of gapmers did not reduced efficacy of shRNA-mediated knock-down. Lamin B served as loading control. (**B**) p21 levels were assessed in cells co-expressing p21-targeting gapmer and EBNA3A shRNA. p21-targeting gapmers effectively reduced accumulation of p21. Immunoblots represent both a short exposure (p21 light) and a longer exposure (p21 dark). GAPDH served as loading control. (**C**) Live Sal cells were counted at indicated time points using Trypan blue exclusion, and harvested at day 4 post transfection for protein analysis.

Figure 3.6: p21 accumulation mediates growth arrest following EBNA3A knock-down



EBNA3C is not required for proliferation of Wp-R

Since all currently known functions of EBNA3A are shared by EBNA3C, and both are required for B cell immortalization and proliferation of LCLs [40,123,129], we asked whether EBNA3C is similarly required for the growth of Wp-R. To address this question, we generated Wp-R cell lines, Oku tetR-3C, in which EBNA3C is conditionally expressed. Two clones expressed an EBNA3C-targeting shRNA produced from a tetracycline-regulated promoter capable of efficiently expressing small RNAs in the presence of Dox. Indeed, we observed that following addition of Dox to the growth medium, EBNA3C was efficiently knocked-down (**Fig. 3.7 A,B**). Knock-down was reversible, and removal of Dox from the medium resulted in restoration of EBNA3C levels (**Fig. 3.7 C**).

To determine whether EBNA3C expression contributes to proliferation of Wp-R, we monitored growth over time of Oku tetR-3C following EBNA3C knock-down. No significant changes in growth were detected in the absence of EBNA3C compared to controls, indicating that EBNA3C is not required for proliferation of Wp-R. These results were surprising considering the requirement for the related family member, EBNA3A, in Wp-R, and support our hypothesis that EBNA3A performs a novel function not shared by EBNA3C. Since previous studies have shown serum deprivation induces apoptosis in BL cells [449], we examined whether EBNA3C confers any growth advantage to Wp-R under growth limiting conditions. We observed reduced proliferative capacity of Oku tetR-3C maintained in 1% serum following EBNA3C knock-down (**Fig. 3.8**). These data suggested that, while EBNA3C is not required for proliferation, EBNA3C can promote survival of Wp-R.

Figure 3.7: Conditional knock-down of EBNA3C in Oku

Oku cells that stably express TetR protein were transfected with EBNA3C shRNA and put under G418 (800 mg/mL) selection. Two clones obtained following selection, clone 1(**A**) and clone 2 (**B**), were treated with Dox (1 μg/mL) to evaluate their ability to conditionally express EBNA3C. Cells were harvested at indicated time points, and in both clones, knock-down of EBNA3C was observed following addition of Dox.

(**C**) Dox was removed from the cell medium and cells were washed with PBS. Following removal of Dox , EBNA3C expression was gradually restored over time. Lamin B served as a loading control.

Oku tetR-3C clones that conditionally express EBNA3C shRNA were generated by Satheesh Cheeyancheri Chencheri.

Figure 3.7: Conditional knock-down of EBNA3C in Oku following Dox addtion

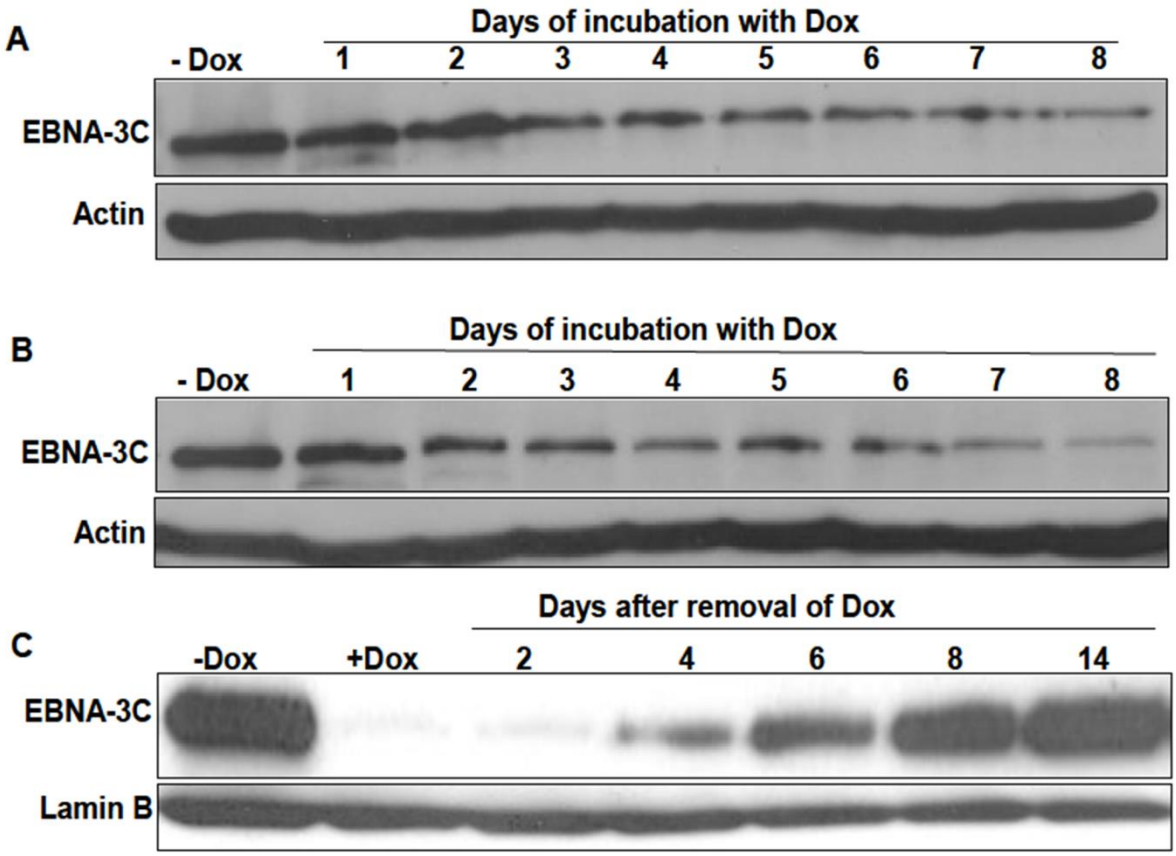
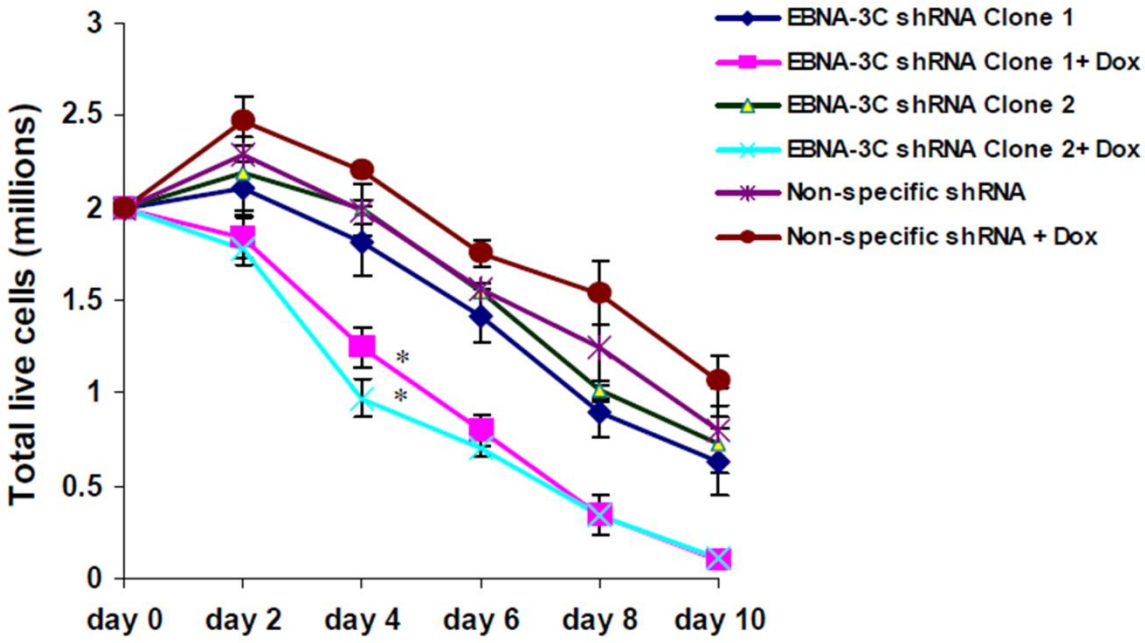


Figure 3.8: EBNA3C expression promotes survival of Wp-R under growth limiting conditions

Oku tetR-3C (Clone 1) cells, either in the presence or absence of Dox, were evaluated for growth in 1% serum. Cells were maintained in Dox for 8 days prior to growth analysis to ensure maximal knock-down of EBNA3C. Oku cells expressing non-specific shRNA were used a control. $2x10^6$ cells were seeded in 10 mL medium, and total live cells were counted every 2 days using Trypan blue exclusion.

Growth results presented include 2 independent experiments performed by Satheesh Cheeyancheri Chencheri.

Figure 3.8: EBNA3C expression promotes survival of Wp-R under growth limiting conditions



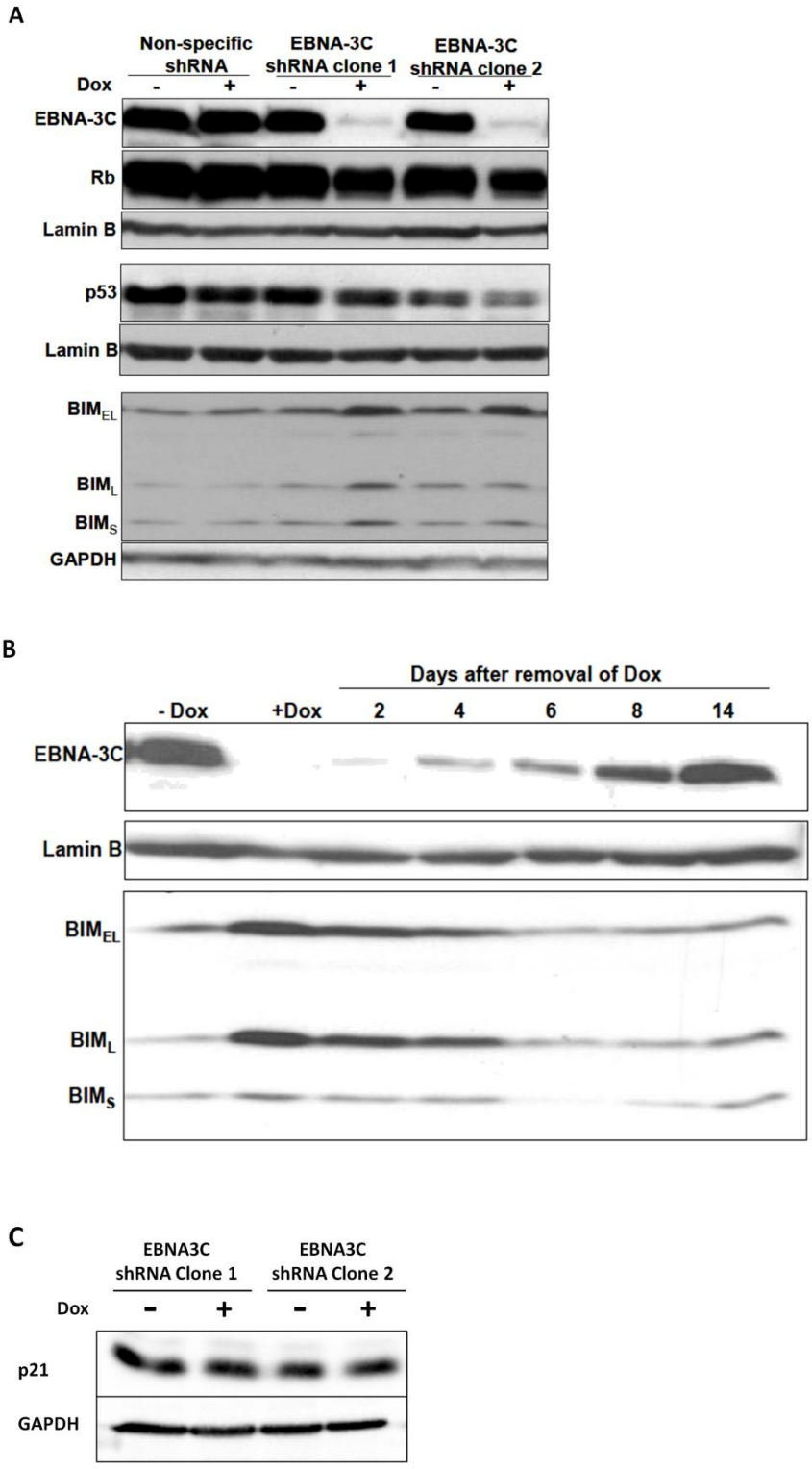
EBNA3C represses expression of Bim in Wp-R

EBNA3A and EBNA3C cooperatively repress the pro-apoptotic factor, Bim, in LCLs [136,190]. We investigated whether EBNA3C maintains this function in Wp-R, potentially contributing to enhanced EBNA3C-mediated survival. Following knock-down of EBNA3C we observed increased protein expression of Bim (**Fig. 3.9 A**). Furthermore, withdrawal of Dox from the growth medium and subsequent restoration of EBNA3C expression, was accompanied by a return of Bim expression to a repressed state (**Fig. 3.9 B**). EBNA3C-mediated repression of Bim likely contributes to survival of Wp-R, however this probably occurs cooperatively with EBNA3A, as we previously determined EBNA3A also represses Bim in Wp-R [150]. In contrast to previously reported data, we did not detect significant changes in expression of p53 or Rb proteins in the presence or absence of EBNA3C, nor did we identify a role for EBNA3C in the regulation of p21, suggesting a novel function for EBNA3A in repression of p21 (**Fig. 3.9 A,C**).

Figure 3.9: EBNA3C represses Bim expression

Oku tetR-3C were maintained in Dox for at least 8 days and then harvested for lysate. (**A**) Bim (3 isoforms: extra long (EL), long (L), short (S)), p53, and Rb expression were analyzed by immunoblot. Lamin B and GAPDH both served as loading controls. (**B**) Restoration of EBNA3C expression, following removal of Dox, repressed expression of Bim. Cell were washed in PBS and moved to growth medium without Dox. (**C**) Oku tetR-3C cells cultured in the presence and absence of Dox were examined for p21 protein expression. GAPDH served as a loading control.

Figure 3.9: EBNA3C represses Bim expression



BHRF1 promotes survival of Wp-R

As overexpression of Myc is a potent inducer of apoptosis [158], we asked whether BHRF1 counteracts Myc-induced apoptosis and promotes survival of Wp-R. We used BHRF1-targeting shRNAs to knock-down BHRF1 expression in Wp-R cell lines, Oku and Sal. Expression of BHRF1 shRNA in Oku lead to diminished proliferative capacity and reduced cell viability compared to control cells (**Fig. 3.10**). Unexpectedly, no significant effect on growth in Sal cells was observed following transfection with BHRF1 shRNA, despite efficient knock-down of BHRF1 protein expression (**Fig. 3.11 A,C**). However, we determined BHRF1 knock-down does impact cell growth and viability in Sal under growth limiting conditions (1% serum) (**Fig. 3.11 B**). Our data suggested BHRF1 does contribute to the survival of Wp-R, although the degree of dependence on BHRF1 expression varies depending on Wp-R cell line.

Figure 3.10: BHRF1 knock-down results in decreased proliferation in Oku

$3x10^6$ Oku were transfected with control, non-functional, or BHRF1-specific shRNA. 48 h post transfection cells were seeded at $3.5x10^5$/mL and put under G418 selection. Live cells were counted at indicated time points, and fed and re-seeded at $3.5x10^5$/mL every 2 days.

Figure 3.10: BHRF1 knock-down results in decreased proliferation in Oku

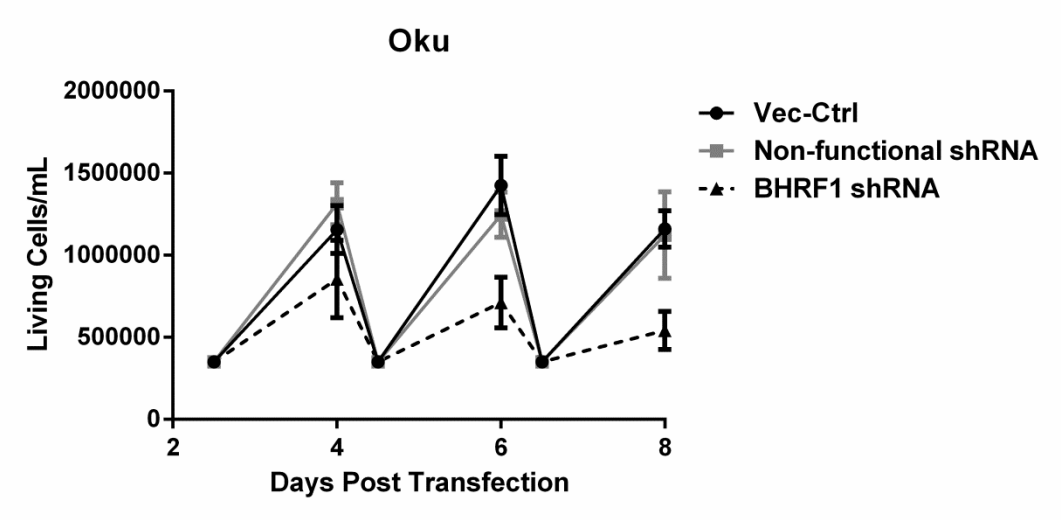
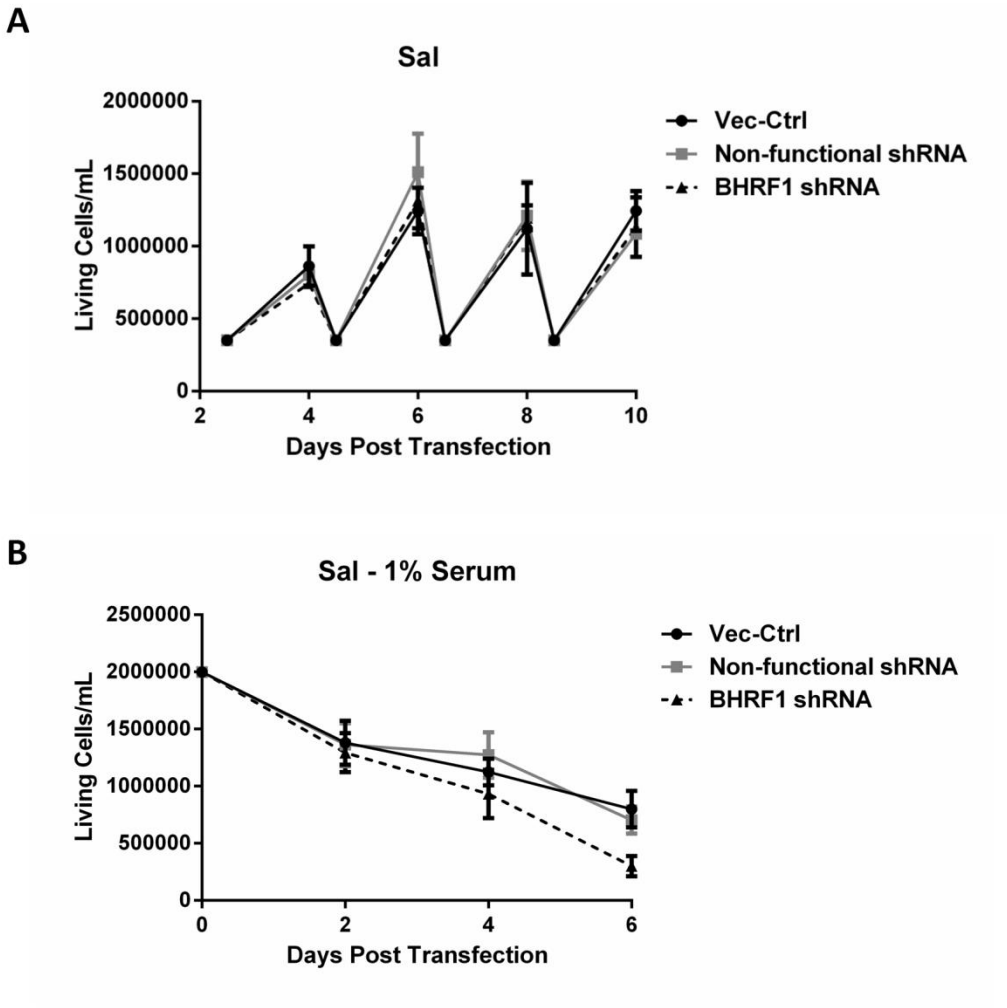


Figure 3.11: BHRF1 promotes survival of Sal under growth limiting conditions

(**A**) $2x10^6$ Sal cells were transfected with indicated shRNA plasmids. 48 h post transfection cells were seeded at $3.5x10^5$/mL and put under G418 selection. Live cells were counted at indicated time points, and fed and re-seeded at $3.5x10^5$/mL every 2 days. (**B**) Sal were transfected as described above and seeded in 10 mL of growth medium containing 1% serum. Live cells were counted every 2 days as indicated. (**C**) Cells were collected and lysed at day 6 post transfection for immunoblot analysis of BHRF1 expression. GAPDH was used as a loading control.

BHRF1 immunoblots performed by Satheesh Cheeyancheri Chencheri.

Figure 3.11: BHRF1 promotes survival of Sal under growth limiting conditions



3.3 Discussion

Both Latency I and Wp-R BL contain a Myc translocation leading to over expression of the proto-oncogene [48,174]. The relatively high percentage of BL tumors expressing Wp-R latency (20%) could indicate that a selective advantage exists for this unique EBV genome. We hypothesized EBV latency-associated proteins expressed in Wp-R, but not Latency I BL, contribute to enhanced oncogenic potential of these cells. To begin, we evaluated whether the EBV latency-associated proteins, EBNA3A, EBNA3C, and BHRF1, contribute to the pathogenesis of Wp-R. We determined EBNA3A is required for the proliferation of Wp-R, while EBNA3C is dispensable. Although the only currently known functions of EBNA3A are shared by EBNA3C, these data suggest that EBNA3A likely does have functions distinct from EBNA3C. In Wp-R, EBNA3C may promote survival by repressing Bim, thereby countering Myc-induced apoptosis. We determined BHRF1 also contributes to survival of Wp-R, although individual Wp-R cell lines differed in their dependence on BHRF1.

Previous reports concluded that EBNA3A maintains growth of LCLs via repression of $p14^{ARF}$ and $p16^{INK4a}$ [129], however our data suggest this is not a primary role of EBNA3A in Wp-R. Following transfection of Wp-R cells with EBNA3A-specific shRNAs, we did not observe changes in $p14^{ARF}$ expression, while $p16^{INK4a}$ was undetectable. Since EBNA3A knock-down results in rapid cessation of growth, we investigated additional cell cycle regulators that could be mediating the G0/G1 cell cycle arrest. We observed a shift in Rb phosphorylation from a predominantly hyperphosphorylated (inactive) to a hypophosphorylated (active) state. These

findings suggested EBNA3A could be positively regulating cyclin/CDK complexes, thereby permitting Rb phosphorylation. Indeed, we found that following knock-down of EBNA3A, protein expression of the cyclin-dependent kinase inhibitor, p21, is increased. We found p21 accumulation was a key mediator of cell cycle arrest, as knock-down of p21 restored cell growth. These data provided a novel function of EBNA3A to repress p21 expression in Wp-R. Additionally EBNA3A-mediated repression of p21, which we determined was conserved in LCLs, is also novel in that it is a function not shared by EBNA3C.

Our observation that EBNA3C is not required for the proliferation of Wp-R is in stark contrast to the role EBNA3C plays in LCLs. Loss of EBNA3C in this cellular context results in cessation of growth, failure to progress through the G1/S restriction point, and reduced overall cell viability [117,450]. Since the functions EBNA3 proteins have in Wp-R have not been studied, there could be several reasons for the differing requirements for EBNA3C in Wp-R and LCLs. It is possible EBNA3C is non-essential for growth of Wp-R since it does not participate in p21 regulation with EBNA3A, and $p16^{INK4a}$, a known target of EBNA3C, is silenced in these tumors, rendering EBNA3C dispensable. Additionally, functions attributed to EBNA3C have been identified in cells expressing EBNA2, a major transcriptional regulator of both viral and cellular genes. EBNA3C can inhibit EBNA2-mediated transactivation of cellular genes via its RBP-Jκ-binding domain, but EBNA3C can also cooperate with EBNA2 to activate LMP promoters [451], demonstrating significant interplay between EBNA3C and EBNA2 in transcriptional regulation. Wp-R tumors, however, do not express EBNA2 and have likely acquired additional mutations to compensate for the lack of EBNA2-

mediated gene regulation. Our data could suggest the role of EBNA3C in promoting B cell proliferation is dependent on EBNA2, thus explaining why EBNA3C does not maintain this function in Wp-R.

We determined BHRF1 contributes to the survival of Wp-R, although the level of contribution varies between cell lines. In Oku, BHRF1 knock-down induced cell death, accompanied by reduced proliferative capacity, whereas in Sal, BHRF1 knock-down only affected cell viability under growth limiting conditions. One potential explanation for this could be elevated expression of truncated EBNA-LP in Sal compared to Oku [184]. Expression of truncated EBNA-LP confers increased resistance to apoptosis [186], and if Sal already express robust levels of this anti-apoptotic factor, these cells may be less dependent on BHRF1 to mediate cell survival.

Here, we examined contributions of EBV latency-associated proteins to the pathogenesis of Wp-R BL. We determined that while EBNA3C and BHRF1 promote survival of Wp-R, and likely contribute to the increased oncogenic potential of Wp-R compared to Latency I BL, EBNA3A expression was absolutely essential for the proliferation of Wp-R. It is possible, however, EBNA3C and BHRF1 play more vital roles in Wp-R *in vivo*. We identified a novel function of EBNA3A to repress p21, and determined that EBNA3A-mediated repression of this cell cycle inhibitor is what facilitates cell cycle progression in Wp-R. In the next chapters, we continued our studies on Wp-R oncogenesis by focusing on EBNA3A and the mechanisms by which it promotes proliferation and repression of p21.

3.4 Materials and Methods

Cell Culture

Sal and Oku are human EBV-positive BL cell lines that maintain Wp-R latency. All B cells were grown in RPMI 1640 (Hyclone, Thermo Scientific, MA) supplemented with 10% fetal bovine serum (FBS). For transfection, cells were grown in roller bottles and used while in log phase of growth (0.8-1.2x10^6 cells/mL). To generate Oku tetR-3C, we transfected Oku, which stably maintain the tetracycline repressor (TetR) protein, with Tet-regulatable plasmids expressing EBNA3C shRNA. Stable lines were produced following blasticidin (3 μg/mL, Invitrogen) and G418 (800 μg/mL, Gibco) selection. Addition of Dox (1 μg/mL) to cell medium of antibiotic resistant clones resulted in efficient knock-down of EBNA3C protein expression.

Plasmids

The EBNA3A-targeting shRNA, 3AshRNA-1490, was described previously [150], and is expressed from the *oriP*-GFP vector (Vec-Ctrl), which contains a neomycin-resistance gene, and the EBV origin of latent DNA replication, *oriP*, allowing for stable, episomal maintenance of the plasmid by EBNA-1 in EBV-positive cells.

shRNAs to target EBNA3C and BHRF1 were designed using the BLOCK-iT™ RNAi Designer from Invitrogen: EBNA3C - (GCATTTCGAAAGGCACAAATA); BHRF1 - (GGAGATAATTGAACGAATTC). These shRNA constructs were sub-cloned into the *Xba*I site of an *oriP*-GFP vector.

Transfection

B cells were transfected by Amaxa nucleofection (Lonza) in solution V using program G16. $5x10^6$ cells (collected in log phase of growth, $8.0x10^5$-$1.2x10^6$/mL) were transfected with 5 μg of plasmid DNA and plated in a 12-well plate with 0.5 mL conditioned media and 0.5 mL fresh RPMI growth medium per well. At 48 h post transfection cells were seeded at $3.5x10^5$ cells/mL in 10mL RPMI growth medium containing 800μg/mL G418 (Invitrogen, NY). Total live cells were counted every 2 days and re-seeded at a concentration of $3.5x10^5$ cells/mL until collected.

Cell harvesting and immunoblotting

Cells were collected and lysed in a derivative of Laemmli sample buffer [452], and sonicated. Proteins were transferred to PVDF membranes following separation by SDS-PAGE. Antibodies used for immunoblotting are as followed: EBNA3A and -3C sheep serum (Exalpha); $p21^{WAF1/CIP1}$ (12D1) and Bcl-XL (Cell Signaling, MA); p53 (DO-1), Zta (BZ1) and Lamin B (M20) (Santa Cruz, CA); Bim (559685) and GAPDH (BD Pharmingen); BHRF1 (MAD8188) (Millipore, MA).

Akata induction

EBV-positive Akata cells that maintain Latency I were seeded at $5x10^5$ cells/mL and treated with 100 μg/mL of human anti-IgG antibody (ICN/Cappel) per mL of culture. Cells were induced for 2 days and then harvested for immunoblot analysis.

Gapmer gymnosis

LNA longRNA Gapmers were supplied by Exiqon. Gapmer sequences are as follows (Uppercase letters indicate LNA and lowercase letters indicate phosphorothioated bases) : Control -(ACCagggcgtatctctccATA), p21 -(TCCgcggggagCTCC) [448]. Gapmers were added to cell medium at day 0 for a final concentration of 100 nM. Cells were transfected with respective plasmids at day 2 and gapmer was readministered. From then on, cells were seeded as described above along with additional doses of gapmer every 2 days.

Propidium iodide staining and cell cycle analysis

$1x10^6$ transfected Sal cells were harvested for propidium iodide (PI) staining. Cells were fixed in 1% paraformaldehyde for 1 hour at 4°C, washed in PBS, and resuspended in 70% ethanol for 2 hours. Cells were washed again and resuspended in PBS containing 40 μg/mL PI (Invitrogen, CA), and 200 μg/mL RNase A (Invitrogen), and incubated for 30 minutes at room temperature in the dark. Following PI staining, cells were analyzed on FACScan Flow Cytometer (BD Biosciences, NJ). Cell cycle analysis was performed based on DNA content of either GFP-positive cells or total cells using ModFit LT software.

Chapter 4: EBNA3A Activates Expression of MicroRNA-155 in B cells

4.1 Introduction

EBV is a ubiquitous human herpesvirus that persists for the life of its host by establishing latent infection predominantly in B lymphocytes [453]. Lifelong infection is achieved by expressing a series of latency programs in which latency-associated genes that promote cell proliferation and survival are progressively down-regulated in order to ensure persistent infection. EBV contributes to both lymphoid and epithelial cell malignancies, and latent infection plays an etiologic role in approximately 98% of cases of eBL [454,455]. Whereas the majority of EBV-positive BL displays the Latency I phenotype, about 20% of BL tumors exhibit the intermediate latency program, Wp-R latency [23]. Wp-R cells contain a unique EBV genome in which the region encoding EBNA2 and a segment of EBNA-LP are deleted. As a result, there is distinct pattern of latency in which expression of truncated EBNA-LP, EBNA1, the EBNA3 family of proteins, and BHRF1 is observed. EBNA2 and the LMPs are not expressed, likely due to loss of EBNA2-mediated activation of LMP promoters [48,53,54].

MicroRNAs are involved in a broad range of diseases, such as cancer [269,456], and to date, miR-155 is one of the most highly implicated microRNAs in the development of cancer [234,256,271,457–460]. Elevated levels of miR-155 have been observed in multiple types of cancer, including lymphomas, and in transgenic mouse models, B cell-targeted overexpression of this miRNA leads to the formation of B-cell

malignancies [271]. EBV infection of resting primary B cells is a potent inducer of miR-155 expression, and in fact miR-155 is the most abundantly expressed miRNA in LCLs. EBV-mediated activation of miR-155 also contributes to the activated phenotype of EBV-positive B cells and is important for efficient episomal maintenance of the EBV genome [282]. miR-155 is robustly expressed in EBV-infected B cells displaying Latency III, but not in those displaying Latency I, suggesting that latency-associated proteins expressed during Latency III activate miR-155 [283–285]. Inhibition of miR-155 in LCLs, but not Latency I BL or EBV-negative B cells, results in abrupt G1 cell cycle arrest, as well as increased apoptotic cell death, demonstrating that miR-155 is required for the proliferation of LCLs [288]. However, neither miR-155 expression levels nor the contribution of miR-155 to the pathogenesis of Wp-R have been previously investigated.

We demonstrated that knock-down of EBV latent protein EBNA3A in Wp-R BL results in rapid arrest of cell growth in the G1 phase of the cell cycle, followed by increased apoptosis (**Chapter 3**) [150]. Additionally, we observed EBNA3A represses p21 in both Wp-R and LCLs, thereby promoting hyperphosphorylation of Rb and progression through the cell cycle [150]. Since this phenotype is strikingly similar to that observed following inactivation of miR-155, and miR-155 has also been implicated in p21 and Rb regulation via targeting of Smad proteins and subsequent disruption of TGFβ signaling pathways [364,461], we asked whether EBNA3A plays a role in induction of miR-155 expression. Here, we show that EBNA3A is both necessary and sufficient for miR-155 activation, though the contribution of miR-155 to Wp-R pathogenesis remains unclear.

4.2 Results

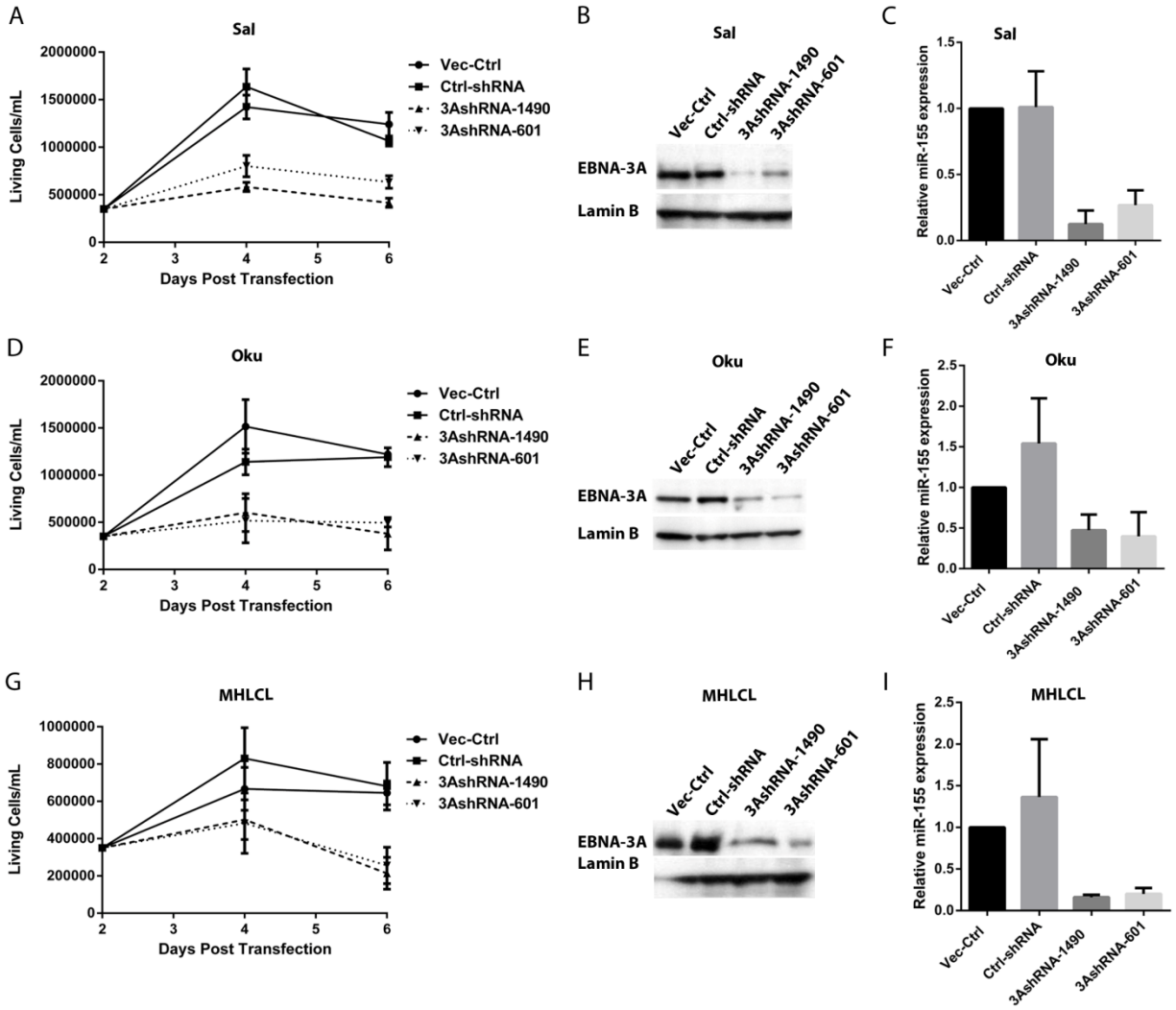
EBNA3A positively regulates miR-155 expression in both Wp-R and LCLs

To determine whether EBNA3A plays a role in regulation of miR-155 expression, we used two distinct shRNAs (3AshRNA-1490 and 3AshRNA-601) that target different regions of EBNA3A, both of which effectively knock-down its expression [150]. The shRNAs are expressed from a vector containing the EBV origin of latent DNA replication, *ori*P, which allows for stable episomal maintenance of the plasmid in EBNA1-expressing cells and a selectable marker to allow selective proliferation of transfected cells. Introduction of EBNA3A-specific shRNAs into either LCLs or two Wp-R cell lines, Sal and Oku, resulted in specific knock-down of EBNA3A, and a concomitant loss of proliferation compared to empty vector (Vec-Ctrl) or a non-targeting control shRNA (Ctrl-shRNA) as we recently reported [150] (**Fig. 4.1 A-B,D-E,G-H**). We next measured miR-155 levels in these cells following EBNA3A knock-down and observed that miR-155 levels were significantly reduced in each cell line transfected with EBNA3A-targeting shRNAs, compared to cells transfected with Vec-Ctrl or Ctrl-shRNA. These data suggested that EBNA3A contributes to miR-155 activation in both Wp-R and LCLs (**Fig. 4.1 C,F,I**).

Figure 4.1: EBNA3A positively regulates miR-155 expression in both Wp-R and LCLs.

Sal, Oku, and MHLCL were transfected with empty vector (Vec-Ctrl), non-targeting control shRNA (Ctrl-shRNA), or two different EBNA3A-specific shRNAs (3AshRNA-1490 and 3AshRNA-601). Transfected cells were evaluated for cell proliferation (**A**,**D**,**G**), EBNA-3A expression by immunoblot analysis at day 6 post transfection (**B**,**E**,**H**) and relative levels of miR-155 measured by qPCR in indicated cell line at day 6 post transfection (**C**,**F**,**I**). Values were normalized to the small cellular nuclear RNA, U6. Growth curves depict rapid loss of proliferation following knock-down of EBNA3A in all cell lines tested, while control cells continue to proliferate. Cells were seeded at $3.5x10^5$ on day 2 post transfection, and then re-seeded at $3.5x10^5$ on day 4 post transfection, following the day 4 cell count. Values are represented as living cells/mL. Cells were transfected with indicated shRNA expression vectors and harvested at day 6 post transfection for immunoblot analysis of EBNA3A and Lamin B. Transfection of both 3AshRNA-1490 and -601 resulted in efficient knock-down of EBNA3A. Lamin B served as a loading control.

Figure 4.1: EBNA3A positively regulates miR-155 expression in both Wp-R and LCLs.



EBNA3A is sufficient to induce miR-155 expression in B cells

To determine whether EBNA3A expression alone is sufficient to induce miR-155, we used BJAB-tetoff-3A, an EBV-negative B cell lymphoma line which conditionally expresses EBNA3A. Upon addition of doxycycline (Dox), EBNA3A expression is gradually downregulated (**Fig.4.2 C**), allowing changes in miR-155 levels to be monitored in the presence and absence of EBNA3A. We observed increased miR-155 in BJAB cells expressing EBNA3A compared to the same cell line in the absence of EBNA3A, suggesting EBNA3A is sufficient for induction of miR-155. (**Fig. 4.2 A**). Following addition of Dox, and subsequent shut-off of EBNA3A expression (which was fully achieved by day 14 post addition of Dox), miR-155 expression was reduced to levels similar to those measured in parental BJAB cells (**Fig. 4.2 A,C**).

Both LMP1 and EBNA2 regulate miR-155 expression, albeit modestly [282,286,287], and we confirmed these findings in BJAB lines stably expressing either LMP1 or EBNA2 (**Fig. 4.2 D,I**). No single EBV protein may be sufficient to induce miR-155 to the levels observed in LCLs, but rather the cooperation of several EBV latency-associated proteins is likely required to achieve full activation. To test this hypothesis, we assessed whether EBNA3A-mediated regulation of miR-155 could be synergistically enhanced in the presence of EBNA2 using BJAB-E2-teton-3A cells, an EBV-negative B cell lymphoma line that stably expresses EBNA2 and conditionally expresses EBNA3A in the presence of Dox. While both EBNA2 and EBNA3A alone were sufficient to increase miR-155 expression, the combined expression of both

proteins further boosted miR-155 to levels higher than when either protein is expressed alone (**Fig. 4.2 D**).

To determine whether EBNA3A-meditated regulation of miR-155 is B cell-specific, we transfected MCF7, an EBV-negative breast cancer line, with an EBNA3A expression vector. Expression of EBNA3A in MCF7 did not significantly alter levels of miR-155 compared to pSG5 empty vector control (**Fig. 4.2 G**). EBNA3A-mediated activation of miR-155 is likely B cell-specific, thus EBNA3A does not maintain this function in MCF7, which likely lacks the necessary cellular context.

EBNA3A does not significantly activate expression of the miR-155 primary transcript, BIC

miR-155 is processed from a non-coding RNA exon transcribed from the BIC gene located on chromosome 21 [256]. The BIC gene was originally described as a common site for viral DNA integration of avian sarcoma leukosis virus proviral DNA, leading to lymphomas in chickens [255,256]. In humans, BIC RNA levels are frequently elevated in various B cell malignancies compared to normal lymphoid tissue [457,460]. An exception is observed in cases of BL, where very little BIC or miR-155 is detected [285]. *In vitro*, BIC expression can be induced in BL cells upon B cell receptor stimulation, however miR-155 levels remain negligible. Although, these data suggested that BL cell lines have a blockade in processing BIC into mature miR-155 [462], a recent study failed to confirm these findings [463]. MiR-155 levels can likely be regulated both by transcription of the BIC primary transcript and processing of the mature microRNA.

To determine whether EBNA3A-mediated regulation of BIC transcription is the mechanism for miR-155 induction, we measured expression of BIC in both Wp-R and LCLs transfected with EBNA3A-targeting shRNAs. Despite the changes EBNA3A knock-down caused in miR155 (**Fig. 4.1**), there were no significant changes in BIC expression in cells transfected with either EBNA3A-targeting shRNA, suggesting that EBNA3A does not influence transcription of BIC (**Fig. 4.3**). In fact, Sal and MHLCL transfected with 3AshRNA-601 displayed elevated levels of BIC, in contrast to miR-155 levels. BIC was also measured in BJAB-tetoff-3A and BJAB-E2-teton-3A in the presence and absence of EBNA3A. We did not observe significant changes in BIC levels when EBNA3A was expressed in BJAB-E2-teton-3A, providing further evidence that EBNA3A does not regulate the BIC transcript to enhance miR-155 expression (**Fig. 4.2 E**). However, when EBNA3A was expressed in BJAB-tetoff-3A, not only did BIC levels not correlate with the increase we observed in miR-155 expression, but instead they were reduced (**Fig. 4.2 B**). If EBNA3A functions to enhance processing of miR-155 rather than transcription of its primary transcript, BIC, then low levels of BIC could be caused by depletion of the full-length transcripts via increased processing into mature miR-155. The differing results obtained in these two cell lines could be due to higher expression levels of EBNA3A in BJAB-tetoff-3A, compared to BJAB-E2-teton-3A, that enhance processing of miR155 to a greater extent resulting in the observed depletion of BIC.

Figure 4.2: EBNA3A is sufficient to induce miR-155 expression in B cells.

(**A**) miR-155 expression was measured by qPCR in BJAB-tetoff-3A cells, and values were normalized to U6. miRNA expression from day 0 and day 14 post dox addition was compared to the EBV-negative B cell lymphoma line, BJAB. (**B**) qPCR was used to assess BIC expression in BJAB-tetoff-3A. Values were normalized to the ribosomal protein, RPLP0. (**C**) BJAB-tetoff-3A cells were harvested at the indicated time points for immunoblot analysis of EBNA3A expression. BJAB and Oku cells served as negative and positive controls, respectively. Lamin B served as a loading control. (**D**) Relative miR-155 expression in BJAB-E2-teton-3A measured by qPCR harvested at the indicated time points for immunoblot analysis of EBNA3A and EBNA2 expression. Lamin B served as a loading control. (**E**) BIC expression, relative to BJAB, was measured by qPCR in BJAB-E2-teton-3A cells and values were normalized to RPLP0. (**F**) BJAB-E2-teton-3A cells were harvested at indicated times post addition of Dox for immunoblot analysis of EBNA3A and EBNA2 levels. Lamin B served as a loading control. (**G**) MCF7 were transfected with an empty vector control (pSG5) or either an EBNA3A (pSG5 3A) or EBNA-3C (pSG5 3C) expression vector. miR-155 expression was measured 72 h post transfection. Values were normalized to U6. (**H**) Immunoblot analysis of MCF7 transfected with EBNA3A and -3C expression vectors. Cells were harvested 72 h post transfection. Lamin B served as a loading control. (**I**) miR-155 expression was measured in EBV-negative BJAB lines that stably express either LMP1 or EBNA2. (**J**) Confirmation of LMP1 and EBNA2 expression in BJAB lines by immunoblot analysis.

Figure 4.2: EBNA3A is sufficient to induce miR-155 expression in B cells.

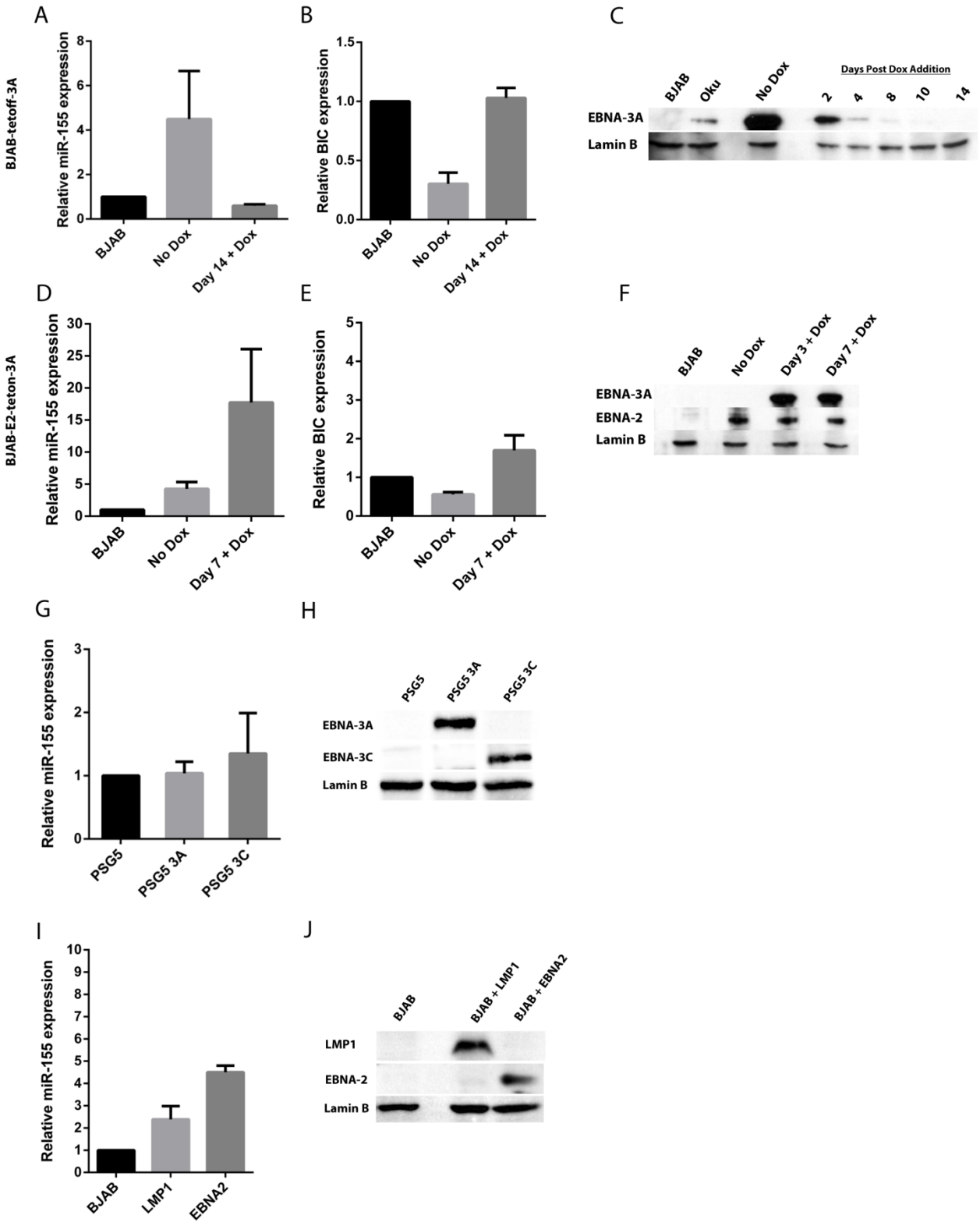
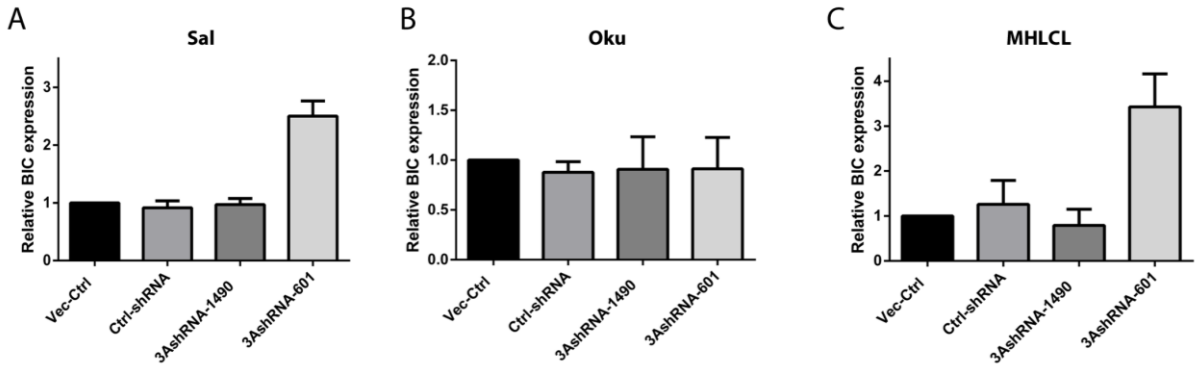


Figure 4.3: EBNA3A does not increase expression of miR-155 primary transcript, BIC.

Sal (**A**), Oku (**B**), and MHLCL (**C**) were transfected with the indicated shRNAs and harvested at day 6 post transfection. BIC expression was measured by qPCR, and values were normalized to RPLP0.

Figure 4.3: EBNA3A does not increase expression of miR-155 primary transcript, BIC.



EBNA3C does not regulate miR-155

A significant level of cooperation exists between the EBNA3 proteins to mediate transcriptional regulation. Although multiple unique functions of EBNA3C have been identified, most known functions of EBNA3A to date are shared with EBNA3C. To determine whether regulation of miR-155 is a unique function of EBNA3A, we utilized a Wp-R cell line, Oku tetR-3C, in which EBNA3C is conditionally expressed. This cell line expresses an EBNA3C-targeting shRNA expressed from a tetracycline-regulated promoter that directs small RNA production in the presence of Dox. Indeed, we observed that following addition of Dox to the growth medium, EBNA3C levels were reduced (**Fig. 4.4 E**). However, there were no significant changes in miR-155 expression, suggesting that EBNA3C did not contribute to miR-155 regulation in Wp-R (**Fig. 4.4 D**). To examine whether LCLs behaved similarly, we used ER/EBNA3C, an LCL that expresses EBNA3C fused to a 4-hydroxytamoxifen (4HT)-dependent mutant estrogen receptor that is active only in the presence of estrogen. Following removal of estrogen, miR-155 levels remained unchanged at day 24 (**Fig. 4.4 A**), even though EBNA3C was inactivated and ultimately degraded [129,450], (**Fig. 4.4 C**). These data indicate that EBNA3C does not affect miR-155 expression in Wp-R or LCLs.

Loss of EBNA3A results in cessation of proliferation and cell death over time, and miR-155 expression levels correlate with the activation state and proliferative capacity of B cells. We next investigated whether reduced miR-155 expression following EBNA3A knock-down was related specifically to the absence of EBNA3A rather than a general consequence of loss of cellular proliferation and cell death. Due to the essential role of EBNA3C in the proliferation of LCLs, ER/EBNA3C LCLs

cease to proliferate following removal of estrogen supplementation [129], (**Fig. 4.4 B**). The finding that miR-155 levels are unaffected by the loss of EBNA3C in this LCL suggests not only that EBNA3C is not involved in miR-155 regulation, but also that the miR-155 reduction we observe following knock-down of EBNA3A is specifically a function of EBNA3A rather than a reflection of the proliferation state of these cells.

miR-155 is required for proliferation of LCLs, but dispensable for proliferation of Wp-R BL

Because miR-155 is essential for the growth of LCLs [288], and the effects of loss of functional miR-155 resemble the loss of EBNA3A, EBNA3A's role in promoting cellular proliferation could be mediated through enhanced miR-155 expression. Although EBNA3A can induce miR-155 in Wp-R, it is unclear whether miR-155 provides any significant proliferative advantage in these tumor cells. To address this question, we used a miR-155-specific microRNA sponge (sp_155), which specifically and stably blocks the activity of miR-155 via overexpression of an mRNA encoding multiple copies of its target site, and monitored growth over time relative to transduction with a control vector (miR_Ctrl). The sp_155 and miR_Ctrl also express GFP, which was used to evaluate transduction efficiency (~40-60%), and puromycin resistance, to select transduced cells. Inactivation of miR-155 did not significantly alter the growth of either Wp-R cell line tested; in contrast, MHLCL cells expressing the miR-155 sponge displayed attenuated proliferation (**Fig. 4.5 A-C**), as previously observed [288]. Additionally, we observed increased levels of p21, previously shown to be an indirect target of miR-155 [364], following miR-155 knock-down in MHLCL, but

not in Sal or Oku (**Fig. 4.5 D**). p15 and p53 levels did not change in any cell line tested.

Notably, levels of miR-155 in Wp-R cells lines were higher than those in Latency I BL, but much less than those observed in LCLs (**Fig. 4.5 E**). It is likely that the levels of miR-155 in Wp-R are insufficient to meet a threshold that would confer a substantial proliferative advantage in these cells. EBNA3A-mediated regulation of miR-155 may be a function that is physiologically significant to the growth of LCLs, but not Wp-R.

Figure 4.4: miR-155 expression is not regulated by EBNA-3C.

(**A**) miR-155 expression was measured in ER/EBNA3C LCL following removal of 4HT. Values were normalized to U6. (**B**) Following removal of 4HT, ER/EBNA3C LCL ceased to proliferate over time compared to cells cultured with 4HT. Cells were fed as needed with fresh 15% RPMI (usually every 3 days). (**C**) Immunoblot analysis of EBNA3C expression in ER/EBNA3C cells harvested at time points indicated. Oku served as a positive control for EBNA3C expression. The size difference between EBNA3C protein detected in ER/EBNA3C LCL and Oku is due to the addition of a 4HT-dependent mutant estrogen receptor fused to EBNA3C expressed in ER/EBNA3C LCL. EBNA3C is degraded over time in ER/EBNA3C LCL following 4HT removal. (**D**) miR-155 expression was measured in Oku tetR-3C in cells that had been cultured with and without Dox. (**E**) Knock-down of EBNA3C was evaluated by immunoblot analysis in Oku tetR-3C cells cultured with and without Dox. Addition of Dox to culture medium activates expression of an EBNA3C-targeting shRNA expression, resulting in efficient and sustainable knock-down of EBNA3C. Wild-type Oku cells were also evaluated as a positive control. Lamin B served as a loading control.

Figure 4.4: EBNA3C does not regulate miR-155.

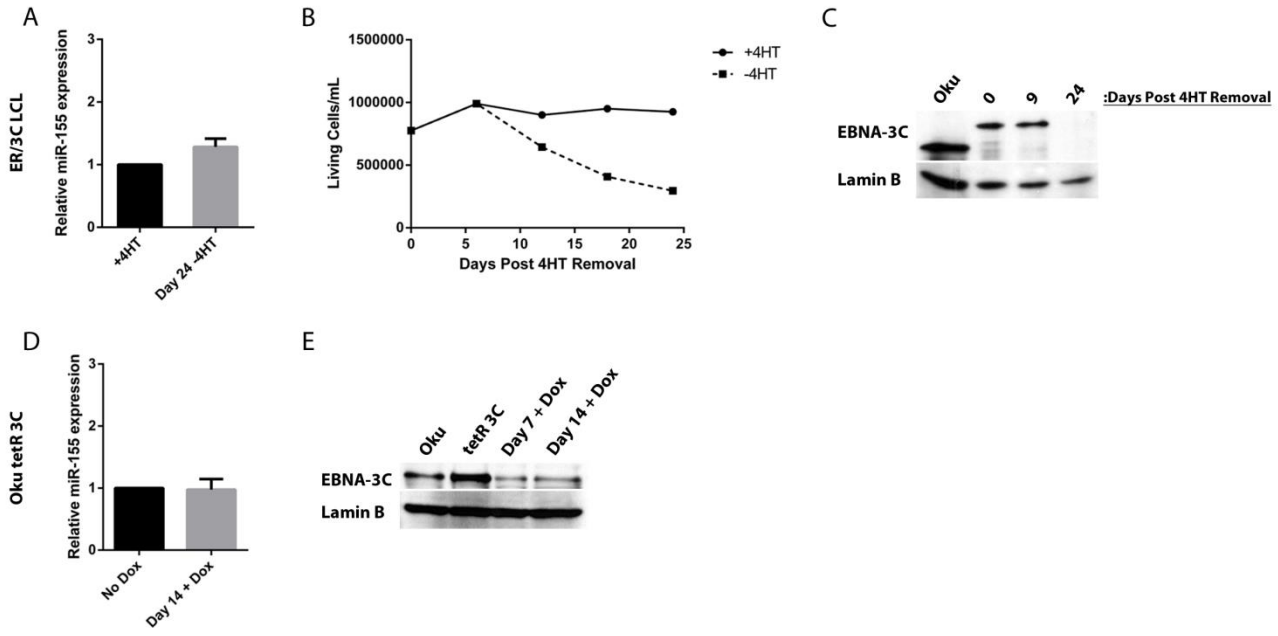
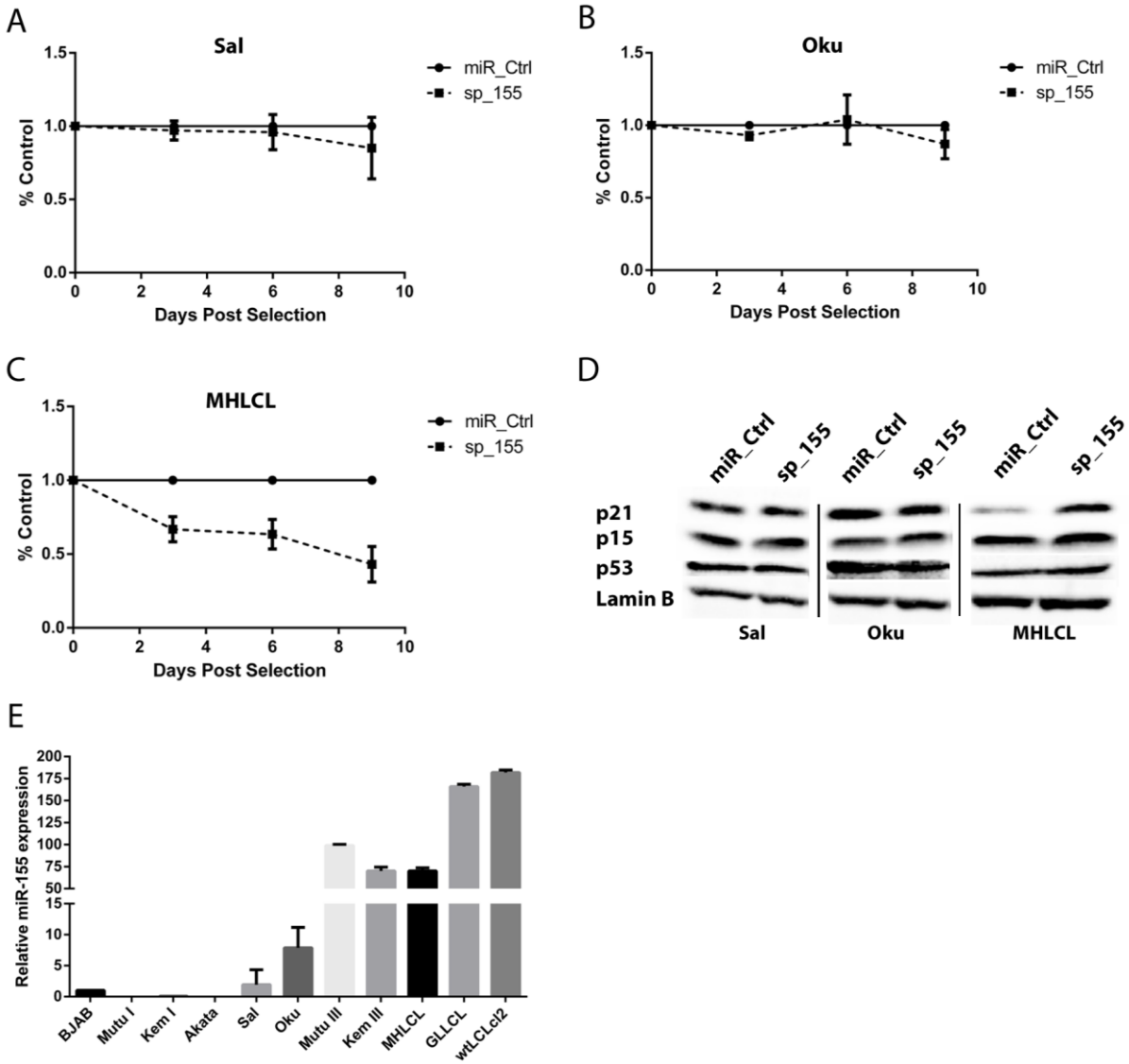


Figure 4.5: miR-155 is required for proliferation of LCLs, but is dispensable for proliferation of Wp-R.

Sal (**A**), Oku (**B**), and MHLCL (**C**) were transduced with either an empty vector control (miR_Ctrl) or a miR-155-specific sponge (sp_155) to functionally inactivate this miRNA. Growth data for cells expressing sp_155 is represented as a percentage of control cell growth. (**D**) Protein expression of p21, p15, and p53 was evaluated by immunoblot analysis. Samples are from day 9 post transduction. Lamin B served as a loading control. Protein analysis for each cell line was performed independently, and represented exposure times for specific proteins of interest are not identical between cell lines. (**E**) miR-155 expression in a panel of B cell lines. BJAB is an EBV-negative cell line. Mutu I, Kem I, and Akata display Latency I. Sal and Oku display Wp-R latency. Mutu III, Kem III, MHLCL, GLLCL, and wtLCLcl2 display Latency III.

Figure 4.5: miR-155 is required for proliferation of LCLs, but is dispensable for proliferation of Wp-R.



4.3 Discussion

EBV infection of B cells induces expression of miR-155, a microRNA with oncogenic potential in a variety of different cell types [234,256,271,457–460]. Our initial interest in the contribution of miR-155 to Wp-R was prompted by the phenotype observed in LCLs following miR-155 knock-down [288]. Following transduction of a miR-155-specific sponge, these cells arrest in G1 and do not progress through the cell cycle [288]. Additionally, miR-155 knock-down results in increased apoptotic cell death. This phenotype was similar to that observed following knock-down of EBNA3A in Wp-R and LCLs [118,150]. Moreover, we observed that EBNA3A represses p21 in both Wp-R and LCLs, thereby promoting hyperphosphorylation of Rb and progression through the cell cycle [150]. miR-155 has also been implicated in p21 regulation via targeting of Smad proteins and subsequent disruption of TGFβ signaling pathways [364]. We therefore investigated whether EBNA3A regulates miR-155 expression, and found that EBNA3A was indeed sufficient to activate miR-155. Our results also suggested a potential mechanism by which EBNA3A represses p21 in a p53-independent manner in LCLs via regulation of miR-155.

The level of miR-155 in EBV-infected B cells correlates with the specific latency state [283–285]. miR-155 expression in Latency III is more robust than that detected in Latency I, suggesting that EBV proteins expressed during Latency III induce miR-155. LMP1 and EBNA2 were previously shown to promote miR-155 expression [282,286,287], however neither protein alone has proven sufficient to fully activate miR-155 to the levels observed in LCLs. A likely scenario is that multiple EBV latent proteins cooperatively regulate miR-155 expression. A similar situation

occurs for the B cell activation antigen CD23. Both CD23 surface and mRNA expression in BJAB are cooperatively increased by coexpression of LMP1 and EBNA2, compared to expression of either protein alone [464]. Coordinated efforts of latency proteins, including EBNA3A, are likely to promote maximal expression of miR-155 in order to stimulate proliferation of B cells. However, our results indicate that at least one protein expressed during Latency III, EBNA3C, did not contribute to regulation of miR-155. This finding was surprising because it implied that regulation of miR-155 is a unique function of EBNA3A that is not shared by EBNA3C. Until recently, most of the known targets of the EBNA3 proteins such as Bim, p16 and p14 were co-regulated by EBNA3A and EBNA3C. Recently, we demonstrated a unique function of EBNA3A, to promote hyperphosphorylation of Rb by repression of cell cycle inhibitor, p21 [150]. Another recent study suggested EBNA3C could repress p21 protein expression [264], however results from our laboratory failed to corroborate a role for EBNA-3C in p21 regulation (***Chapter 3***). Since the number of genes uniquely regulated by either EBNA3A or EBNA3C are few, our data are significant and suggest that, in keeping with the requirement of both proteins for immortalization, the EBNA3 proteins have unique functions.

miR-155 is essential for proper B cell activation, and contributes to the activated state of EBV-infected B cells [267,465]. Interestingly, two other members of the herpesvirus family, KSHV and MDV, encode functional orthologs of cellular miR-155 [290,291]. Although EBV does not encode a viral microRNA equivalent of miR-155, its ability to induce cellular expression of miR-155 suggests an important and conserved role in lymphotropic herpesvirus biology. In this study we provided further evidence

to support previous findings that miR-155 is required for the proliferation of LCLs, and further investigate miR-155 expression and function in Wp-R cells, which display a distinct pattern of latency. Wp-R cells expressed an intermediate level of miR-155 compared to Latency III and Latency I cells (**Fig 4.5 E**). Because Wp-R latency does not express the full complement of EBV latent proteins, these cells likely lack the ability to fully activate miR-155 expression. Unlike in LCLs, miR-155 does not contribute significantly to the growth of Wp-R, perhaps because the amount of miR-155 in these cells is insufficient to confer a proliferative advantage. Alternatively, Wp-R cells may have accumulated additional mutations that contribute to proliferation, rendering miR-155 dispensable. Wp-R expression of EBNA3A and -3C also promotes proliferation and survival via mechanisms independent of miR-155 induction. Unlike in Wp-R, EBNA3A-mediated activation of miR-155 may only promote growth in LCLs

4.4 Materials and Methods

Cell Culture

Sal and Oku are human EBV-positive BL cell lines that maintain Wp-R latency. MHLCL is an EBV-immortalized lymphoblastoid B cell line. The BJAB-tetoff-3A cell line is a BJAB (EBV-negative B cell lymphoma) cell line created to express a tet-off EBNA3A responsive gene in which addition of Dox results in loss of EBNA3A expression. BJAB-E2-teton-3A is a BJAB cell line that stably expresses EBNA-2 and conditionally expresses EBNA3A in the presence of Dox. All B cells were grown in RPMI 1640 (Hyclone, Thermo Scientific, MA) supplemented with 10% fetal bovine serum (FBS). For ER/EBNA-3C LCL, 15% FBS was used. Medium for BJAB-tetoff-3A cells included 800 μg/mL G418 (Invitrogen, NY) and 0.5 μg/mL puromycin (Sigma, MO) and for BJAB-E2-teton-3A cells included 800 μg/mL G418 and 300 μg/mL hygromycin (Millipore, MA). Culture medium for ER/EBNA-3C LCL included 400 nM 4-Hydroxytamoxifen (4HT) (Sigma, MO). For transfection, cells were grown in roller bottles and used while in log phase of growth (0.8-1.2x10^6 cells/mL). MCF7 cells were maintained in EMEM (Lonza, MD) supplemented with 10% FBS and 0.01mg/mL bovine insulin (Sigma #I1882). 293T cells was grown in DMEM (Lonza, MD) containing 10% FBS.

Plasmids

The EBNA3A shRNAs -1490 and -601 target different sites within EBNA3A mRNA and have been previously described [150]. A BLAST search was performed to

verify the shRNAs would not target other viral or cellular sequences. A control, non-targeting shRNA, (Ctrl-shRNA) (GACTTCTGAATGAGACAACATCGAAATGTTGTCTCATTCAGAAGTC) was also generated as previously reported [150]. These shRNAs are expressed from the *oriP*-GFP vector, which contains a neomycin-resistance gene, and the EBV origin of latent DNA replication, *oriP*, allowing for stable, episomal maintenance of the plasmid by EBNA-1 in EBV-positive cells. The pMSCV-puro-GFP-miR-CTRL and pMSCV-puro-GFP-miR-155-SPONGE vectors were kind gifts from Dr. Erik Flemington of Tulane University School of Medicine.

Transfection

Sal, Oku, and MHLCL cells were transfected by Amaxa nucleofection (Lonza) in solution V using program G16. Prior to transfection, cells were seeded at $3.5x10^5$ cells/mL and maintained in roller bottles for 48 h until they had reached log phase of growth ($8.0x10^5$-$1.2x10^6$). $5x10^6$ cells were transfected with 5 μg of plasmid DNA and plated in a 12-well plate with 0.5 mL conditioned media and 0.5 mL fresh RPMI growth medium per well. 24 h post transfection, cells were fed with 1.5 mL fresh growth media as needed. At 48 h post transfection, GFP expression was observed by fluorescence microscopy, and cells were seeded at $3.5x10^5$ cells/mL in 10mL RPMI growth medium containing 800 μg/mL G418, and thereafter re-seeded every other day as indicated until harvested. Transfection of MCF7 and 293T was carried out using Fugene 6 (Promega, WI) according to the manufacturer's instructions.

Retroviral transduction

Retroviral particles were produced by transient co-transfection of pMSCV vectors with pMD-gag-pol and pCAG-LRD into 293T cells using Fugene 6 reagent (Promega, WI). Supernatant was collected after 72 h, filtered through 0.45 μm filters and used immediately for transduction. For Sal, Oku, and MHLCL, transduction was performed by suspending $1x10^6$ cells in 1 mL of viral supernatant and 8 μg/mL polybrene (Millipore, MA). Cells were plated into a 12-well plate and centrifuged for 90 minutes at 900 rpm. 24 h post transduction, cells were washed and plated with fresh RPMI growth medium. 72 h post transduction, GFP expression was monitored, and cells were split to $3.5x10^5$ in fresh RPMI growth medium with 0.2 μg/mL puromycin. Transduction efficiency for Sal and Oku was approximately 30-50%, and for MHLCL was approximately 40-60%.

RNA isolation and qRT-PCR

Total RNA was prepared using Qiagen RNeasy kit (74104). Isolated RNA was reverse transcribed to generate cDNA using iScript cDNA synthesis kit (Bio-Rad, 170-8891). BIC primary transcript and RPLP0 were amplified with iQ SYBR Green supermix (Bio-Rad, 170-8880) using the following primers: As described previously [286], BIC forward (5'-CTCTAATGGTGGCACAAA-3') and BIC reverse (5'-TGATAAAAACAAACATGGGCTTGAC-3'); RPLP0 forward (5'-AGATGCAGCAGATCCGCAT-3') and RPLP0 reverse (5'-GTGGTGATACCTAAAGCCTG-3'). miRNA was isolated using mirVana miRNA isolation kit (Ambion AM1560) and cDNA synthesized using Taqman microRNA

reverse transcription kit (Applied Biosystems, 4366596). Mature miR-155 expression was measured using the Taqman Universal master mix (Applied Biosystems, 4440043) and Taqman microRNA assays primer sets (Applied Biosystems, 4427975) for miR-155 (Assay ID #002623) and U6 (Assay ID #001973). Expression of BIC and miR-155 was determined using the comparative C_T method ($2^{-\Delta\Delta C_T}$).

Immunoblotting

Cells were harvested by centrifugation, washed with phosphate buffered saline (PBS), followed by lysis in a derivative of Laemmli sample buffer [452]**,** and sonicated. Proteins were separated by SDS-PAGE, and then transferred onto PVDF membranes. The following primary antibodies were used for immunoblotting: α-EBNA3A and -3C sheep serum (Exalpha); p53 (DO-1) and Lamin B (M20) (Santa Cruz, CA); $p21^{WAF1/CIP1}$ (12D1), and $p15^{INK4B}$ (Cell Signaling, MA); LMP1 (S12) [466]; EBNA-2 (PE2) [467].

Chapter 5: EBNA3A represses p53-mediated expression of the cell cycle inhibitor $p21^{WAF1/CIP1}$

5.1 Introduction

EBV is a human herpesvirus linked to the development of tumors of both lymphoid and epithelial origin [453]. Lifelong infection of predominantly B lymphocytes is achieved by establishment of a series of latency programs. Upon initial infection of naive B cells, EBV expresses 9 latency-associated proteins (Latency III) that promote cellular proliferation and survival. Subsequently, the highly restricted latency programs, Latency I and Latency 0, serve as the long-term reservoir of the virus [23].

EBV latent infection plays an etiologic role in approximately 98% of endemic BL cases [454,455]. Classical EBV-associated BL displays Latency I, in which expression of EBNA1 is driven by the Q promoter. A unique subset of BL tumors, Wp-R, exhibit a distinct pattern of latency driven solely from the W promoter [23]. Wp-R cells contain an EBV genome in which the region coding for EBNA2 and a segment of EBNA-LP are deleted. Also, in contrast to classical BL, Wp-R cells express BHRF1, a viral homolog of BCL2 predominantly produced during lytic replication. The Wp-R deletion brings the BHRF1 gene closer to Wp enabling latency-driven expression of BHRF1 [53,54].

In both Latency I and Wp-R BL tumors, the c-Myc oncogene is translocated to a highly transcriptionally active Ig locus [454]. C-Myc efficiently drives proliferation; however c-Myc also can be potent inducer of apoptosis by inducing ARF [468], which binds to and inhibits HDM2 leading to stabilization of p53 [410,411,469,470]. Transgenic

mice expressing Ig heavy-chain enhancer (Eμ)-driven c-Myc develop B cell lymphomas and survive up to 6 months [163]. Soon after birth, these mice exhibit polyclonal proliferation of B cells in bone marrow and peripheral blood, however Myc-induced apoptosis likely contributes to an initial delay of lymphomagenesis and extends survival [471]. F1 offspring of Eμ-*Myc* mice crossed with transgenic mice that overexpress anti-apoptotic Bcl-2 (Eμ-*Bcl-2*) significantly accelerates lymphoma development, suggesting that suppression of Myc-driven apoptosis is important to promote cellular proliferation [472]. Indeed, in Latency I BL, the ARF-HDM2-p53 pathway is frequently mutated [160,163]. In contrast, Wp-R tumors contain wild-type p53, but even so, are resistant to caspase-mediated apoptosis and more tumorigenic in xenograft assays than Latency I BL [184,190,473].

It is not uncommon for pathogens to hijack cellular pathways to promote their replication and survival. Oncogenic DNA viruses, such as HPV, encode proteins that target and degrade p53 [432,434]. Although there is little evidence linking EBV to direct degradation of p53, we predicted that EBV gene products expressed in Wp-R, such as EBNA3A, likely inhibit p53 signaling pathways to promote cell survival in the presence of high c-Myc levels.

EBNA3A promotes G1/S transition by repressing the cell cycle inhibitor, p21, resulting in hyperphosphorylation of Rb and progression through the cell cycle (**Chapter 3**) [150]. This function of EBNA3A is conserved in LCLs, which also contain wild-type p53 but display Latency III (full repertoire of EBV latency-associated proteins expressed). Following DNA damage, wild-type p53, but not mutant, upregulates p21, a vital component of the p53-mediated growth arrest pathway

[320,321,474]. p21 can also be regulated by p53-independent signaling [364,475]. Here we investigated whether EBNA3A repression of p21 is p53-dependent or –independent, and whether this mechanism is transcriptional or post-transcriptional. Since the specific mechanisms by which EBNA3A represses p21 protein expression remain unclear, we asked whether this occurs by a p53-dependent or –independent mechanism, and whether that mechanism is transcriptional or post-transcriptional. We demonstrate that EBNA3A inhibits p53-mediated transcriptional activation of p21. Inhibition of p53 activity may also allow EBNA3A to suppress induction of additional p53-induced apoptotic effectors such as Bim [135,476].

5.2 Results

EBNA3A represses transcription of p21

We previously demonstrated that EBNA3A represses p21 protein production in Wp-R and LCLs (**Chapter 3**) [150]. To investigate the mechanism of inhibition, we measured p21 mRNA levels following EBNA3A knock-down. A Wp-R cell line and an LCL, Sal and MHLCL respectively, were transfected with either an EBNA3A-specific shRNA (3AshRNA-1490) or empty vector control (Vec-Ctrl), as previously described [150]. EBNA3A loss resulted in higher levels of p21 mRNA in both cell lines tested (**Fig. 5.1 A**).

To determine whether the increased accumulation of p21 mRNA following EBNA3A knock-down resulted from enhanced transcription of the p21 gene, levels of unspliced primary transcripts of p21 were assayed. Total RNA isolated from Sal and MHLCL, transfected with either 3AshRNA-1490 or Vec-Ctrl, was amplified using two different intron-specific primers for nascent p21 transcripts (507 and 7011, **Fig. 5.1 B**). Levels of p21 primary transcript were increased in both cell lines following EBNA3A knock-down, suggesting enhanced transcription of the gene locus (**Fig. 5.1 A**). Additionally, because p21 primary transcripts and levels of mature p21 mRNA (assessed using cross-exon junction primers, **Fig. 5.1 B**) were elevated to the same extent, EBNA3A likely does not affect splicing of p21 mRNA. To further investigate the mechanism, we manipulated EBNA3A levels in cells co-transfected with a p21 promoter luciferase reporter vector. When EBNA3A levels were reduced, p21

promoter activity increased (**Fig. 5.1 C**), supporting a role for EBNA3A in regulating p21 expression at the level of transcription.

Alternative p21 transcripts are repressed by EBNA3A

The p21 gene locus encodes 8 alternative transcripts produced from 3 unique transcriptional start sites [353,354] (**Fig. 2.9**). Currently little is known about unique biological roles for each of these alternative transcripts. Elevated expression of $p21^{alt\text{-}a}$ has been measured in models of chronic hypoxic stress, while p21B, which contains a unique open reading frame encoding a non-homologous protein, has been proposed to function during apoptosis rather than cell cycle arrest [353,477]. The transcripts vary in their dependence on p53 levels, likely due to the positioning of transcriptional sites with respect to p53 response elements [354]. While additional transcription factors such as Sp1/Sp3 and Smad family proteins [478,479] also contribute to selective p21 transcript induction (e.g. Smads predominantly activate the classical $p21^{Var1}$ promoter), all of the p21 transcripts are induced following stress-induced activation of p53.

Analysis of p21 alternative transcript levels could provide insight into the mechanism of EBNA3A-mediated regulation of p21 expression. If transcripts from a specific transcriptional start site were particularly induced following EBNA3A knock-down, we could identify potential regulatory elements proximal to that promoter. To investigate whether particular p21 transcript variants were regulated differentially by EBNA3A, we measured transcript levels by qPCR in cells transfected with 3AshRNA-1490 or Vec-Ctrl. When EBNA3A was reduced, all of the p21 transcripts increased

to about the same extent, indicating that EBNA3A represses expression of all known variants encoded by the p21 gene locus (**Fig. 5.2**).

Figure 5.1: EBNA3A expression represses transcription of the p21 gene.

(**A**) Sal and MHLCL were transfected with empty vector (Vec-Ctrl) or EBNA3A-targeting shRNA (3AshRNA-1490). Transfected cells were harvested at day 4 post transfection and subjected to qPCR analysis to measure relative levels of mature p21 mRNA and unspliced p21 primary transcripts. Primers 507 (specific for intron 1) and 7011 (specific for intron 2) were designed as previously described [480], and named for their position relative to the classic p21 transcriptional start site. Values were normalized to the ribosomal protein, RPLP0. (**B**) Schematic representation of the p21 gene locus and the primers used to amplify specific intronic sequences. Mature p21 transcripts were amplified using primers that cross exons 2 and 3, as indicated by horizontal arrows. Exons are depicted as numbered gray boxes. (**C**) Sal and MHLCL were co-transfected with either Vec-Ctrl or 3AshRNA-1490, along with the p21 promoter reporter vector, WWP-Luc (p21/WAF1 promoter). Luciferase activity was measured on a BioTek Synergy H1 multi-mode plate reader and relative luminescence was normalized to Renilla activity.

Figure 5.1: EBNA3A expression represses transcription of the p21 gene.

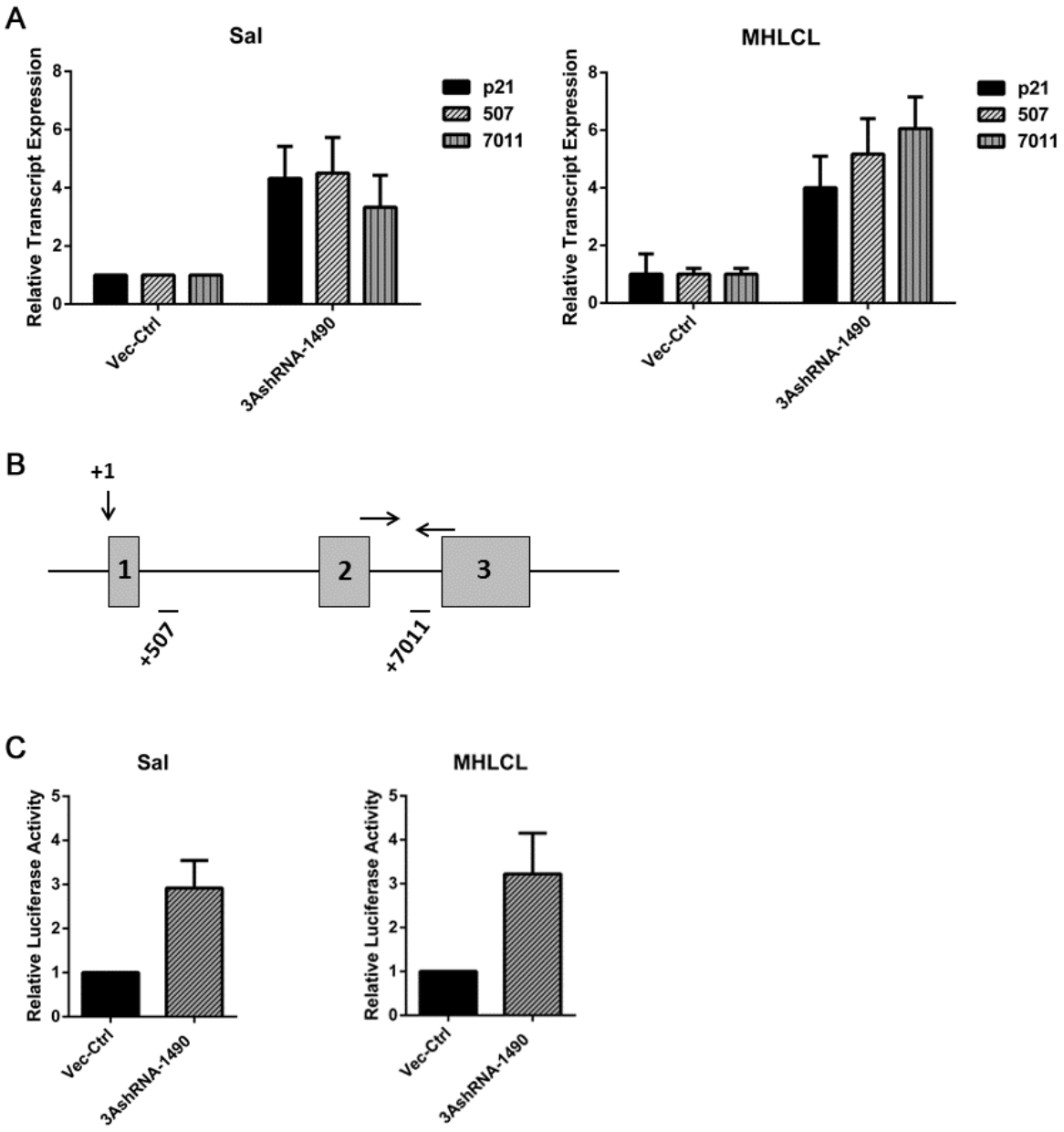
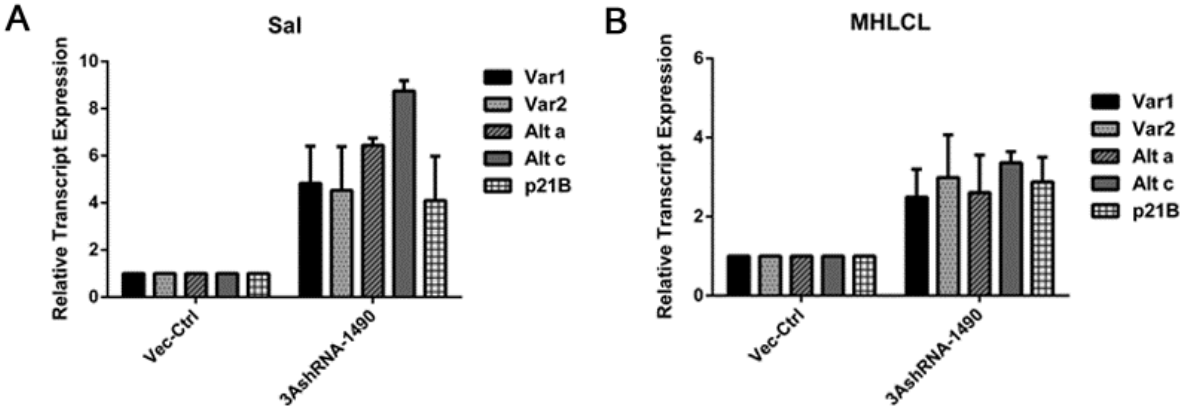


Figure 5.2: EBNA3A represses expression of p21 alternative transcripts.

Sal (**A**) and MHLCL (**B**) were transfected with either Vec-Ctrl or 3AshRNA-1490 and harvested at day 4 post transfection. Alternative transcript levels were measured by qPCR and normalized to RPLP0.

Figure 5.2: EBNA3A represses expression of p21 alternative transcripts.



EBNA3A-mediated p21 repression occurs via a p53-dependent mechanism

p21 is the predominant downstream effector of growth arrest by p53 [320,478,481]. Because each p21 transcript variant measured increased following EBNA3A knock-down (**Fig. 5.2**), and p53 can regulate expression of all p21 transcript variants [354], we hypothesized that EBNA3A represses p21 via a p53-dependent mechanism. In support of this hypothesis, we observed increased p53 protein expression when EBNA3A levels are reduced, suggesting EBNA3A might impact p53 activity (**Fig. 5.3**). To investigate whether the increased levels of p21 observed following EBNA3A knock-down were due to a p53-dependent or -independent mechanism, we inhibited p53 activity with the chemical inhibitor pifithrin-α (PFTα). In cells transfected with EBNA3A-targeting shRNA, PFTα treatment rescued cell growth and prevented p21 accumulation compared to DMSO-treated controls (**Fig. 5.3 A,B**). PFTα treatment also increased hyperphosphorylation of Rb, as expected in cells with reduced levels of p21, an inhibitor of cyclin/cdk-mediated Rb phosphorylation (**Fig. 5.3 B**) [380,381] Accordingly, p21 induction and subsequent cell cycle arrest following EBNA3A knock-down is due, at least in part, to a p53-dependent mechanism.

To directly investigate whether EBNA3A impacts p53 activity, we co-expressed 3AshRNA-1490 or Vec-Ctrl with a p53-responsive reporter construct (p50-2). This construct contains 2 copies of the p53-response element isolated from the muscle-specific creatine kinase (MCK) promoter and inserted upstream of a minimal promoter luciferase reporter gene [482]. Following EBNA3A knock-down in Wp-R, luciferase activity was increased, indicative of enhanced p53 activity (**Fig. 5.3 C**). We

conclude that EBNA3A repressed the transcriptional activity of p53, thus inhibiting induction of the downstream effector, p21.

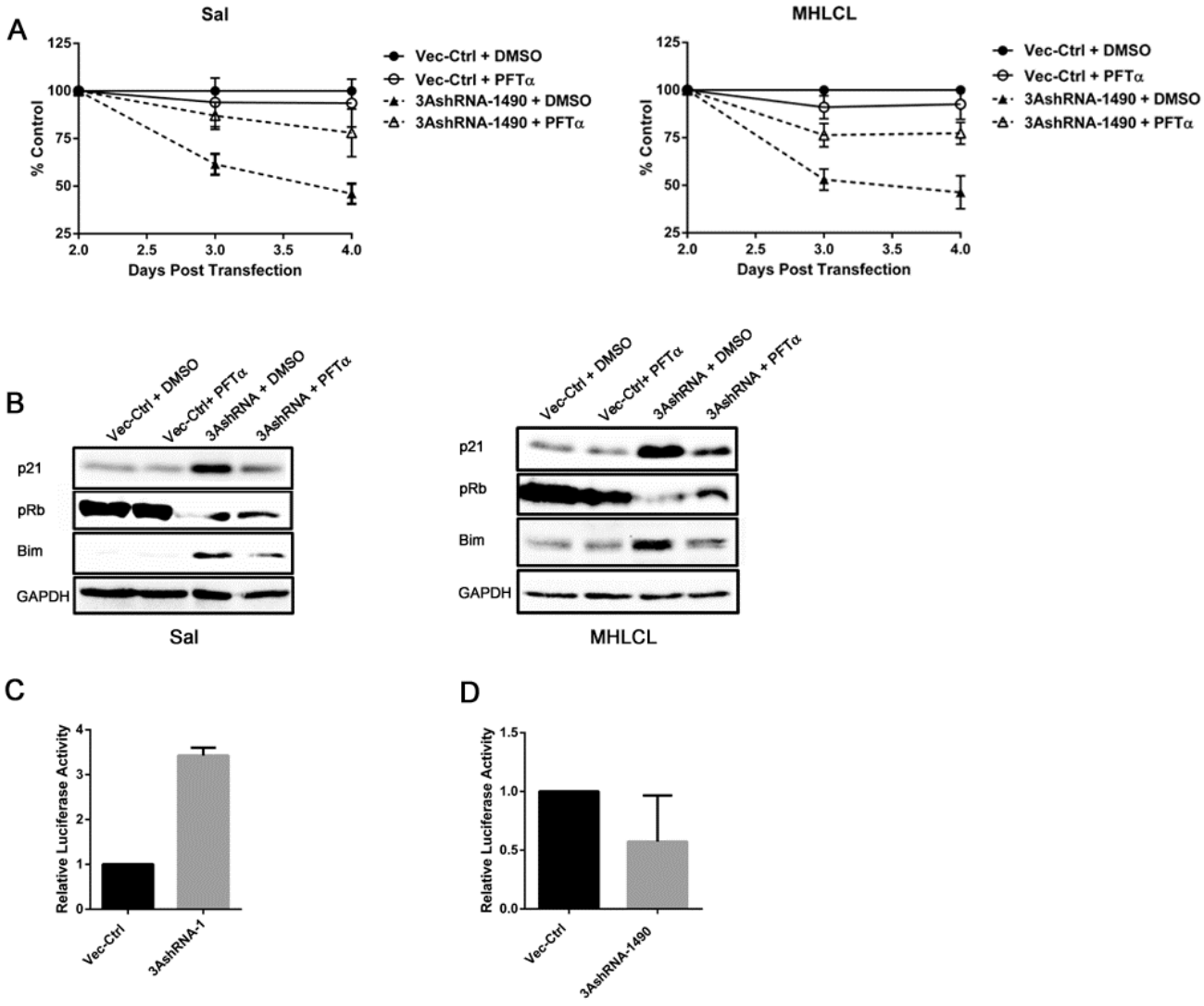
These data suggest that EBNA3A-mediated repression of p53 activity has the potential to inhibit multiple targets of p53 signaling. EBNA3A cooperates with EBNA3C to repress the pro-apoptotic protein Bim [135]. Since Bim can be a downstream target of p53, although not a direct one [483], we investigated whether EBNA3A regulated expression of Bim was p53-dependent, similarly to p21. In EBNA3A shRNA-transfected cells, PFTα treatment prevented accumulation of Bim, indicating that EBNA3A-mediated regulation of Bim levels is also by a p53-dependent mechanism (**Fig. 5.3 B**).

We next investigated whether EBNA3A repression of p53 occurs at the transcriptional level. The p53 promoter has a putative binding site for RBP-Jκ, a component of the Notch receptor signaling pathway, and it was shown that RBP-Jκ binds to and represses p53 gene expression [122]. EBNA3A can regulate target genes by interacting with the cellular proteins, RBP-Jκ and CtBP, and RBP-Jκ specifically functions as a transcriptional repressor via its interaction with EBNA-3A [120,484,485]. Thus, we predicted that EBNA3A may directly repress p53 transcription by interacting with RBP-Jκ in the p53 promoter. Using a p53 promoter luciferase reporter construct containing the RBP-Jκ binding site, we assayed p53 promoter activity in the presence or absence of EBNA3A. In Wp-R, EBNA3A knock-down did not significantly alter activity of the p53 promoter, leading us to conclude EBNA3A does not repress p53 transcription (**Fig. 5.3 D**). Instead, EBNA3A may regulate p53 at a subsequent stage of production.

Figure 5.3: EBNA3A represses p53-dependent activation of p21 expression.

(**A**) Sal and MHLCL were transfected with indicated shRNA or control expression vector and treated with DMSO or 30μM PFTα beginning at day 0. Cells were seeded at $3.5x10^5$ on day 2 post transfection and counted each day until harvest at day 4. Values are represented as a percentage of respective control growth. (**B**) Sal and MHLCL cells collected at day 4 were subjected to immunoblot analysis of proteins indicated. GAPDH served as a loading control. (**C**) p53 activity was measured in Sal by co-transfecting the p53-responsive reporter vector, p50-2, and either Vec-Ctrl or 3AshRNA-1490. Luciferase activity was measured on a BioTek Synergy H1 multi-mode plate reader and relative luminescence was normalized to Renilla. (**D**) p53 promoter activity was assessed in Sal cells co-transfected with the 1.7kb p53 promoter-luciferase vector [122], and either Vec-Ctrl or 3AshRNA-1490. Activity of the p53 promoter in cells expressing 3AshRNA-1490 is indicated relative to Vec-Ctrl cells. All luciferase activity was normalized to Renilla.

Figure 5.3: EBNA3A represses p53-dependent activation of p21 expression.



Levels of HDM2, a negative regulator of p53, are diminished following EBNA3A knock-down

HDM2 is a ubiquitin ligase that promotes degradation of p53 and suppresses its transcriptional activity [406,486]. As a result, HDM2 is critical for regulation of basal p53 levels during normal cellular conditions, and is often mutated or aberrantly expressed in human cancers [407]. Thus, we asked whether EBNA3A expression regulates protein levels of HDM2. Levels of HDM2 were reduced in Sal and MHLCL cells transfected with EBNA3A shRNA (**Fig. 5.4**). Loss of this negative regulator could contribute to increased p53 stability, resulting in increased p53 expression observed following EBNA3A knock-down (**Fig. 5.4**). These data indicate EBNA3A promotes expression of HDM2, providing a potential pathway by which EBNA3A may enhance p53 degradation.

EBNA3A contributes to phosphorylation of p21 in LCLs, but not in Wp-R

Several phosphorylation sites of p21 have been identified. The best characterized of which is T145, a substrate for the serine/threonine kinase Akt [343]. Phosphorylation at T145 has profound consequences for p21 function, including decreased inhibition of cyclin/cdk complex activity, loss of the ability to interact with and inhibit PCNA, and relocalization from the nucleus to cytoplasm, enabling interaction with an alternative subset of binding partners [345]. In the cytoplasm p21 inhibits the pro-apoptotic kinase ASK1, facilitates assembly of cyclin D1/cdk4 complexes, and binds procaspase 3, blocking its proteolytic activation [347,487]. Additionally, cytoplasmic localization of p21 has been shown to alter the half-life of

p21 [373]. Thus, although traditionally considered to be anti-proliferative, p21 has vital pro-proliferative and survival roles when post-translationally modified [343].

We evaluated whether changes in phosphorylation of p21 could affect the ability of EBNA3A to promote cellular proliferation. Using a phospho-specific antibody for p21 T145, we compared levels of phospho-p21 in cells transfected with 3AshRNA-1490 or Vec-Ctrl. In the Wp-R cell line, Sal, EBNA3A knock-down resulted in no changes in phospho-p21 levels. In contrast, EBNA3A knock-down reduced phosphorylated p21 levels in MHLCL, consistent with the anti-proliferative phenotype observed following loss of EBNA3A (**Fig. 5.5**). Additionally, we measured Akt protein levels in cells transfected with EBNA3A shRNA and Vec-Ctrl. In Sal, Akt levels were unaffected regardless of the presence or absence of EBNA-3A. In MHLCL, although total amounts of Akt did not change, EBNA-3A knock-down reduced the amount of phosphorylated (active) Akt (**Fig. 5.5**). These results suggest EBNA3A activates Akt in LCLs, but not Wp-R, which may subsequently phosphorylate p21 to favor its pro-proliferative activity. It is possible in LCLs EBNA-3A cooperates with additional proteins expressed during Latency III, to regulate Akt activation. LMP1, an EBV latent protein expressed during Latency III but not Wp-R, was previously found to stimulate phosphotidylinositol 3-kinase (PI3K) activity leading to phosphorylation and activation of Akt [488].

Figure 5.4: HDM2 expression is decreased following EBNA3A knock-down.

HDM2 and p53 protein levels were evaluated by immunoblot analysis of Sal (**A**) and MHLCL (**B**) transfected with Vec-Ctrl or 3AshRNA-1490 and harvested at day 4 post transfection. GAPDH and Lamin B served as loading controls.

Figure 5.4: HDM2 expression is decreased following EBNA3A knock-down.

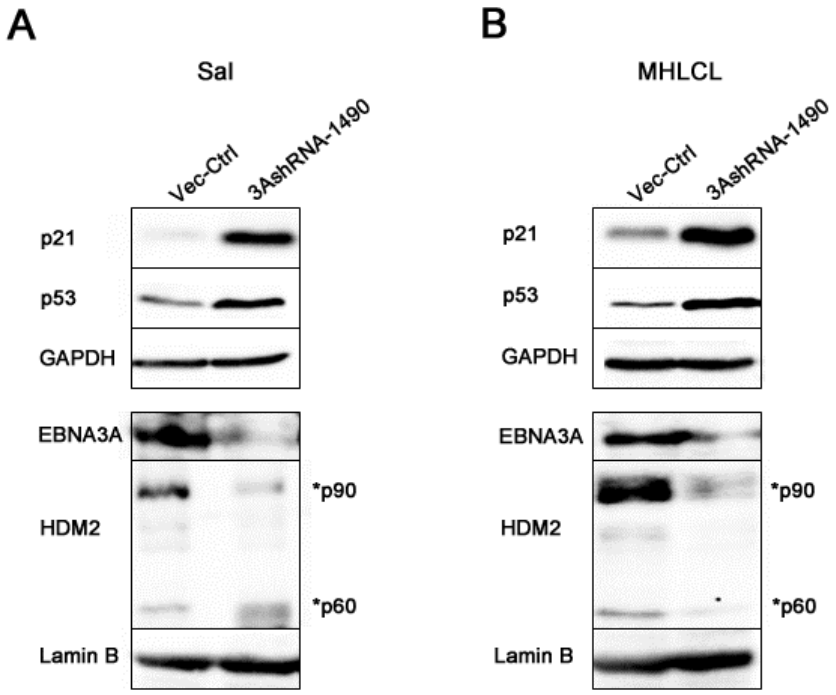
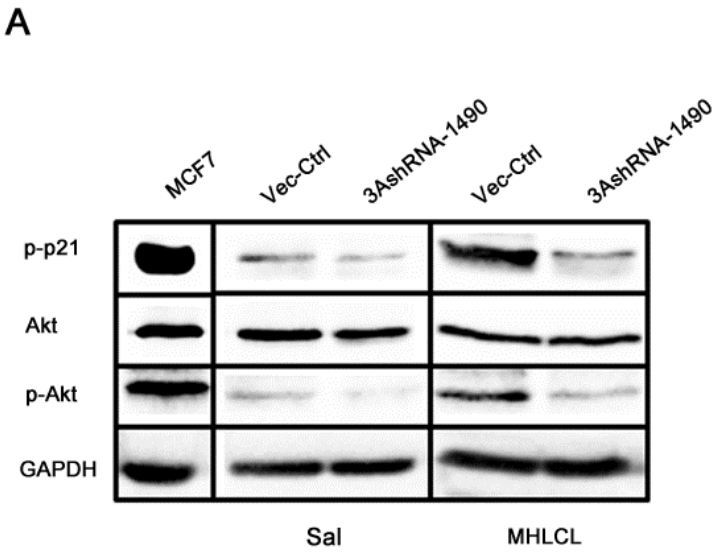


Figure 5.5: Differences in EBNA3A-mediated p21 phosphorylation of LCLs and Wp-R.

Phosphorylated p21 and Akt levels were evaluated by immunoblot analysis in Sal and MHLCL transfected with Vec-Ctrl or 3AshRNA-1490 and harvested at day 4 post transfection. Untransfected MCF7 cells served as a positive control for phospho-specific expression of p21 and Akt. GAPDH served as loading control.

Figure 5.5: Differences in EBNA3A-mediated p21 phospho-regulation of LCLs and Wp-R.



5.3 Discussion

Previously we reported that EBNA3A-mediated repression of p21 is required for the continued proliferation of Wp-R BL (**Chapter 3**) [150], but the mechanism by which this occurs had not been investigated. Here, we demonstrate that EBNA3A repressed p21 at the level of transcription, resulting in decreased levels of the p21 primary transcript and p21 promoter activity. Furthermore, p21 primary transcripts and mature p21 mRNA levels were similarly elevated, indicating that EBNA3A regulates transcription of the gene locus rather than splicing of the mRNA.

Additionally, we assessed levels of p21 transcript variants, and found all p21 variants measured were increased following EBNA3A knock-down. Little is known about the biological roles played by these alternative transcripts, however, with the exception of p21B which has a unique open reading frame, all of these variants encode an identical p21 protein [353,354]. The p21 alternative transcripts are conserved across species, indicating there is likely some biological significance [489]. It is possible that variations in 5' non-coding regions of transcript variants could contribute to differences in post-transcriptional stability or translational efficiency. Alternatively, transcripts produced from one promoter may function to compensate when another promoter is silenced. Methylation of the primary p21 promoter is implicated in certain cancers [490]. The elevated expression of p21B in our system following EBNA3A knock-down is notable. Few studies have focused on expression patterns and functions of the p21B alternative transcript, although it was proposed to be less abundantly expressed than the other transcripts and to play a role in apoptosis rather than cell cycle arrest [353]. Both Wp-R and LCLs transfected with EBNA3A shRNA exhibit increased apoptotic cell death [150]. Perhaps elevated levels of p21B play a role

in this cell death, as we measured readily detectable levels of this transcript. Selective inhibition of the alternative transcripts should reveal their individual contributions to the proliferative capacity of Wp-R and LCLs.

EBNA3A-mediated repression of p21 is p53-dependent. Not only did EBNA3A inhibit p53 proteins levels and transcriptional activity, but treatment of EBNA3A shRNA-transfected cells with a p53 chemical inhibitor (PFTα) prevented accumulation of p21 and rescued cell growth. These data suggest that EBNA3A inhibits p53 to repress transactivation of downstream p53 target genes, including p21.

Although growth of EBNA3A shRNA-transfected cells treated with PFTα was not restored to control levels, p21 protein also never decreased to control levels, so an intermediate growth rescue is not unexpected. Failure to reduce p21 expression to levels of control transfected cells following PFTα treatment could be due to technical limitations of the assay, although we cannot eliminate the possibility that some level of p21 activation is p53-independent. For example, EBNA3A itself could directly target p21 though the RBP-Jκ response element in the p21 promoter.

EBNA3A-mediated repression of p53 activity may serve as a multifunctional strategy to overcome wild-type p53 and inhibit multiple p53-targeted mediators of cell cycle arrest and apoptosis, thus promoting cellular proliferation. Indeed we observed a correlation between p53 activity and Bim accumulation following EBNA3A knock-down (**Fig. 5.3 B**). PFTα treatment prevented accumulation of Bim compared to DMSO-treated cells, indicating EBNA3A repressed Bim levels, at least in part, by inhibiting p53 signaling.

In both Wp-R and LCLs, we detected increased p53 protein expression following EBNA3A knock-down. There are multiple mechanisms by which EBNA3A could affect p53, however, EBNA3A did not repress transcription of the p53 gene (**Fig. 5.3 D**). EBNA3A could alter p53 protein stability, potentially by regulating HDM2, a critical negative regulator of p53. In the absence of EBNA3A, protein expression of HDM2 was reduced, correlating with elevated p53 protein expression. These data suggest EBNA3A might promote HDM2 stabilization to enhance HDM2 protein expression and subsequent degradation of p53. Previously, EBNA3C was shown to bind and stabilize HDM2 in LCLs, likely by acting as a deubiquitinating enzyme (DUB) [439]. EBNA3A could act similarly or cooperatively with EBNA3C as a DUB to regulate HDM2.

Post-translational modification stabilizes p53 and regulates its activity. Although there is substantial *in vitro* evidence of several phosphorylation sites in p53, the consequences of phosphorylation of the these residues on p53 function are still poorly understood [491]. Evidence suggests phosphorylation of p53 may extend the half-life of p53, alter its cellular localization, prevent its interaction with HDM2, as well as enhance p53 DNA binding activity [492–495]. Even though p53 phosphorylation is not absolutely essential for DNA damage-induced p53 activity, it very likely contributes to stabilization and optimal function of p53 [496]. Future studies could investigate whether changes in p53 phosphorylation status play a role in EBNA3A-mediated regulation of p53 activity.

Our work implicates EBNA3A in regulating p21 phosphorylation in LCLs, but not in Wp-R. Perhaps an EBV latent protein expressed in LCLs but not Wp-R is

required to cooperate with EBNA3A for phosphorylation of p21. One candidate is LMP1, which enhances Akt-mediated phosphorylation of p21 in Latency III [488]. Alternatively, Wp-R tumors may have acquired additional mutations rendering regulation of p21 phosphorylation cascades dispensable. It is possible that EBNA3A can not only repress p53-mediated transcriptional activation of p21 to promote cellular proliferation, but also promotes phosphorylation of basal p21 levels to support a proliferative phenotype.

5.4 Materials and Methods

Cell Culture

The Sal cell line is a human EBV-positive BL that maintains Wp-R latency. MHLCL is an EBV-immortalized lymphoblastoid B cell line displaying Latency III. All cells were grown in RPMI 1640 (Hyclone, Thermo Scientific, MA) supplemented with 10% fetal bovine serum (FBS). For transfection, cells were grown in roller bottles and used while in log phase of growth (0.8-1.2x10^6 cells/mL).

Plasmids

The EBNA3A-targeting shRNA, 3AshRNA-1490, was described previously [150], and is expressed from the *oriP*-GFP vector (Vec-Ctrl), which contains a neomycin-resistance gene, and the EBV origin of latent DNA replication, *oriP*, allowing for stable, episomal maintenance of the plasmid by EBNA-1 in EBV-positive cells. The p21 promoter luciferase reporter vector, WWP-Luc (p21/WAF1 promoter), was a gift from Bert Vogelstein (Addgene plasmid #16451) [320]. Dr. Gerard Zambetti of St. Jude Children's Research Hospital kindly provided the p53-responsive reporter construct (p50-2). As previously described [122], the 1.7kb p53 promoter-luciferase vector contains a 1672 bp KpnI-HindIII fragment cloned from the murine genome and inserted into a pGL3-basic luciferase vector. The 1.7kb p53 promoter vector was a kind gift from Dr. David Reisman at the University of South Carolina.

Transfection

Sal and MHLCL cells were transfected by Amaxa nucleofection (Lonza) in solution V using program G16. Prior to transfection, cells were seeded at $3.5x10^5$ cells/mL and maintained in roller bottles for 48 h until they had reached log phase of growth ($8.0x10^5$-$1.2x10^6$). $5x10^6$ cells were transfected with 5 μg of plasmid DNA and plated in a 12-well plate with 0.5 mL conditioned media and 0.5 mL fresh RPMI growth medium per well. 24 h post transfection, cells were fed with 1.5 mL fresh growth media as needed. At 48 h post transfection, GFP expression was observed by fluorescence microscopy, and cells were seeded at $3.5x10^5$ cells/mL in 10mL RPMI growth medium containing 800μg/mL G418 (Invitrogen, NY), and thereafter re-seeded every other day as indicated until collected. Cells were harvested for mRNA and protein analysis at day 4 post-transfection.

For PFTα experiments, cells were transfected with shRNA as described above. At day 0, transfected cells were treated with either DMSO or 30 μM PFTα, and retreated every 48 h until collection.

Immunoblotting

Cells were harvested by centrifugation, washed with phosphate buffered saline (PBS), followed by lysis in a derivative of Laemmli sample buffer [452]**,** and sonicated. Proteins were separated by SDS-PAGE, and then transferred onto PVDF membranes. The following primary antibodies were used for immunoblotting: EBNA3A sheep serum (Exalpha); p53 (DO-1), p-p21 (T145), HDM2, GAPDH

(FL335), and Lamin B (M20) (Santa Cruz, CA); $p21^{WAF1/CIP1}$ (12D1), Rb (4H1), pRb (S807/811), Akt, p-Akt (T308) (Cell Signaling, MA); Bim (BD Pharmingen, CA).

RNA isolation and qRT-PCR

Total RNA was prepared using Qiagen RNeasy kit (74104). Isolated RNA was reverse transcribed to generate cDNA using iScript cDNA synthesis kit (Bio-Rad, 170-8891). Mature p21 mRNA and RPLP0 were amplified using the following primers : p21 forward (5'-GGATTAGGGCTTCCTCTTGGA-3') and p21 reverse (5'-GCAGACCAGCATGACAGATTT-3'); RPLP0 forward (5'-AGATGCAGCAGATCCGCAT-3') and RPLP0 reverse (5'-GTGGTGATACCTAAAGCCTG-3'). Primary p21 transcripts (+507, +7011) were amplified using primers as previously described [480]. Alternative transcripts (Var1, Var2, Alt a, Alt c) were amplified using primers previously described in [354] and p21B primers in [497]. Expression of target transcripts was determined using the comparative C_T method ($2^{-\Delta\Delta C_T}$), relative to RPLP0.

Luciferase Assay

Cells were transfected with indicated shRNA as described above, along with 2 μg of p21 promoter or p50-2 reporter constructs (3 μg for 1.7kb p53 promoter-luciferase) and 2 μg of Renilla vector. At day 3 post-transfection, cells were collected by centrifugation, washed with phosphate-buffered saline (PBS), and then lysed in 100μl of Cell Culture Lysis Reagent or *Renilla* assay lysis buffer (Promega). 20μl of the lysate was mixed with either Luciferase or Renilla Assay Reagent (Promega) and

assayed for luminescence for 10 s on a BioTek Synergy H1 multi-mode plate reader using Gen5 software. Relative luciferase activity was normalized to Renilla.

Chapter 6: Overall discussion

6.1 Association between EBV and the development of Wp-R

The link between EBV and BL was established over 50 years ago, and since then, the contribution of EBV latency-associated proteins to lymphomagenesis has remained a rapidly expanding topic of study. Recent studies examining the loss of the EBV episome in established B cell lines provided key insight into roles of EBV in Latency I BL and Wp-R [191]. We and others have shown that EBV confers little apoptotic resistance to Latency I BL, but it provides significant apoptotic resistance and promotes proliferation in Wp-R cell lines [150,191].

Mutations in p53 as well as Rb pathways are commonly observed in Latency I BL [160], relieving the requirement for EBV latency-associated protein expression. In a mouse model of Myc-induced BL, the necessity of p53 and $p14^{ARF}$ inactivation was established [163]. Like LCLs, Wp-R tumors contain wild-type, transcriptionally active p53 [190]. Our data also suggest that Rb function is wild-type because G1/S cell cycle arrest is concomitant with a shift in hyperphosphorylated Rb to hypophosphorylated Rb (**Fig. 3.4**) [150]. Chemical inhibition of p53 in Wp-R cells also enhanced Rb phosphorylation and reduced levels of the downstream effector, p21 (**Fig. 5.3**). Thus, it is likely that one or more latency-associated gene products are critical to circumvent the activity of these tumor suppressor proteins.

DNA tumor viruses, such as adenovirus, HPV, and SV40, encode oncogenes whose products interact with cell cycle regulatory machinery. Adenovirus E1A, HVP E7, and SV40 large T antigen all possess a motif that binds and inhibits Rb [434,435,498].

Cellular p53 activity is inhibited by adenovirus E1B, HPV E6, and SV40 small T antigen [432,433,499,500]. Furthermore, E6 and E7 target p53 and Rb for degradation, respectively [501,502]. KSHV, a γ-herpesvirus similar to EBV, expresses latency-associated nuclear antigen (LANA), which directly binds to p53 and Rb and inhibits tumor suppressor activity [436,437]. In contrast, there is currently little convincing data demonstrating a role for EBV latent proteins in directly altering p53 or Rb activities. EBNA-LP was reported to co-localize with p53 and Rb, however the function of these proteins did not appear to be inhibited by this interaction with EBNA-LP [503,504]. EBNA3C is also implicated in degradation of p53 and Rb, but in Wp-R, we observed readily detectable basal levels of p53 and Rb that were not significantly altered by decreased expression of endogenous EBNA3C (***Chapter 3***).

In xenograft assays, Wp-R cells are more tumorigenic than Latency I BL cells [150]. We determined EBV proteins expressed during Wp-R latency contributed to proliferation and survival of these tumors (***Chapter 3***). Apoptotic resistance in Wp-R is largely credited to expression of the anti-apoptotic protein, BHRF1, although EBNA3C likely contributes under certain conditions. Whereas EBNA3A and EBNA3C cooperatively repress Bim, exogenous expression of the EBNA3s in Latency I BL cell lines does not significantly influence resistance to apoptosis [54]. However, in many Latency I BL cell lines epigenetic silencing of the Bim promoter is observed, possibly negating the requirement for the EBNA3s [136,137]. Nevertheless we propose that BHRF1 is a predominant mediator that counteracts apoptosis in Wp-R.

To date, individual roles of EBNA3 proteins have remained elusive, as most functions attributed to these proteins are shared between them. Both EBNA3A and

EBNA3C are required for B cell immortalization and proliferation, yet one cannot compensate for the other, suggesting each may have at least one unique function [40,117,118]. Our studies in Wp-R allowed us to identify an essential role for EBNA3A in cellular proliferation, whereas EBNA3C was dispensable (**Fig. 3.1**). Due to the dramatic and abrupt loss of proliferation following shRNA-mediated knock-down of EBNA3A, we focused subsequent studies on examining this unique function of EBNA3A in Wp-R.

6.2 The requirement of EBNA3A for Wp-R proliferation

EBNA3A knock-down in Wp-R resulted in rapid G1 cell cycle arrest followed by apoptosis (**Fig. 3.1, 3.2, 3.3**). Due to a chromosomal translocation, c-Myc is overexpressed in all forms of BL. Since Myc expression is associated with promoting cell cycle transition and proliferation, it was possible that EBNA3A regulates levels of Myc in these cells. However, our previous work did not detect any changes in Myc protein levels in the presence or absence of EBNA3A [150]. Another key regulator of the G1/S transition is Rb, and following EBNA3A knock-down we observed a shift in phosphorylation state of Rb from predominantly hyper- to hypophosphorylated (**Fig. 3.4**). This result suggests that EBNA3A regulates activity or expression of cyclin/CDK complexes, although we did not observe EBNA3A-mediated regulation of cyclin or CDK protein expression [150]. Notably, KSHV encodes K cyclin, a functional homolog of cyclin D that interacts with CDK4/6 [438,505,506]. Although EBV does not encode its own viral cyclin, it has been proposed that the virus regulates expression of cellular cyclin/CDK complexes. LMP1 and EBNA2, which are not expressed in Wp-R,

activate expression of cyclin D1 and D2, respectively [507,508]. Additionally, overexpressing EBNA3C in HEK293 and BJAB cells appears to stabilize cyclin D1 [148]. In Wp-R, however, protein expression of both cyclin D1 and cyclin D2 is undetectable, probably due to promoter methylation and the lack of LMP1, providing one possible explanation for why EBNA3C is non-essential for growth of these cells [149,150,508].

Since cyclin/CDK protein levels are unaffected by EBNA3A, we reasoned that the activity of these complexes may be inhibited instead. CKIs are potent inhibitors of cyclin/CDK-mediated phosphorylation of Rb [509]. In LCLs, EBNA3A and EBNA3C repress expression of the CKIs p14 and p16, however EBNA3A-mediated p14 repression is minimal in these cells [129]. In Wp-R, we did not detect p16, suggesting that p16 in these tumors is epigenetically silenced as is often observed in Latency I BL [171], and p14 levels were unaltered after EBNA3A knock-down [150]. These data may reveal why EBNA3C was not required for proliferation of Wp-R.

In the CIP/KIP family of CKIs, levels of p21, but not p27 or p57, increased in the absence of EBNA3A [150] (**Fig. 3.4**). The depletion of Rb observed at late time points following EBNA3A knock-down, is consistent with a role for p21 as the principle mediator of Rb hyperphosphorylation in Wp-R [150]. Colon carcinoma cell lines exhibit a similar link to p21 activity and changes in total Rb levels [510]. Following prolonged p21-induced cell cycle arrest, hypophosphorylated Rb is less stable and serves as a substrate for caspase cleavage [511].

Previous studies in LCLs implicated a crucial role for EBNA3C in repressing p14 and p16 to promote cellular proliferation, and following inactivation of EBNA3C,

shRNA-mediated knock-down of both p14 and p16 fully restores growth [129]. However knock-down of p14 and p16 in this context is not sufficient to rescue growth after loss of EBNA3A. As in Wp-R, it is likely that EBNA3A-mediated repression of p21 is also required for proliferation in LCLs [129].

To evaluate the importance of EBNA3A-mediated p21 repression, we used p21-specific LNA longRNA GapmeRs to knock-down p21 expression in cells transfected with EBNA3A shRNA. The enhanced proliferation demonstrated that p21 was indeed the mediator of cell cycle arrest following EBNA3A knock-down (**Fig. 3.6**).

6.3 Mechanism of p21 repression

In addition to increases in p21 protein expression, we observed similar (~4-fold) increases in p21 mRNA following EBNA3A knock-down (**Fig. 5.1 A**). Regulation of mRNA levels occurs at several stages in production, including transcription, splicing, transport, and/or mRNA stability. Given that levels of the unspliced p21 primary transcript increased following EBAN3A knock-down, it is likely that EBNA3A does not affect post-transcriptional events but rather regulates transcription of p21. Consistent with this notion, knock-down of EBNA3A resulted in increased p21 promoter activity (**Fig. 5.1**).

The p21 gene locus contains 3 transcriptional start sites, yielding 8 unique p21 transcripts ($p21^{Var1}$,$p21^{Var2}$, $p21^{alt\text{-}a}$, $p21^{alt\text{-}a'}$, $p21^{alt\text{-}b}$, $p21^{alt\text{-}c,}$ p21B, p21C) [353,354]. These alternative p21 transcripts are conserved across multiple species, as expected if there is a biological role for each transcript [489]. Generation of multiple transcripts could be advantageous if transcripts produced from one promoter function to

compensate when another promoter is silenced, as is often observed in cancer [490]. Alternatively, differences in 5' non-coding regions of transcript variants could regulate post-transcriptional stability or translational efficiency. Precise biological roles have yet to be assigned to the individual transcripts. However there is aberrant p21^{alt-a} expression in a model of chronic hypoxic stress, and p21B, which contains a unique open reading frame encoding a non-homologous protein, may be active during apoptosis rather than cell cycle arrest [353,477]. Because the level of each p21 transcript increased following EBNA3A knock-down, EBNA3A likely regulates expression of the entire p21 gene locus, rather than specific alternative transcripts (**Fig. 5.2**). Notably, we detected robust levels of p21B, previously described as one of the least abundant p21 transcripts [353]. The non-canonical function of p21B may contribute to apoptosis induction following EBNA3A knock-down. Given the readily detectable expression of p21B in Wp-R, EBNA3A-mediated repression of this transcript could be particularly important for survival of Wp-R. In future studies, shRNA specific for p21B could be used to examine the exact contribution of this transcript to Wp-R survival.

p21 is a major transcriptional target of p53, and both basal expression and stress-induced upregulation of p21 are mostly p53 dependent [320,474,478,481]. Different cellular stresses trigger specific and dynamic p53-binding patterns within the p21 locus, thereby resulting in diverse p21 variant expression profiles all of which can vary on their dependence of p53 levels. However, additional transcription factors, including Sp1/Sp3, STAT5, and SMAD3/4/5, also contribute to regulation of p21 expression.

We asked whether EBNA3A-mediated repression of p21 transcription is due to a p53-dependent or -independent mechanism. However, as we will elaborate, EBNA3A activation of p21 may be both p53-dependent and -independent. Previous studies have indicated multiple p21 pathways can be at play in identical cellular backgrounds. HCT116 cells with wild-type p53 or p53 homozygous deletion (-/-) will similarly activate p21 expression following γ-irradiation or chemotherapeutics such as Doxorubicin [510,512]. These data could reflect redundant pathways for p21 induction within the same cell, or conversely, in the absence of p53, compensating pathways are triggered to activate p21.

6.4 p53-independent p21 activation: miR-155 regulation

Our previous work indicated a potential p53-independent mechanism of p21 induction at work in an atypical Wp-R cell line, P3HR1. This line exhibits Wp-R latency; however, in contrast to other Wp-R cell lines, the p53 gene in P3HR1 is mutated [167]. As is observed in Sal and Oku, EBNA3A knock-down in P3HR1 resulted in loss of proliferation and induction of apoptosis. Furthermore, p21 accumulation was detected in P3HR1 following EBNA3A knock-down, implicating a potential p53-independent pathway of p21 activation in the absence of functional p53 (Unpublished data).

The oncogenic miR-155 plays an important role in LCLs, with inactivation resulting in G1 cell cycle arrest and induction of apoptosis. These results were strikingly similar to the phenotype we observe when EBNA3A is knocked-down in both Wp-R and LCLs, leading us to ask whether EBNA3A regulates miR-155 to

promote B cell proliferation [129,150]. miR-155 targets Smad5, a transcription factor involved in TGFβ signaling pathways, and in LCLs, overexpression of miR-155 confers resistance to TGFβ-mediated cell cycle inhibitory effects and p21 induction [364]. miR-155 represses expression of Smad5, thereby inhibiting Smad5-mediated activation of the p21 promoter [364]. We hypothesized that EBNA3A represses p21 independently of p53 by activating miR-155 expression.

Indeed, EBNA3A positively regulated miR-155 in both Wp-R and LCLs, although levels if miR-155 in Wp-R were much lower than in LCLs (**Fig. 4.1, 4.5**). In LCLs, but not Wp-R, EBNA3A regulation of miR-155 was required for proliferation (**Fig. 4.5**) [288]. Consistent with these data, when miR-155 was inactivated p21 levels increased in LCLs, but not Wp-R (**Fig. 4.5**). It is possible that the levels of miR-155 in Wp-R are below a threshold necessary to confer a proliferative advantage. We suspect relatively low levels of miR-155 in Wp-R, compared to LCLs, are insufficient to target and repress Smad5, which leave p21 levels unchanged. Alternatively, Wp-R cells could have acquired additional mutations that compensate for low levels of miR-155, and these cells may have become dependent on p53-mediated mechanisms of p21 regulation. Notably, P3HR1 cells have much higher levels of miR-155 compared to other Wp-R lines [286]. EBNA3A regulation of miR-155 in this Wp-R line could explain the p53-independent mechanism of p21 repression we observed (*Unpublished data*), which is not conserved in typical Wp-R lines.

We propose that EBNA3A contributes, at least in part, to p21 repression by regulating miR-155 in LCLs. In Wp-R, EBNA3A maintains the ability to promote miR-155 expression; however, unlike LCLs, miR-155 is not required for proliferation and

does not inhibit p21. The contribution of additional EBV latency-associated proteins not expressed in Wp-R may be necessary for full miR-155 activation and subsequent contribution to B cell proliferation. Indeed, co-expression of EBNA2 and EBNA3A in BJAB activated miR-155 more robustly than when either protein was expressed alone (**Fig. 4.2 D**).

miR-155: p53 cross-talk

miR-155 targeting of Smad proteins could regulate the p21 promoter independently of p53, however we cannot rule out cross-talk between miR-155 and p53 in our system. Studies of inflammatory responses in microglia implicated a role for p53 in miR-155 activation. These findings may be perplexing in that p53, a canonical tumor suppressor, would not be expected to activate an oncogenic miRNA. The role of p53 in the modulation of microglia inflammatory responses could be complex, occuring via microRNA-dependent regulation of c-Maf, a transcription factor known to promote anti-inflammatory responses [513–515]. miR-155 targets and represses c-Maf expression to support a pro-inflammatory environment. The mechanism by which p53 activates miR-155 is unknown; however, the miR-155 promoter does not contain a consensus p53 binding site. p53 has been previously shown to promote processing and post-transcriptional maturation of miRNAs, so it is possible p53 enhances miR-155 activity in this way [516]. However in our studies, we observed increased p53 concomitant with decreased miR-155 expression following EBNA3A knock-down, and therefore p53 is not likely to directly activate miR-155 in LCLs or Wp-R.

Alternatively, miR-155 is implicated in repressing p53 activity indirectly through targeting of SOCS1[517]. SOCS1 binds to p53, enhancing p53 phosphorylation and transcriptional activity [517]. Given this potential for significant cross-talk between miR-155 and p53, we cannot rule out that the miR-155-mediated repression of p21 observed in LCLs is p53-dependent. In future studies, the role of p53 in miR-155 activation could be addressed by inhibiting p53 with PFTα and measuring miR-155 levels.

miR-155 and p21 phosphorylation

Phosphorylation of multiple different residues can modulate p21 stability, subcellular localization, and binding partner affinities. Phosphorylated p21 is often associated with growth promoting functions, in contrast to its canonical role in cell cycle arrest. We determined LCLs contain appreciable levels of phosphorylated p21, which decrease following EBNA3A knock-down (**Fig. 5.5**). Additionally, Akt, the kinase primarily responsible for p21 phosphorylation, was active in LCLs. In the absence of EBNA3A, levels of p-Akt were reduced, implicating a role for EBNA3A in Akt activation and subsequent phosphorylation of p21 (**Fig. 5.5**). LCLs contain higher basal levels of p21 than Wp-R, however if p21 is predominantly phosphorylated in LCLs, that may explain why LCLs proliferate despite robust p21 expression.

Aberrant miR-155 expression contributes to lymphomagenesis by dysregulating Akt signaling. miR-155 targets and represses two negative regulators of the PI3K-Akt pathway, SHIP1 and p85α (PIK3R1), resulting in constitutive activation of this signaling pathway in multiple types of lymphoma [518,519]. It is possible

that EBNA3A-mediated activation of miR-155 promotes activation of Akt, leading to increased phosphorylation of p21 and a pro-growth program.

How does EBNA3A regulate miR-155?

Currently the mechanism by which EBNA3A promotes miR-155 expression is unknown. All of the EBNA3s have been established to regulate transcription, though not through direct DNA binding [520]. Instead, EBNA3s bind cellular transcription factors, such as RBP-Jκ [521]. EBNA3A and EBNA3C also recruit cellular factors involved in histone modification, such as HDACs, histone acetyltransferases (HATs), CtBP, and polycomb group repressor complexes [127,128,136,485,522,523]. This recruitment of HDACs contributes to EBNA3A and EBNA3C-mediated silencing of the Bim and p16 promoters [128,136]. Additionally, EBNA3A and EBNA3C regulate gene expression by modulating chromatin looping between distal regulatory elements and promoters [524,525]. It is possible that EBNA3A mediates alterations in chromatin architecture, bringing enhancer elements proximal to the miR-155 gene. EBNA3A could also recruit co-activators such as the HAT, p300 [522]. Future studies should address whether EBNA3A causes any epigenetic changes in the miR-155 locus. Additionally, miR-155 can be activated by NF-κB binding to promoter response elements, so EBNA3A, along with EBNA2 which activates the LMP1 promoter, might potentiate LMP1-mediated NF-κB induction and transactivation of miR-155 in LCLs [451].

EBV also interferes with the germinal center response by inhibiting B cell lymphoma 6 (BCL6) [139,526,527]. BCL6 not only represses NF-κB, and potentially NF-κB-mediated activation of miR-155, but BCL6 also directly binds to and represses the

miR-155 promoter [528,529]. Future studies should investigate whether EBNA3A-mediated BCL6 inhibition indirectly leads to miR-155 activation.

Our data suggest that EBNA3A promotes miR-155 processing from precursor RNA made from the BIC locus (***Chapter 4***). microRNA processing can be regulated at multiple steps, including regulation of Drosha and Dicer expression, essential Drosha/Dicer binding proteins, and nuclear export of pre-miRNAs to the cytoplasm. B cell lymphomas and colorectal cancer exhibit reduced levels of the tumor suppressor miRNAs, miR-143 and miR-145, due to processing defects [530,531]. Aberrant expression of Dicer and Drosha has been observed in several types of cancer including non-small-cell-lung cancer (NSCLC) as well as cervical cancer [532,533]. Even so, direct regulation of processing proteins may not always be the most effective strategy to regulate a miRNA because it would produce global effects on general miRNA processing. Regulation of processing can also involve targeting of miRNA-specific factors that inhibit or promote processing of precursors [534–538]. This strategy allows for regulation of specific miRNAs without affecting global miRNA expression. While future studies should examine overall levels of Drosha and Dicer, EBNA3A could enhance miR-155 processing by influencing specific regulatory factors. Alternatively, EBNA3A might impact the stability of mature miR-155 to enhance expression. Further investigations should also evaluate whether EBNA3A regulates expression of additional microRNAs besides miR-155.

Previously, EBNA3A and EBNA3C were thought to share all functions and binding partners. However we determined EBNA3C does not regulate miR-155, identifying a unique role for EBNA3A (**Fig. 4.4**). In the absence of direct DNA binding

activity, EBNA3A could regulate gene expression by interacting with currently unidentified cellular factors that do not bind EBNA3C.

6.5 p53-dependent p21 activation

We initially investigated EBNA3A-mediated regulation of miR-155 to test a potential p53-independent mechanism of p21 repression. Although miR-155 could be playing a role in p21 expression in LCLs, we determined p21 was not regulated by miR-155 in Wp-R. Instead, following EBNA3A knock-down p53 appeared to activated p21. The fact that all p21 transcripts were repressed by EBNA3A suggested that p53 activated transcription because, although the individual promoters regulating p21 transcript variants can be differentially regulated by various transcription factors, p53 response elements are located proximal to each transcriptional start site [353,354,489]. Because the p21 promoter contains an RBP-Jκ binding site, we cannot rule out the possibility that EBNA3A, at least in part, directly represses p21 transcription via interactions with RBP-Jκ [121]. However, this mechanism is less likely because EBNA3C also shares the ability to bind RBP-Jκ, but does not regulate p21 expression.

The fact that the chemical inhibitor of p53, PFTα, prevented p21 accumulation following EBNA3A knock-down suggests p53-mediated regulation (**Fig. 5.3**). This conclusion is supported by preliminary experiments using a dominant negative p53 construct (p53DN) in conjunction with EBNA3A shRNA (*Data not shown*). Using a p53-responsive reporter construct, we determined EBNA3A represses transcriptional activity of p53 (**Fig. 5.3**). EBNA3A-mediated repression of p53 activity could serve

as a multifunctional strategy to inhibit multiple effectors downstream of p53, in addition to p21. We demonstrated that EBNA3A regulates Bim (which can be downstream of p53, though not a direct target [483]) in a p53-dependent manner (**Fig. 5.3**).

EBNA3A-mediated repression of p53 has the potential to impact expression of any p53-inducible gene, however there is likely to be considerable selectively of downstream targets. We did not detect changes in levels of the other p53-regulated proteins tested, cyclin D, p14, and p16 [150,539]. Epigenetic silencing of p16 likely contributes to p53-nonresponsiveness in Wp-R, and epigenetic modulation could play a role in selective regulation of other target genes. Although the regulation of p53 transcriptional activity has been extensively studied, the basis of p53 target gene selectivity remains incompletely understood [540]. Whether a target is regulated can depend on the cell type, specific stressor, and stability of the p53 transcriptional machinery [541–543]. Previous studies identified specific factors important for selectivity of p53-regulated genes, including apoptosis-stimulating protein of p53 (ASPP), cellular apoptosis susceptibility (CAS), hematopoietic zinc finger (HZF), and cyclin-dependent kinase 8 (CDK8) [544–547].

Another intriguing question is why EBNA3C did not play a role in regulation of p53 activity or repression of p21. EBNA3A and EBNA3C are currently thought to cooperatively regulate gene expression by interacting with the same binding partners, and possibly interacting with each other [136]. In LCLs, EBNA3C is implicated in regulating p53 activity, but in Wp-R we determined EBNA3C does not affect p53 activity and is not required for proliferation [440,548,549]. Perhaps in Wp-R a cofactor

required for EBNA3C to interact with p53 is silenced or mutated in Wp-R, or somehow EBNA3C activity is specifically inhibited.

How does EBNA3A regulate p53 activity?

The p53 gene can be directly repressed by RBP-Jκ binding to the RBP-Jκ consensus sequence in the p53 promoter [122]. Because EBNA3A interacts with RBP-Jκ, that interaction could have mediated transcriptional repression of p53. However, EBNA3A did not alter activity of a p53 promoter in reporter gene assays (**Fig. 5.3**). Furthermore, we detected only minor changes in p53 mRNA levels following EBNA3A knock-down. We conclude EBNA3A does not regulate p53 expression at the level of transcription. These data were consistent with previous reports in LCLs suggesting EBV may not directly affect p53 transcription [550].

We propose that EBNA3A represses p53 at the protein level, perhaps by regulating expression of HDM2, which would contribute to p53 stabilization (**Fig. 5.4**). After HDM2 binds to p53, HDM2 acts as a ubiquitin ligase to promote proteasomal degradation of p53 [406,486]. HDM2 is overexpressed in a variety of cancers, including B cell lymphomas, myeloid leukemias, glioblastomas, and leiomyosarcomas [551]. Since many of these tumors maintain wild-type p53 expression, HDM2 likely enhances tumor growth by degradation of p53. However, tumors that both overproduce HDM2 and have p53 mutations, seemingly redundant mutations, are more aggressive than those with only one of these properties [552]. HDM2 likely has many p53-independent roles in growth regulation, such as degradation of Rb [553]. The HDM2 gene expresses two alternative transcripts from two independent promoters,

only one of which is regulated by p53 [554–556]. p53 transactivates expression of HDM2, thereby generating a negative feedback loop to regulate its expression [557,558]. EBNA3A could stimulate expression of HDM2 by activating transcription, possibly at the p53-independent promoter. EBNA3A might associate with a transcription factor required for HDM2 expression, or could recruit co-activators such as HATs.

Alternatively, EBNA3A may stabilize HDM2 protein levels. HDM2 was previously shown to be a substrate for Akt [559–561]. Phosphorylation of specific residues stabilizes HDM2 by inhibiting self-ubiquitination as well as enhances HDM2-mediated degradation of p53 [559,560]. EBNA3A-mediated activation of Akt in LCLs could contribute to increased HDM2 levels and subsequent degradation of p53. A previous study identified a role for EBNA3C in direct binding to HDM2, resulting in HDM2 stabilization [439]. It was suggested that EBNA3C is a deubiquitinating enzyme, deubiquitinating HDM2 prevents proteasomal degradation [439]. EBNA3A could have an activity similar to that of EBNA3C. Additionally, herpesvirus-associated ubiquitin-specific protease (HAUSP), an important regulator of HDM2 and p53 ubiquitination, is targeted by EBNA1 resulting in decreased p53 levels [562–566]. The ability of EBNA3A to either function similarly to HAUSP or to target HAUSP activity could be investigated in relation to EBNA3A-mediated regulation of HDM2 and p53 protein expression. Future studies should also further investigate HDM2, including mRNA levels and promoter activity. Knock-down of HDM2 in cells transfected with EBNA3A shRNA will be crucial to examine whether targeting of HDM2 is required for EBNA3A-mediated repression of p53.

6.6 Concluding remarks

Currently, Wp-R is estimated to account for 20% of all EBV-associated BL. However, it is possible that the incidence of Wp-R BL may be even higher. The original studies that characterized BL as Latency I distinguished these tumors by the absence of EBNA2 expression. Because the EBV genome in Wp-R carries a deletion in EBNA2, any Wp-R BL could have been classified as Latency I. Wp-R tumors were shown to display increased tumorigenesis in SCID mice compared to Latency I BL, suggesting that Wp-R is a highly aggressive form of EBV-associated BL [150]. If the same is true in humans, the prognosis for individuals with Wp-R BL in endemic areas, where malaria-driven immunosuppression is a constant cofactor and access to health care may not be optimal, could be poor.

EBNA3A contributes significantly to the pathogenesis of Wp-R BL. While EBNA3C and BHRF1 expression may enhance survival of Wp-R cells, EBNA3A plays a critical role in driving proliferation. Further studies should focus on exact mechanisms by which EBNA3A modulates oncogenic miRNA signaling pathways and overcomes cell cycle checkpoints to promote cell cycle progression.

Appendix

A.1 Ki67 expression following EBNA3A knock-down

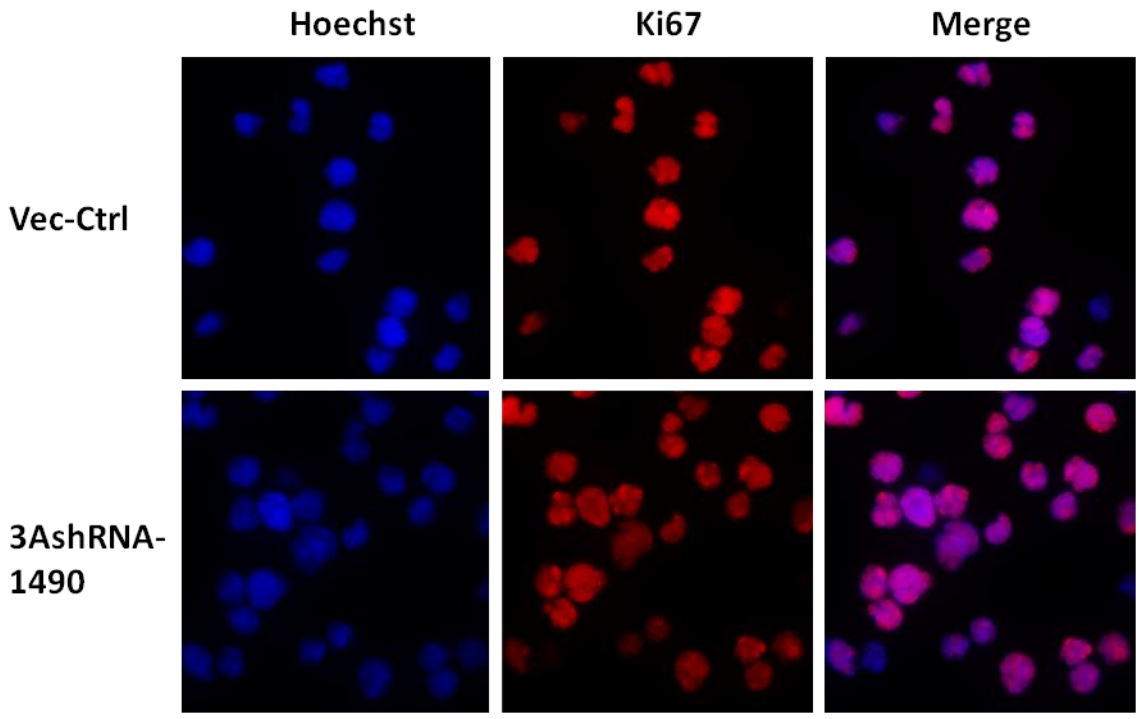
Ki67 is a nuclear protein present in proliferating cells and absent in quiescent or terminally differentiated cells [567]. Ki67 expression is specifically observed during G1, S, G2, and M phases of the cell cycle, but not in G0 at which point cells have left the cell cycle [568]. While Ki67 is often used as a proliferation marker, this marker does not only indicate active division, but the potential of a cell to divide. This means that cells perturbed in such a way to cause a stall within the cell cycle remain Ki67-positive. For example, when DNA synthesis is blocked by hydroxyurea administration, or cell cycle arrest is induced via exogenous expression of p21, cells can maintain Ki67 expression [569]. Cells that continue to express Ki67 likely still have the potential to proliferate and may ultimately do so following removal of the block.

Since we observe G1/S cell cycle arrest following EBNA3A knock-down, we asked whether these cells maintain Ki67 expression. We determined Wp-R cells remain Ki67-positive in the absence of EBNA3A (**Fig. 7.1**). These data suggest there is a block in cell cycle progression following downregulation of EBNA3A rather than entry into a true quiescent state.

Figure A.1: EBNA3A shRNA-transfected cells maintain expression of Ki67

Sal cells were harvested 6 days post transfection with the indicated DNA, fixed with 10% buffered formalin phosphate, and analyzed for Ki67 expression by immunofluorescent staining. After incubation with a secondary antibody conjugated to Alexa 594, cells were stained with Hoechst to identify cell nuclei. Both Vec-Ctrl and 3AshRNA-140-transfected cells were positive for Ki67 expression.

Figure A.1: EBNA3A shRNA-transfected maintain expression of Ki67



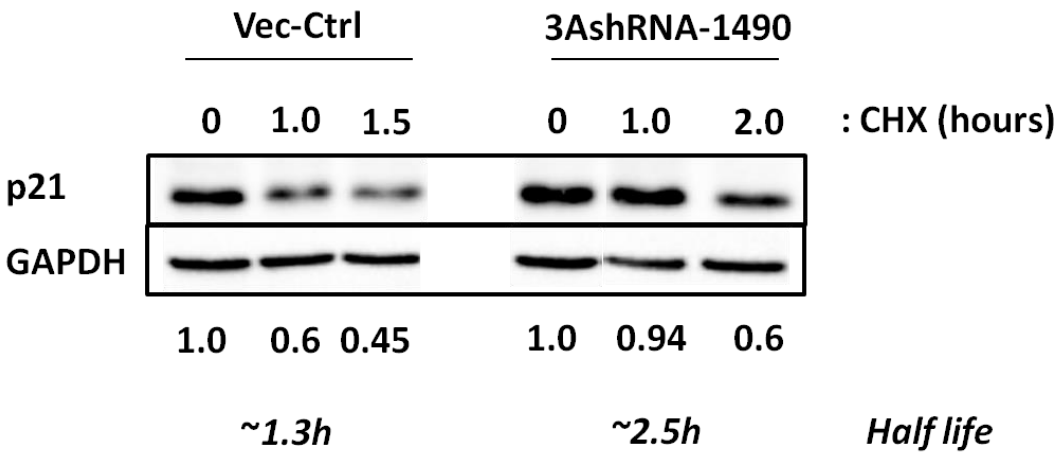
A.2 EBNA3A expression affects p21 protein stability in Wp-R

p21 is a short-lived protein degraded in a proteasome-dependent manner [365–367]. However, basal turnover does not always require ubiquitination, as the 20S proteasome can bind directly to the C terminus of p21 to mediate degradation [368–370]. Posttranslational modifications as well as subcellular localization have been shown to contribute to regulation of p21 protein stability [373,570]. Our data indicate a role for EBNA3A in repression of p21 transcription to promote cellular proliferation. We asked whether EBNA3A expression can also influence protein stability of p21 by assessing its half-life in the presence and absence of EBNA3A. Following transfection of EBNA3A shRNA, cells were treated with cycloheximide (CHX) to inhibit protein synthesis and enable p21 half-life calculations (**Fig. 7.2**). We determined p21 stability is increased after EBNA3A knock-down (~2.5h) compared to control transfected cells (~1.3h). Enhanced stability may contribute to the elevated p21 levels observed following EBNA3A knock-down. Our data implicated a role for EBNA3A in p21 phosphorylation (***Chapter 5***), which could be playing a role in alterations to p21 half-life. Additionally, HDM2 can directly promote proteasome-dependent degradation of p21 [571]. We showed decreased HDM2 protein expression in the absence of EBNA3A (***Chapter 5***), which likely contributes to enhanced p53 stability, but could also promote stabilization of p21.

Figure A.2: Alterations in p21 half-life following EBNA3A knock-down

At day 6 post transfection with indicated DNA, Sal cells were treated with 10μg/ml CHX for indicated lengths of time. Cells were harvested and lysates resolved on 13% SDS-PAGE for immunoblotting for p21 and GAPDH expression. Immunoblots were quantified using Bio-Rad ChemiDocMP imaging system and Image Lab image capture and analysis software (values indicated below figure). Increased half-life calculations were observed in cells transfected with EBNA3A shRNA compared to Vec-Ctrl.

Figure A.2: Alterations in p21 half-life following EBNA3A knock-down



A.3 CtBP-binding domain of EBNA3A is not required for repression of p21 in MCF7

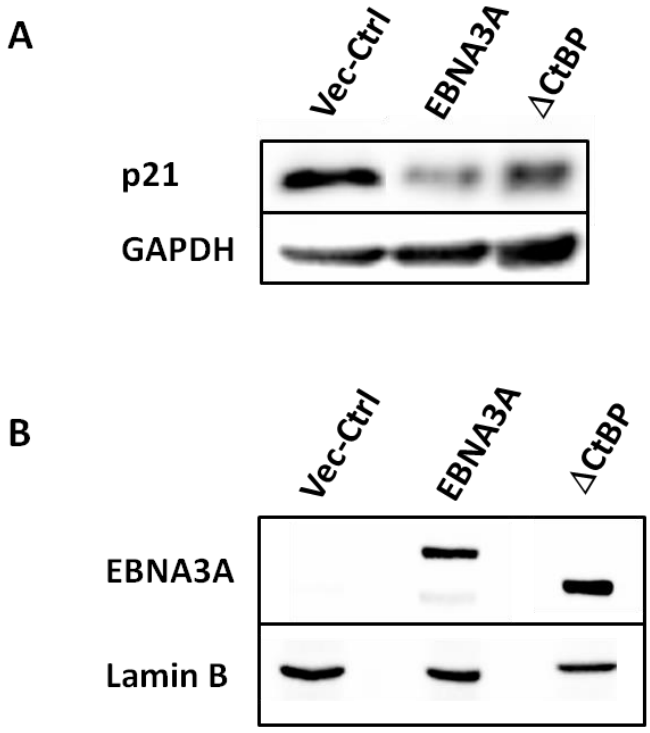
To determine whether EBNA3A represses expression of p21 in the absence of additional EBV latency-associated proteins, we transfected MCF7, a breast cancer cell line that displays wild-type p53, with an EBNA3A expression vector. We measured decreased p21 protein expression in the presence of EBNA3A compared to vector control transfected cells, indicating EBNA3A expression is sufficient to repress p21 in MCF7 cells (**Fig. 7.3 A**).

Since CtBP is one of only two cellular proteins known to interact with EBNA3A, and the CtBP-binding domain was shown to be essential for Bim repression in B cells, we sought to determine whether this domain is similarly required for regulation of p21. We generated an EBNA3A expression vector containing a small deletion in the CtBP-binding domain (ΔCtBP), and transfected this vector into MCF7 to evaluate changes in p21 protein levels (**Fig. 7.3 B**). Cells expressing EBNA3AΔCtBP displayed decreased p21 levels compared to control transfected cells, suggesting the CtBP-binding domain is not completely required for EBNA3A-mediated repression of p21. It is possible that EBNA3A interactions with RBP-Jκ play a role in p21 repression, a possibility that remains to be investigated.

Figure A.3: CtBP-binding domain is not essential for EBNA3A-mediated repression of p21 in MCF7

MCF7 cells were transfected with an empty pSG5 vector (Vec-Ctrl), pSG5-EBNA3A (EBNA3A), or pSG5-EBNA3AΔCtBP (ΔCtBP), and harvested after 72 h. Lysates were resolved on 13% (**A**) and 7% (**B**) SDS-PAGE for immunoblot analysis. $2.5x10^5$ total cells were loaded per lane. p21 (**A**) and EBNA3A (**B**) levels were evaluated, and GAPDH and Lamin B served as loading controls.

Figure A.3: CtBP-binding domain is not essential for EBNA3A-mediated repression of p21 in MCF7



A.4 EBNA3A does not contribute to Smad activation in LCLs

Transforming growth factor (TGF) β cytokines are involved in numerous cellular functions, including regulation of cellular proliferation, differentiation, apoptosis, and tumorigenesis [572]. In B and T cells, TGFβ signaling has immunosuppressive functions and inhibits proliferation via growth inhibitory signals transduced through TGFβ receptors, R-I, R-II, and R-III [573,574]. TGFβ unresponsiveness is observed in many human cancers and is believed to contribute to tumor formation [575]. The Smad family of proteins are the major intracellular TGFβ signal effectors, activated by TGFβ receptors following ligand stimulation [576]. Phosphorylated Smads form heteromeric complexes and translocate to the nucleus where they activate transcription of target genes [576,577].

TGFβ treatment in EBV-positive BL cell lines results in growth inhibition, induction of apoptosis, and EBV lytic reactivation [578]. Smad phosphorylation and induction of p21 is observed following TGFβ stimulation in these cells, however LCLs were found to be refractory to TGFβ treatment. While LCLs maintained expression of Smad family proteins and TGFβ R-I, they did not express R-II [578]. EBV latency-associated proteins expressed in Latency III may contribute to downregulation of TGFβ R-II and thus Smad-mediated activation of genes involved in growth arrest, such as p21. There is some evidence LMP1 plays a role in regulation of TGFβ-mediated responses and transcriptional activity of Smad proteins, but this mechanism is unclear [579,580].

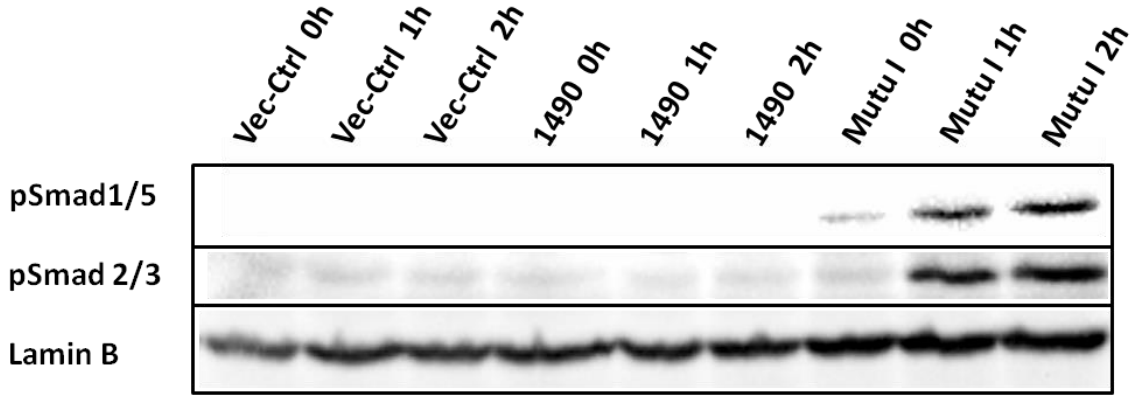
We asked whether EBNA3A can contribute to regulation of TGFβ signaling in LCLs. If so, this could additionally provide an example of p53-independent regulation

of p21 mediated by EBNA3A. MHLCLs were transfected with either Vec-Ctrl or 3AshRNA-1490 and treated with TGFβ to evaluate changes in Smad phosphorylation and activation. We observed a lack of TGFβ-mediated Smad activation regardless of EBNA3A expression, suggesting EBNA3A is not involved in regulating R-II expression or activity in LCLs (**Fig. 7.4**). We confirmed TGFβ responsiveness in the BL cell line, Mutu I, which display increased Smad phosphorylation in response to ligand stimulation (**Fig. 7.4**).

Figure A.4: EBNA3A does not contribute to Smad activation in LCLs

MHLCL were transfected with Vec-Ctrl or 3AshRNA-1490, and treated with TGFβ (5ng/mL) at day 4 post transfection. Cells were harvested for immunoblot analysis of phosphorylated Smad proteins. Smad activation was not observed in MHLCL in the presence or absence of EBNA3A. Mutu I, a Latency I BL cell line previously shown to express TGFβ R-II and respond to growth inhibitory activity of TGFβ treatment, were similarly treated with TGFβ. This served as a positive control for Smad activation in response to TGFβ. Lamin B served as a loading control.

Figure A.4: EBNA3A does not contribute to Smad activation in LCLs



References

1. Burkitt, D. A sarcoma involving the jaws in African children. *Br. J. Surg.* **46,** 218–23 (1958).

2. Burkitt, D. & O'Conor, G. T. Malignant lymphoma in African children. I. A clinical syndrome. *Cancer* **14,** 258–69

3. Epstein, M. A., Achong, B. G. & Barr, Y. M. Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma. *Lancet* **1,** 702–3 (1964).

4. Adams, A. & Lindahl, T. Epstein-Barr virus genomes with properties of circular DNA molecules in carrier cells. *Proc. Natl. Acad. Sci. U. S. A.* **72,** 1477–81 (1975).

5. Hayward, S. D. & Kieff, E. DNA of Epstein-Barr virus. II. Comparison of the molecular weights of restriction endonuclease fragments of the DNA of Epstein-Barr virus strains and identification of end fragments of the B95-8 strain. *J. Virol.* **23,** 421–9 (1977).

6. Baer, R. *et al.* DNA sequence and expression of the B95-8 Epstein—Barr virus genome. *Nature* **310,** 207–211 (1984).

7. Sadler, R. H. & Raab-Traub, N. Structural analyses of the Epstein-Barr virus BamHI A transcripts. *J. Virol.* **69,** 1132–41 (1995).

8. Pfeffer, S. *et al.* Identification of virus-encoded microRNAs. *Science* **304,** 734–6 (2004).

9. Henle, G., Henle, W. & Diehl, V. Relation of Burkitt's tumor-associated herpes-ytpe virus to infectious mononucleosis. *Proc. Natl. Acad. Sci. U. S. A.* **59,** 94–101 (1968).

10. Diehl, V., Henle, G., Henle, W. & Kohn, G. Demonstration of a herpes group virus in cultures of peripheral leukocytes from patients with infectious mononucleosis. *J. Virol.* **2,** 663–9 (1968).

11. Temple, R. M. *et al.* Efficient replication of Epstein-Barr virus in stratified epithelium in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 16544–9 (2014).

12. Frangou, P., Buettner, M. & Niedobitek, G. Epstein-Barr virus (EBV) infection in epithelial cells in vivo: rare detection of EBV replication in tongue mucosa but not in salivary glands. *J. Infect. Dis.* **191,** 238–42 (2005).

13. Greenspan, J. S. *et al.* Replication of Epstein-Barr virus within the epithelial cells of oral ‘hairy’ leukoplakia, an AIDS-associated lesion. *N. Engl. J. Med.* **313,** 1564–71 (1985).

14. Strauch, B., Andrews, L. L., Siegel, N. & Miller, G. Oropharyngeal excretion of Epstein-Barr virus by renal transplant recipients and other patients treated with immunosuppressive drugs. *Lancet (London, England)* **1,** 234–7 (1974).

15. Gerber, P., Lucas, S., Nonoyama, M., Perlin, E. & Goldstein, L. I. Oral excretion of Epstein-Barr virus by healthy subjects and patients with infectious mononucleosis. *Lancet (London, England)* **2,** 988–9 (1972).

16. Kurth, J. *et al.* EBV-infected B cells in infectious mononucleosis: viral strategies for spreading in the B cell compartment and establishing latency. *Immunity* **13,** 485–95 (2000).

17. Anagnostopoulos, I., Hummel, M., Kreschel, C. & Stein, H. Morphology, immunophenotype, and distribution of latently and/or productively Epstein-Barr virus-infected cells in acute infectious mononucleosis: implications for the interindividual infection route of Epstein-Barr virus. *Blood* **85,** 744–50 (1995).

18. Steven, N. M. *et al.* Immediate early and early lytic cycle proteins are frequent targets of the Epstein-Barr virus-induced cytotoxic T cell response. *J. Exp. Med.* **185,** 1605–17 (1997).

19. Hochberg, D. *et al.* Acute infection with Epstein-Barr virus targets and overwhelms the peripheral memory B-cell compartment with resting, latently infected cells. *J. Virol.* **78,** 5194–204 (2004).

20. Laichalk, L. L. & Thorley-Lawson, D. A. Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo. *J. Virol.* **79,** 1296–307 (2005).

21. Young, L. S. & Rickinson, A. B. Epstein-Barr virus: 40 years on. *Nat. Rev. Cancer* **4,** 757–68 (2004).

22. Henle, W., Diehl, V., Kohn, G., Zur Hausen, H. & Henle, G. Herpes-type virus and chromosome marker in normal leukocytes after growth with irradiated Burkitt cells. *Science* **157,** 1064–5 (1967).

23. Rowe, M. *et al.* Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt’s lymphoma cells. *EMBO J.* **6,** 2743–51 (1987).

24. Rowe, M., Lear, A. L., Croom-Carter, D., Davies, A. H. & Rickinson, A. B. Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. *J. Virol.* **66,** 122–31 (1992).

25. Masucci, M. G. Epstein-Barr virus oncogenesis and the ubiquitin-proteasome system. *Oncogene* **23,** 2107–15 (2004).

26. Hurley, E. A. & Thorley-Lawson, D. A. B cell activation and the establishment of Epstein-Barr virus latency. *J. Exp. Med.* **168,** 2059–75 (1988).

27. Tierney, R., Kirby, H., Nagra, J., Rickinson, A. & Bell, A. The Epstein-Barr virus promoter initiating B-cell transformation is activated by RFX proteins and the B-cell-specific activator protein BSAP/Pax5. *J. Virol.* **74,** 10458–67 (2000).

28. Speck, S. H., Pfitzner, A. & Strominger, J. L. An Epstein-Barr virus transcript from a latently infected, growth-transformed B-cell line encodes a highly repetitive polypeptide. *Proc. Natl. Acad. Sci. U. S. A.* **83,** 9298–302 (1986).

29. Allday, M. J., Crawford, D. H. & Griffin, B. E. Epstein-Barr virus latent gene expression during the initiation of B cell immortalization. *J. Gen. Virol.* **70 (Pt 7),** 1755–64 (1989).

30. Rogers, R. P., Woisetschlaeger, M. & Speck, S. H. Alternative splicing dictates translational start in Epstein-Barr virus transcripts. *EMBO J.* **9,** 2273–7 (1990).

31. Woisetschlaeger, M., Yandava, C. N., Furmanski, L. A., Strominger, J. L. & Speck, S. H. Promoter switching in Epstein-Barr virus during the initial stages of infection of B lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1725–9 (1990).

32. Nitsche, F., Bell, A. & Rickinson, A. Epstein-Barr virus leader protein enhances EBNA-2-mediated transactivation of latent membrane protein 1 expression: a role for the W1W2 repeat domain. *J. Virol.* **71,** 6619–28 (1997).

33. Harada, S. & Kieff, E. Epstein-Barr virus nuclear protein LP stimulates EBNA-2 acidic domain-mediated transcriptional activation. *J. Virol.* **71,** 6611–8 (1997).

34. Johannsen, E., Miller, C. L., Grossman, S. R. & Kieff, E. EBNA-2 and EBNA-3C extensively and mutually exclusively associate with RBPJkappa in Epstein-Barr virus-transformed B lymphocytes. *J. Virol.* **70,** 4179–83 (1996).

35. Robertson, E. S., Lin, J. & Kieff, E. The amino-terminal domains of Epstein-Barr virus nuclear proteins 3A, 3B, and 3C interact with RBPJ(kappa). *J. Virol.* **70,** 3068–74 (1996).

36. Pope, J. H., Horne, M. K. & Scott, W. Identification of the filtrable leukocyte-transforming factor of qimr-wil cells as herpes-like virus. *Int. J. Cancer* **4,** 255–260 (1969).

37. Mannick, J. B., Cohen, J. I., Birkenbach, M., Marchini, A. & Kieff, E. The Epstein-Barr virus nuclear protein encoded by the leader of the EBNA RNAs is important in B-lymphocyte transformation. *J. Virol.* **65,** 6826–37 (1991).

38. Kaye, K. M., Izumi, K. M. & Kieff, E. Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9150–4 (1993).

39. Hammerschmidt, W. & Sugden, B. Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes. *Nature* **340,** 393–7 (1989).

40. Tomkinson, B., Robertson, E. & Kieff, E. Epstein-Barr virus nuclear proteins EBNA-3A and EBNA-3C are essential for B-lymphocyte growth transformation. *J. Virol.* **67,** 2014–25 (1993).

41. Cohen, J. I., Wang, F. & Kieff, E. Epstein-Barr virus nuclear protein 2 mutations define essential domains for transformation and transactivation. *J. Virol.* **65,** 2545–54 (1991).

42. Young, L. S. & Murray, P. G. Epstein-Barr virus and oncogenesis: from latent genes to tumours. *Oncogene* **22,** 5108–21 (2003).

43. Kutok, J. L. & Wang, F. Spectrum of Epstein-Barr virus-associated diseases. *Annu. Rev. Pathol.* **1,** 375–404 (2006).

44. Fields, B. N., Knipe, D. M. & Howley, P. M. *Fields' Virology, Volume 1*. (Lippincott Williams & Wilkins, 2007).

45. Yin, Y., Manoury, B. & Fåhraeus, R. Self-inhibition of synthesis and antigen presentation by Epstein-Barr virus-encoded EBNA1. *Science* **301,** 1371–4 (2003).

46. Levitskaya, J. *et al.* Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. *Nature* **375,** 685–8 (1995).

47. Hochberg, D. *et al.* Demonstration of the Burkitt's lymphoma Epstein-Barr virus phenotype in dividing latently infected memory cells in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 239–44 (2004).

48. Kelly, G., Bell, A. & Rickinson, A. Epstein-Barr virus-associated Burkitt lymphomagenesis selects for downregulation of the nuclear antigen EBNA2. *Nat. Med.* **8,** 1098–104 (2002).

49. Woisetschlaeger, M. *et al.* Role for the Epstein-Barr virus nuclear antigen 2 in viral promoter switching during initial stages of infection. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 3942–6 (1991).

50. Abbot, S. D. *et al.* Epstein-Barr virus nuclear antigen 2 induces expression of the virus-encoded latent membrane protein. *J. Virol.* **64,** 2126–34 (1990).

51. Wang, F., Tsang, S. F., Kurilla, M. G., Cohen, J. I. & Kieff, E. Epstein-Barr virus nuclear antigen 2 transactivates latent membrane protein LMP1. *J. Virol.* **64,** 3407–16 (1990).

52. Fåhraeus, R., Jansson, A., Ricksten, A., Sjöblom, A. & Rymo, L. Epstein-Barr virus-encoded nuclear antigen 2 activates the viral latent membrane protein promoter by modulating the activity of a negative regulatory element. *Proc. Natl. Acad. Sci. U. S. A.* **87,** 7390–4 (1990).

53. Austin, P. J., Flemington, E., Yandava, C. N., Strominger, J. L. & Speck, S. H. Complex transcription of the Epstein-Barr virus BamHI fragment H rightward open reading frame 1 (BHRF1) in latently and lytically infected B lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **85,** 3678–82 (1988).

54. Kelly, G. L. *et al.* An Epstein-Barr virus anti-apoptotic protein constitutively expressed in transformed cells and implicated in burkitt lymphomagenesis: the Wp/BHRF1 link. *PLoS Pathog.* **5,** e1000341 (2009).

55. Humme, S. *et al.* The EBV nuclear antigen 1 (EBNA1) enhances B cell immortalization several thousandfold. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 10989–94 (2003).

56. Wang, J., Lindner, S. E., Leight, E. R. & Sugden, B. Essential elements of a licensed, mammalian plasmid origin of DNA synthesis. *Mol. Cell. Biol.* **26,** 1124–34 (2006).

57. Rawlins, D. R., Milman, G., Hayward, S. D. & Hayward, G. S. Sequence-specific DNA binding of the Epstein-Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. *Cell* **42,** 859–68 (1985).

58. Yates, J. L., Warren, N. & Sugden, B. Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. *Nature* **313,** 812–5

59. Yates, J., Warren, N., Reisman, D. & Sugden, B. A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. *Proc. Natl. Acad. Sci. U. S. A.* **81,** 3806–10 (1984).

60. Ohno, S., Luka, J., Lindahl, T. & Klein, G. Identification of a purified complement-fixing antigen as the Epstein-Barr-virus determined nuclear antigen (EBNA) by its binding to metaphase chromosomes. *Proc. Natl. Acad. Sci. U. S. A.* **74,** 1605–9 (1977).

61. Sample, J., Henson, E. B. & Sample, C. The Epstein-Barr virus nuclear protein 1 promoter active in type I latency is autoregulated. *J. Virol.* **66,** 4654–61 (1992).

62. Sugden, B. & Warren, N. A promoter of Epstein-Barr virus that can function during latent infection can be transactivated by EBNA-1, a viral protein required for viral DNA replication during latent infection. *J. Virol.* **63,** 2644–9 (1989).

63. Reisman, D. & Sugden, B. trans activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. *Mol. Cell. Biol.* **6,** 3838–46 (1986).

64. Drotar, M. E. *et al.* Epstein-Barr virus nuclear antigen-1 and Myc cooperate in lymphomagenesis. *Int. J. Cancer* **106,** 388–95 (2003).

65. Kang, M.-S. *et al.* Epstein-Barr virus nuclear antigen 1 does not induce lymphoma in transgenic FVB mice. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 820–5 (2005).

66. Alfieri, C., Birkenbach, M. & Kieff, E. Early events in Epstein-Barr virus infection of human B lymphocytes. *Virology* **181,** 595–608 (1991).

67. Cohen, J. I., Wang, F., Mannick, J. & Kieff, E. Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. *Proc. Natl. Acad. Sci. U. S. A.* **86,** 9558–62 (1989).

68. Grossman, S. R., Johannsen, E., Tong, X., Yalamanchili, R. & Kieff, E. The Epstein-Barr virus nuclear antigen 2 transactivator is directed to response elements by the J kappa recombination signal binding protein. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7568–72 (1994).

69. Friedmann, D. R., Wilson, J. J. & Kovall, R. A. RAM-induced allostery facilitates assembly of a notch pathway active transcription complex. *J. Biol. Chem.* **283,** 14781–91 (2008).

70. Sakai, T. *et al.* Functional replacement of the intracellular region of the Notch1 receptor by Epstein-Barr virus nuclear antigen 2. *J. Virol.* **72,** 6034–9 (1998).

71. Zhao, B. *et al.* RNAs induced by Epstein-Barr virus nuclear antigen 2 in lymphoblastoid cell lines. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 1900–5 (2006).

72. Maier, S. *et al.* Cellular target genes of Epstein-Barr virus nuclear antigen 2. *J. Virol.* **80,** 9761–71 (2006).

73. Kaiser, C. *et al.* The proto-oncogene c-myc is a direct target gene of Epstein-Barr virus nuclear antigen 2. *J. Virol.* **73,** 4481–4 (1999).

74. Wang, F. *et al.* Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23. *Proc. Natl. Acad. Sci. U. S. A.* **84,** 3452–6 (1987).

75. Cordier, M. *et al.* Stable transfection of Epstein-Barr virus (EBV) nuclear antigen 2 in lymphoma cells containing the EBV P3HR1 genome induces expression of B-cell activation molecules CD21 and CD23. *J. Virol.* **64,** 1002–13 (1990).

76. Fuentes-Pananá, E. M. & Ling, P. D. Characterization of the CBF2 binding site within the Epstein-Barr virus latency C promoter and its role in modulating EBNA2-mediated transactivation. *J. Virol.* **72,** 693–700 (1998).

77. Fuentes-Pananá, E. M., Peng, R., Brewer, G., Tan, J. & Ling, P. D. Regulation of the Epstein-Barr virus C promoter by AUF1 and the cyclic AMP/protein kinase A signaling pathway. *J. Virol.* **74,** 8166–75 (2000).

78. Thomas, J. A. *et al.* Immunohistology of Epstein-Barr virus-associated antigens in B cell disorders from immunocompromised individuals. *Transplantation* **49,** 944–53 (1990).

79. Sample, J., Hummel, M., Braun, D., Birkenbach, M. & Kieff, E. Nucleotide sequences of mRNAs encoding Epstein-Barr virus nuclear proteins: a probable transcriptional initiation site. *Proc. Natl. Acad. Sci. U. S. A.* **83,** 5096–100 (1986).

80. Allan, G. J., Inman, G. J., Parker, B. D., Rowe, D. T. & Farrell, P. J. Cell growth effects of Epstein-Barr virus leader protein. *J. Gen. Virol.* **73 (Pt 6),** 1547–51 (1992).

81. Peng, R., Moses, S. C., Tan, J., Kremmer, E. & Ling, P. D. The Epstein-Barr virus EBNA-LP protein preferentially coactivates EBNA2-mediated stimulation of latent membrane proteins expressed from the viral divergent promoter. *J. Virol.* **79,** 4492–505 (2005).

82. Kulwichit, W. *et al.* Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 11963–8 (1998).

83. Wang, D., Liebowitz, D. & Kieff, E. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell* **43,** 831–40 (1985).

84. Wang, D. *et al.* Epstein-Barr virus latent infection membrane protein alters the human B-lymphocyte phenotype: deletion of the amino terminus abolishes activity. *J. Virol.* **62,** 4173–84 (1988).

85. Mosialos, G. *et al.* The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell* **80,** 389–99 (1995).

86. Izumi, K. M. *et al.* The Epstein-Barr virus oncoprotein latent membrane protein 1 engages the tumor necrosis factor receptor-associated proteins TRADD and receptor-interacting protein (RIP) but does not induce apoptosis or require RIP for NF-kappaB activation. *Mol. Cell. Biol.* **19,** 5759–67 (1999).

87. Yang, X., He, Z., Xin, B. & Cao, L. LMP1 of Epstein-Barr virus suppresses cellular senescence associated with the inhibition of p16INK4a expression. *Oncogene* **19,** 2002–13 (2000).

88. Ohtani, N. *et al.* Epstein-Barr virus LMP1 blocks p16INK4a-RB pathway by promoting nuclear export of E2F4/5. *J. Cell Biol.* **162,** 173–83 (2003).

89. Lo, A. K. F. *et al.* Phenotypic alterations induced by the Hong Kong-prevalent Epstein-Barr virus-encoded LMP1 variant (2117-LMP1) in nasopharyngeal epithelial cells. *Int. J. Cancer* **109,** 919–25 (2004).

90. Li, L. *et al.* Latent membrane protein 1 of Epstein-Barr virus regulates p53 phosphorylation through MAP kinases. *Cancer Lett.* **255,** 219–31 (2007).

91. Fries, K. L., Miller, W. E. & Raab-Traub, N. Epstein-Barr virus latent membrane protein 1 blocks p53-mediated apoptosis through the induction of the A20 gene. *J. Virol.* **70,** 8653–9 (1996).

92. Wu, H.-C. *et al.* MDM2 expression in EBV-infected nasopharyngeal carcinoma cells. *Lab. Invest.* **84,** 1547–56 (2004).

93. Longnecker, R., Miller, C. L., Miao, X. Q., Marchini, A. & Kieff, E. The only domain which distinguishes Epstein-Barr virus latent membrane protein 2A (LMP2A) from LMP2B is dispensable for lymphocyte infection and growth transformation in vitro; LMP2A is therefore nonessential. *J. Virol.* **66,** 6461–9 (1992).

94. Longnecker, R., Miller, C. L., Miao, X. Q., Tomkinson, B. & Kieff, E. The last seven transmembrane and carboxy-terminal cytoplasmic domains of Epstein-

Barr virus latent membrane protein 2 (LMP2) are dispensable for lymphocyte infection and growth transformation in vitro. *J. Virol.* **67,** 2006–13 (1993).

95. Kim, O. J. & Yates, J. L. Mutants of Epstein-Barr virus with a selective marker disrupting the TP gene transform B cells and replicate normally in culture. *J. Virol.* **67,** 7634–40 (1993).

96. Caldwell, R. G., Wilson, J. B., Anderson, S. J. & Longnecker, R. Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. *Immunity* **9,** 405–11 (1998).

97. Caldwell, R. G., Brown, R. C. & Longnecker, R. Epstein-Barr virus LMP2A-induced B-cell survival in two unique classes of EmuLMP2A transgenic mice. *J. Virol.* **74,** 1101–13 (2000).

98. Portis, T. & Longnecker, R. Epstein-Barr virus (EBV) LMP2A mediates B-lymphocyte survival through constitutive activation of the Ras/PI3K/Akt pathway. *Oncogene* **23,** 8619–28 (2004).

99. Fruehling, S. & Longnecker, R. The immunoreceptor tyrosine-based activation motif of Epstein-Barr virus LMP2A is essential for blocking BCR-mediated signal transduction. *Virology* **235,** 241–51 (1997).

100. Rovedo, M. & Longnecker, R. Epstein-barr virus latent membrane protein 2B (LMP2B) modulates LMP2A activity. *J. Virol.* **81,** 84–94 (2007).

101. Swaminathan, S., Tomkinson, B. & Kieff, E. Recombinant Epstein-Barr virus with small RNA (EBER) genes deleted transforms lymphocytes and replicates in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 1546–50 (1991).

102. Clemens, M. J. *et al.* Regulation of the interferon-inducible eIF-2 alpha protein kinase by small RNAs. *Biochimie* **76,** 770–8 (1994).

103. Kitagawa, N. *et al.* Epstein-Barr virus-encoded poly(A)(-) RNA supports Burkitt's lymphoma growth through interleukin-10 induction. *EMBO J.* **19,** 6742–50 (2000).

104. Komano, J., Maruo, S., Kurozumi, K., Oda, T. & Takada, K. Oncogenic role of Epstein-Barr virus-encoded RNAs in Burkitt's lymphoma cell line Akata. *J. Virol.* **73,** 9827–31 (1999).

105. Chen, H., Smith, P., Ambinder, R. F. & Hayward, S. D. Expression of Epstein-Barr virus BamHI-A rightward transcripts in latently infected B cells from peripheral blood. *Blood* **93,** 3026–32 (1999).

106. Deacon, E. M. *et al.* Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells. *J. Exp. Med.* **177,** 339–49 (1993).

107. Chiang, A. K., Tao, Q., Srivastava, G. & Ho, F. C. Nasal NK- and T-cell lymphomas share the same type of Epstein-Barr virus latency as nasopharyngeal carcinoma and Hodgkin's disease. *Int. J. Cancer* **68,** 285–90 (1996).

108. Marquitz, A. R., Mathur, A., Nam, C. S. & Raab-Traub, N. The Epstein-Barr Virus BART microRNAs target the pro-apoptotic protein Bim. *Virology* **412,** 392–400 (2011).

109. Kang, D., Skalsky, R. L. & Cullen, B. R. EBV BART MicroRNAs Target Multiple Pro-apoptotic Cellular Genes to Promote Epithelial Cell Survival. *PLoS Pathog.* **11,** e1004979 (2015).

110. Hennessy, K., Fennewald, S. & Kieff, E. A third viral nuclear protein in lymphoblasts immortalized by Epstein-Barr virus. *Proc. Natl. Acad. Sci. U. S. A.* **82,** 5944–8 (1985).

111. Hennessy, K., Wang, F., Bushman, E. W. & Kieff, E. Definitive identification of a member of the Epstein-Barr virus nuclear protein 3 family. *Proc. Natl. Acad. Sci. U. S. A.* **83,** 5693–7 (1986).

112. Ricksten, A. *et al.* BamHI E region of the Epstein-Barr virus genome encodes three transformation-associated nuclear proteins. *Proc. Natl. Acad. Sci. U. S. A.* **85,** 995–9 (1988).

113. Joab, I. *et al.* Mapping of the gene coding for Epstein-Barr virus-determined nuclear antigen EBNA3 and its transient overexpression in a human cell line by using an adenovirus expression vector. *J. Virol.* **61,** 3340–4 (1987).

114. Petti, L. & Kieff, E. A sixth Epstein-Barr virus nuclear protein (EBNA3B) is expressed in latently infected growth-transformed lymphocytes. *J. Virol.* **62,** 2173–8 (1988).

115. Sample, C. & Parker, B. Biochemical characterization of Epstein-Barr virus nuclear antigen 3A and 3C proteins. *Virology* **205,** 534–9 (1994).

116. Speck, S. H., Pfitzner, A. & Strominger, J. L. in *Advances in Viral Oncology* (ed. Klein, G.) 133–150 (Raven Press, 1989).

117. Maruo, S. *et al.* Epstein-Barr virus nuclear protein EBNA3C is required for cell cycle progression and growth maintenance of lymphoblastoid cells. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 19500–5 (2006).

118. Maruo, S., Johannsen, E., Illanes, D., Cooper, A. & Kieff, E. Epstein-Barr Virus nuclear protein EBNA3A is critical for maintaining lymphoblastoid cell line growth. *J. Virol.* **77,** 10437–47 (2003).

119. Dalbiès-Tran, R., Stigger-Rosser, E., Dotson, T. & Sample, C. E. Amino acids of Epstein-Barr virus nuclear antigen 3A essential for repression of Jkappa-mediated transcription and their evolutionary conservation. *J. Virol.* **75,** 90–9 (2001).

120. Zhao, B., Marshall, D. R. & Sample, C. E. A conserved domain of the Epstein-Barr virus nuclear antigens 3A and 3C binds to a discrete domain of Jkappa. *J. Virol.* **70,** 4228–36 (1996).

121. Rangarajan, A. *et al.* Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. *EMBO J.* **20,** 3427–36 (2001).

122. Boggs, K., Henderson, B. & Reisman, D. RBP-Jk binds to and represses transcription of the p53 tumor suppressor gene. *Cell Biol. Int.* **33,** 318–324 (2009).

123. Maruo, S. *et al.* Epstein-Barr virus nuclear protein 3A domains essential for growth of lymphoblasts: transcriptional regulation through RBP-Jkappa/CBF1 is critical. *J. Virol.* **79,** 10171–9 (2005).

124. Lee, S. *et al.* Epstein-Barr virus nuclear protein 3C domains necessary for lymphoblastoid cell growth: interaction with RBP-Jkappa regulates TCL1. *J. Virol.* **83,** 12368–77 (2009).

125. Schaeper, U. *et al.* Molecular cloning and characterization of a cellular phosphoprotein that interacts with a conserved C-terminal domain of adenovirus E1A involved in negative modulation of oncogenic transformation. *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10467–71 (1995).

126. Shi, Y. *et al.* Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature* **422,** 735–8 (2003).

127. Touitou, R., Hickabottom, M., Parker, G., Crook, T. & Allday, M. J. Physical and functional interactions between the corepressor CtBP and the Epstein-Barr virus nuclear antigen EBNA3C. *J. Virol.* **75,** 7749–55 (2001).

128. Skalska, L., White, R. E., Franz, M., Ruhmann, M. & Allday, M. J. Epigenetic repression of p16(INK4A) by latent Epstein-Barr virus requires the interaction of EBNA3A and EBNA3C with CtBP. *PLoS Pathog.* **6,** e1000951 (2010).

129. Maruo, S. *et al.* Epstein-Barr virus nuclear antigens 3C and 3A maintain lymphoblastoid cell growth by repressing p16INK4A and p14ARF expression. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 1919–24 (2011).

130. Strasser, A., Harris, A. W. & Cory, S. bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell* **67,** 889–99 (1991).

131. Opferman, J. T. & Korsmeyer, S. J. Apoptosis in the development and maintenance of the immune system. *Nat. Immunol.* **4,** 410–5 (2003).

132. Strasser, A. The role of BH3-only proteins in the immune system. *Nat. Rev. Immunol.* **5,** 189–200 (2005).

133. Egle, A., Harris, A. W., Bouillet, P. & Cory, S. Bim is a suppressor of Myc-induced mouse B cell leukemia. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 6164–9 (2004).

134. Komano, J., Sugiura, M. & Takada, K. Epstein-Barr virus contributes to the malignant phenotype and to apoptosis resistance in Burkitt's lymphoma cell line Akata. *J. Virol.* **72,** 9150–6 (1998).

135. Anderton, E. *et al.* Two Epstein-Barr virus (EBV) oncoproteins cooperate to repress expression of the proapoptotic tumour-suppressor Bim: clues to the pathogenesis of Burkitt's lymphoma. *Oncogene* **27,** 421–33 (2008).

136. Paschos, K., Parker, G. A., Watanatanasup, E., White, R. E. & Allday, M. J. BIM promoter directly targeted by EBNA3C in polycomb-mediated repression by EBV. *Nucleic Acids Res.* **40,** 7233–46 (2012).

137. Paschos, K. *et al.* Epstein-barr virus latency in B cells leads to epigenetic repression and CpG methylation of the tumour suppressor gene Bim. *PLoS Pathog.* **5,** e1000492 (2009).

138. Tomkinson, B. & Kieff, E. Use of second-site homologous recombination to demonstrate that Epstein-Barr virus nuclear protein 3B is not important for lymphocyte infection or growth transformation in vitro. *J. Virol.* **66,** 2893–903 (1992).

139. White, R. E. *et al.* Extensive co-operation between the Epstein-Barr virus EBNA3 proteins in the manipulation of host gene expression and epigenetic chromatin modification. *PLoS One* **5,** e13979 (2010).

140. Krauer, K. G. *et al.* The Epstein-Barr virus nuclear antigen-6 protein co-localizes with EBNA-3 and survival of motor neurons protein. *Virology* **318,** 280–94 (2004).

141. Calderwood, M. A. *et al.* Epstein-Barr virus and virus human protein interaction maps. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 7606–11 (2007).

142. Kashuba, E. V *et al.* Regulation of transactivation function of the aryl hydrocarbon receptor by the Epstein-Barr virus-encoded EBNA-3 protein. *J. Biol. Chem.* **281,** 1215–23 (2006).

143. Puga, A., Xia, Y. & Elferink, C. Role of the aryl hydrocarbon receptor in cell cycle regulation. *Chem. Biol. Interact.* **141,** 117–30 (2002).

144. Pang, P.-H. *et al.* Molecular mechanisms of p21 and p27 induction by 3-methylcholanthrene, an aryl-hydrocarbon receptor agonist, involved in antiproliferation of human umbilical vascular endothelial cells. *J. Cell. Physiol.* **215,** 161–71 (2008).

145. Koliopanos, A. *et al.* Increased arylhydrocarbon receptor expression offers a potential therapeutic target for pancreatic cancer. *Oncogene* **21,** 6059–70 (2002).

146. Knight, J. S., Sharma, N. & Robertson, E. S. Epstein-Barr virus latent antigen 3C can mediate the degradation of the retinoblastoma protein through an SCF cellular ubiquitin ligase. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 18562–6 (2005).

147. Knight, J. S. & Robertson, E. S. Epstein-Barr virus nuclear antigen 3C regulates cyclin A/p27 complexes and enhances cyclin A-dependent kinase activity. *J. Virol.* **78,** 1981–91 (2004).

148. Saha, A. *et al.* Epstein-Barr virus nuclear antigen 3C facilitates G1-S transition by stabilizing and enhancing the function of cyclin D1. *PLoS Pathog.* **7,** e1001275 (2011).

149. Pokrovskaja, K. *et al.* Phenotype-related differences in the expression of D-type cyclins in human B cell-derived lines. *Cell Growth Differ.* **7,** 1723–32 (1996).

150. Tursiella, M. L. *et al.* Epstein-Barr virus nuclear antigen 3A promotes cellular proliferation by repression of the cyclin-dependent kinase inhibitor p21WAF1/CIP1. *PLoS Pathog.* **10,** e1004415 (2014).

151. White, R. E. *et al.* EBNA3B-deficient EBV promotes B cell lymphomagenesis in humanized mice and is found in human tumors. *J. Clin. Invest.* **122,** 1487–502 (2012).

152. Gottschalk, S. *et al.* An Epstein-Barr virus deletion mutant associated with fatal lymphoproliferative disease unresponsive to therapy with virus-specific CTLs. *Blood* **97,** 835–43 (2001).

153. Taub, R. *et al.* Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. *Proc. Natl. Acad. Sci.* **79,** 7837–7841 (1982).

154. Dalla-Favera, R. *et al.* Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proc. Natl. Acad. Sci.* **79,** 7824–7827 (1982).

155. Hummel, M. *et al.* A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. *N. Engl. J. Med.* **354,** 2419–30 (2006).

156. Felsher, D. W. & Bishop, J. M. Transient excess of MYC activity can elicit genomic instability and tumorigenesis. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3940–4 (1999).

157. Felsher, D. W. & Bishop, J. M. Reversible tumorigenesis by MYC in hematopoietic lineages. *Mol. Cell* **4,** 199–207 (1999).

158. Askew, D. S., Ashmun, R. A., Simmons, B. C. & Cleveland, J. L. Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* **6,** 1915–22 (1991).

159. Lindström, M. S. & Wiman, K. G. Role of genetic and epigenetic changes in Burkitt lymphoma. *Semin. Cancer Biol.* **12,** 381–7 (2002).

160. Sample, J. & Ruf, I. in *Epstein-Barr Virus* (eds. Tselis, A. & Johnson, H.) 187–222 (Informa Healthcare, 2006).

161. Marcu, K. B., Bossone, S. A. & Patel, A. J. myc function and regulation. *Annu. Rev. Biochem.* **61,** 809–60 (1992).

162. Adams, J. M. *et al.* The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* **318,** 533–8

163. Eischen, C. M., Weber, J. D., Roussel, M. F., Sherr, C. J. & Cleveland, J. L. Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. *Genes Dev.* **13,** 2658–69 (1999).

164. Hsu, B. *et al.* Evidence that c-myc mediated apoptosis does not require wild-type p53 during lymphomagenesis. *Oncogene* **11,** 175–9 (1995).

165. Schmitt, C. A., McCurrach, M. E., de Stanchina, E., Wallace-Brodeur, R. R. & Lowe, S. W. INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53. *Genes Dev.* **13,** 2670–7 (1999).

166. Carbone, A. Emerging pathways in the development of AIDS-related lymphomas. *Lancet. Oncol.* **4,** 22–9 (2003).

167. Farrell, P. J., Allan, G. J., Shanahan, F., Vousden, K. H. & Crook, T. p53 is frequently mutated in Burkitt's lymphoma cell lines. *EMBO J.* **10,** 2879–87 (1991).

168. Gaidano, G. *et al.* p53 mutations in human lymphoid malignancies: association with Burkitt lymphoma and chronic lymphocytic leukemia. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5413–7 (1991).

169. Capoulade, C. *et al.* Overexpression of MDM2, due to enhanced translation, results in inactivation of wild-type p53 in Burkitt's lymphoma cells. *Oncogene* **16,** 1603–10 (1998).

170. Lindström, M. S., Klangby, U. & Wiman, K. G. p14ARF homozygous deletion or MDM2 overexpression in Burkitt lymphoma lines carrying wild type p53. *Oncogene* **20,** 2171–7 (2001).

171. Klangby, U. *et al.* p16/INK4a and p15/INK4b gene methylation and absence of p16/INK4a mRNA and protein expression in Burkitt's lymphoma. *Blood* **91,** 1680–7 (1998).

172. Hecht, J. L. & Aster, J. C. Molecular biology of Burkitt's lymphoma. *J. Clin. Oncol.* **18,** 3707–21 (2000).

173. Gregory, C. D. *et al.* Identification of a subset of normal B cells with a Burkitt's lymphoma (BL)-like phenotype. *J. Immunol.* **139,** 313–8 (1987).

174. Magrath, I. The pathogenesis of Burkitt's lymphoma. *Adv. Cancer Res.* **55,** 133–270 (1990).

175. Ruf, I. K. *et al.* Epstein-barr virus regulates c-MYC, apoptosis, and tumorigenicity in Burkitt lymphoma. *Mol. Cell. Biol.* **19,** 1651–60 (1999).

176. Shimizu, N., Tanabe-Tochikura, A., Kuroiwa, Y. & Takada, K. Isolation of Epstein-Barr virus (EBV)-negative cell clones from the EBV-positive Burkitt's lymphoma (BL) line Akata: malignant phenotypes of BL cells are dependent on EBV. *J. Virol.* **68,** 6069–73 (1994).

177. Harris, R. S., Croom-Carter, D. S., Rickinson, A. B. & Neuberger, M. S. Epstein-Barr virus and the somatic hypermutation of immunoglobulin genes in Burkitt's lymphoma cells. *J. Virol.* **75,** 10488–92 (2001).

178. Brady, G., Macarthur, G. J. & Farrell, P. J. Epstein-Barr virus and Burkitt lymphoma. *Postgrad. Med. J.* **84,** 372–7 (2008).

179. Bornkamm, G. W., Hudewentz, J., Freese, U. K. & Zimber, U. Deletion of the nontransforming Epstein-Barr virus strain P3HR-1 causes fusion of the large internal repeat to the DSL region. *J. Virol.* **43,** 952–68 (1982).

180. Rabson, M., Gradoville, L., Heston, L. & Miller, G. Non-immortalizing P3J-HR-1 Epstein-Barr virus: a deletion mutant of its transforming parent, Jijoye. *J. Virol.* **44,** 834–44 (1982).

181. Khanna, R. & Burrows, S. R. Role of cytotoxic T lymphocytes in Epstein-Barr virus-associated diseases. *Annu. Rev. Microbiol.* **54,** 19–48 (2000).

182. Rowe, M. *et al.* Restoration of endogenous antigen processing in Burkitt's lymphoma cells by Epstein-Barr virus latent membrane protein-1: coordinate up-regulation of peptide transporters and HLA-class I antigen expression. *Eur. J. Immunol.* **25,** 1374–84 (1995).

183. Khanna, R., Burrows, S. R., Argaet, V. & Moss, D. J. Endoplasmic reticulum signal sequence facilitated transport of peptide epitopes restores immunogenicity of an antigen processing defective tumour cell line. *Int. Immunol.* **6,** 639–45 (1994).

184. Kelly, G. L. *et al.* Epstein-Barr virus nuclear antigen 2 (EBNA2) gene deletion is consistently linked with EBNA3A, -3B, and -3C expression in Burkitt's lymphoma cells and with increased resistance to apoptosis. *J. Virol.* **79,** 10709–17 (2005).

185. Gregory, C. D. *et al.* Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. *Nature* **349,** 612–4 (1991).

186. Garibal, J. *et al.* Truncated form of the Epstein-Barr virus protein EBNA-LP protects against caspase-dependent apoptosis by inhibiting protein phosphatase 2A. *J. Virol.* **81,** 7598–607 (2007).

187. Pearson, G. R. *et al.* Identification of an Epstein-Barr virus early gene encoding a second component of the restricted early antigen complex. *Virology* **160,** 151–61 (1987).

188. Flanagan, A. M. & Letai, A. BH3 domains define selective inhibitory interactions with BHRF-1 and KSHV BCL-2. *Cell Death Differ.* **15,** 580–8 (2008).

189. Watanabe, A. *et al.* Epstein-Barr virus-encoded Bcl-2 homologue functions as a survival factor in Wp-restricted Burkitt lymphoma cell line P3HR-1. *J. Virol.* **84,** 2893–901 (2010).

190. Anderton, E. *et al.* Two Epstein-Barr virus (EBV) oncoproteins cooperate to repress expression of the proapoptotic tumour-suppressor Bim: clues to the pathogenesis of Burkitt's lymphoma. *Oncogene* **27,** 421–33 (2008).

191. Vereide, D. T. & Sugden, B. Lymphomas differ in their dependence on Epstein-Barr virus. *Blood* **117,** 1977–85 (2011).

192. Lee, R. C., Feinbaum, R. L. & Ambros, V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75,** 843–54 (1993).

193. Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116,** 281–97 (2004).

194. Olsen, P. H. & Ambros, V. The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. *Dev. Biol.* **216,** 671–80 (1999).

195. Wightman, B., Ha, I. & Ruvkun, G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell* **75,** 855–62 (1993).

196. Pillai, R. S. *et al.* Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science* **309,** 1573–6 (2005).

197. Fabian, M. R., Sonenberg, N. & Filipowicz, W. Regulation of mRNA translation and stability by microRNAs. *Annu. Rev. Biochem.* **79,** 351–79 (2010).

198. Friedman, R. C., Farh, K. K.-H., Burge, C. B. & Bartel, D. P. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* **19,** 92–105 (2009).

199. Griffiths-Jones, S., Saini, H. K., van Dongen, S. & Enright, A. J. miRBase: tools for microRNA genomics. *Nucleic Acids Res.* **36,** D154–8 (2008).

200. Bartel, D. P. MicroRNAs: target recognition and regulatory functions. *Cell* **136,** 215–33 (2009).

201. Wang, Y., Keys, D. N., Au-Young, J. K. & Chen, C. MicroRNAs in embryonic stem cells. *J. Cell. Physiol.* **218,** 251–5 (2009).

202. Dostie, J., Mourelatos, Z., Yang, M., Sharma, A. & Dreyfuss, G. Numerous microRNPs in neuronal cells containing novel microRNAs. *RNA* **9,** 180–6 (2003).

203. Xu, P., Vernooy, S. Y., Guo, M. & Hay, B. A. The Drosophila microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. *Curr. Biol.* **13,** 790–5 (2003).

204. Ozsolak, F. *et al.* Chromatin structure analyses identify miRNA promoters. *Genes Dev.* **22,** 3172–83 (2008).

205. Monteys, A. M. *et al.* Structure and activity of putative intronic miRNA promoters. *RNA* **16,** 495–505 (2010).

206. Lee, Y. *et al.* MicroRNA genes are transcribed by RNA polymerase II. *EMBO J.* **23,** 4051–60 (2004).

207. Lee, Y. *et al.* The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425,** 415–9 (2003).

208. Han, J. *et al.* Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell* **125,** 887–901 (2006).

209. Denli, A. M., Tops, B. B. J., Plasterk, R. H. A., Ketting, R. F. & Hannon, G. J. Processing of primary microRNAs by the Microprocessor complex. *Nature* **432,** 231–5 (2004).

210. Han, J. *et al.* The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev.* **18,** 3016–27 (2004).

211. Knight, S. W. & Bass, B. L. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. *Science* **293,** 2269–71 (2001).

212. Ketting, R. F. *et al.* Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. *Genes Dev.* **15,** 2654–9 (2001).

213. Hutvágner, G. *et al.* A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* **293,** 834–8 (2001).

214. Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409,** 363–6 (2001).

215. Park, J.-E. *et al.* Dicer recognizes the 5’ end of RNA for efficient and accurate processing. *Nature* **475,** 201–5 (2011).

216. Tian, Y. *et al.* A phosphate-binding pocket within the platform-PAZ-connector helix cassette of human Dicer. *Mol. Cell* **53,** 606–16 (2014).

217. MacRae, I. J. Structural Basis for Double-Stranded RNA Processing by Dicer. *Science (80-.).* **311,** 195–198 (2006).

218. Tsutsumi, A., Kawamata, T., Izumi, N., Seitz, H. & Tomari, Y. Recognition of the pre-miRNA structure by Drosophila Dicer-1. *Nat. Struct. Mol. Biol.* **18,** 1153–8 (2011).

219. Tabara, H. *et al.* The rde-1 gene, RNA interference, and transposon silencing in C. elegans. *Cell* **99,** 123–32 (1999).

220. Hammond, S. M. Argonaute2, a Link Between Genetic and Biochemical Analyses of RNAi. *Science (80-.).* **293,** 1146–1150 (2001).

221. Mourelatos, Z. *et al.* miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. *Genes Dev.* **16,** 720–8 (2002).

222. Chendrimada, T. P. *et al.* TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* **436,** 740–4 (2005).

223. Miyoshi, K., Tsukumo, H., Nagami, T., Siomi, H. & Siomi, M. C. Slicer function of Drosophila Argonautes and its involvement in RISC formation. *Genes Dev.* **19,** 2837–48 (2005).

224. MacRae, I. J., Ma, E., Zhou, M., Robinson, C. V. & Doudna, J. A. In vitro reconstitution of the human RISC-loading complex. *Proc. Natl. Acad. Sci.* **105,** 512–517 (2008).

225. Betancur, J. G. & Tomari, Y. Dicer is dispensable for asymmetric RISC loading in mammals. *RNA* **18,** 24–30 (2012).

226. Kawamata, T., Seitz, H. & Tomari, Y. Structural determinants of miRNAs for RISC loading and slicer-independent unwinding. *Nat. Struct. Mol. Biol.* **16,** 953–60 (2009).

227. Kawamata, T. & Tomari, Y. Making RISC. *Trends Biochem. Sci.* **35,** 368–76 (2010).

228. Rand, T. A., Petersen, S., Du, F. & Wang, X. Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* **123,** 621–9 (2005).

229. Diederichs, S. & Haber, D. A. Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. *Cell* **131,** 1097–108 (2007).

230. He, L. & Hannon, G. J. MicroRNAs: small RNAs with a big role in gene regulation. *Nat. Rev. Genet.* **5,** 522–31 (2004).

231. Calin, G. A. *et al.* Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 15524–9 (2002).

232. Calin, G. A. *et al.* Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 2999–3004 (2004).

233. Lu, J. *et al.* MicroRNA expression profiles classify human cancers. *Nature* **435,** 834–8 (2005).

234. Volinia, S. *et al.* A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 2257–61 (2006).

235. Calin, G. A. *et al.* A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N. Engl. J. Med.* **353,** 1793–801 (2005).

236. Schetter, A. J. *et al.* MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* **299,** 425–36 (2008).

237. Dillhoff, M., Liu, J., Frankel, W., Croce, C. & Bloomston, M. MicroRNA-21 is overexpressed in pancreatic cancer and a potential predictor of survival. *J. Gastrointest. Surg.* **12,** 2171–6 (2008).

238. Rossi, S. *et al.* microRNA fingerprinting of CLL patients with chromosome 17p deletion identify a miR-21 score that stratifies early survival. *Blood* **116,** 945–52 (2010).

239. Laganà, A. *et al.* Variability in the incidence of miRNAs and genes in fragile sites and the role of repeats and CpG islands in the distribution of genetic material. *PLoS One* **5,** e11166 (2010).

240. Wynendaele, J. *et al.* An illegitimate microRNA target site within the 3’ UTR of MDM4 affects ovarian cancer progression and chemosensitivity. *Cancer Res.* **70,** 9641–9 (2010).

241. Mishra, P. J., Mishra, P. J., Banerjee, D. & Bertino, J. R. MiRSNPs or MiR-polymorphisms, new players in microRNA mediated regulation of the cell: Introducing microRNA pharmacogenomics. *Cell Cycle* **7,** 853–8 (2008).

242. Diederichs, S. & Haber, D. A. Sequence variations of microRNAs in human cancer: alterations in predicted secondary structure do not affect processing. *Cancer Res.* **66,** 6097–104 (2006).

243. Weber, B., Stresemann, C., Brueckner, B. & Lyko, F. Methylation of human microRNA genes in normal and neoplastic cells. *Cell Cycle* **6,** 1001–5 (2007).

244. Saito, Y. *et al.* Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell* **9,** 435–43 (2006).

245. Garzon, R. *et al.* MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. *Blood* **113,** 6411–8 (2009).

246. Fabbri, M. *et al.* MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 15805–10 (2007).

247. Chang, T.-C. *et al.* Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol. Cell* **26,** 745–52 (2007).

248. Thomson, J. M. *et al.* Extensive post-transcriptional regulation of microRNAs and its implications for cancer. *Genes Dev.* **20,** 2202–7 (2006).

249. Nakamura, T., Canaani, E. & Croce, C. M. Oncogenic All1 fusion proteins target Drosha-mediated microRNA processing. *Proc. Natl. Acad. Sci.* **104,** 10980–10985 (2007).

250. Jakymiw, A. *et al.* Overexpression of dicer as a result of reduced let-7 MicroRNA levels contributes to increased cell proliferation of oral cancer cells. *Genes. Chromosomes Cancer* **49,** 549–59 (2010).

251. Merritt, W. M. *et al.* Dicer, Drosha, and outcomes in patients with ovarian cancer. *N. Engl. J. Med.* **359,** 2641–50 (2008).

252. Wang, Y., Medvid, R., Melton, C., Jaenisch, R. & Blelloch, R. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat. Genet.* **39,** 380–5 (2007).

253. Lagos-Quintana, M. *et al.* Identification of tissue-specific microRNAs from mouse. *Curr. Biol.* **12,** 735–9 (2002).

254. Tam, W., Ben-Yehuda, D. & Hayward, W. S. bic, a novel gene activated by proviral insertions in avian leukosis virus-induced lymphomas, is likely to function through its noncoding RNA. *Mol. Cell. Biol.* **17,** 1490–502 (1997).

255. Clurman, B. E. & Hayward, W. S. Multiple proto-oncogene activations in avian leukosis virus-induced lymphomas: evidence for stage-specific events. *Mol. Cell. Biol.* **9,** 2657–64 (1989).

256. Tam, W., Hughes, S. H., Hayward, W. S. & Besmer, P. Avian bic, a gene isolated from a common retroviral site in avian leukosis virus-induced lymphomas that encodes a noncoding RNA, cooperates with c-myc in lymphomagenesis and erythroleukemogenesis. *J. Virol.* **76,** 4275–86 (2002).

257. Van den Berg, A. *et al.* High expression of B-cell receptor inducible gene BIC in all subtypes of Hodgkin lymphoma. *Genes. Chromosomes Cancer* **37,** 20–8 (2003).

258. O'Connell, R. M., Taganov, K. D., Boldin, M. P., Cheng, G. & Baltimore, D. MicroRNA-155 is induced during the macrophage inflammatory response. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 1604–9 (2007).

259. Taganov, K. D., Boldin, M. P., Chang, K.-J. & Baltimore, D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 12481–6 (2006).

260. Masaki, S., Ohtsuka, R., Abe, Y., Muta, K. & Umemura, T. Expression patterns of microRNAs 155 and 451 during normal human erythropoiesis. *Biochem. Biophys. Res. Commun.* **364,** 509–14 (2007).

261. Teng, G. & Papavasiliou, F. N. Shhh! Silencing by microRNA-155. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **364,** 631–7 (2009).

262. Haasch, D. *et al.* T cell activation induces a noncoding RNA transcript sensitive to inhibition by immunosuppressant drugs and encoded by the proto-oncogene, BIC. *Cell. Immunol.* **217,** 78–86

263. Rodriguez, A. *et al.* Requirement of bic/microRNA-155 for normal immune function. *Science* **316,** 608–11 (2007).

264. Banerjee, A., Schambach, F., DeJong, C. S., Hammond, S. M. & Reiner, S. L. Micro-RNA-155 inhibits IFN-gamma signaling in CD4+ T cells. *Eur. J. Immunol.* **40,** 225–31 (2010).

265. Oertli, M. *et al.* MicroRNA-155 is essential for the T cell-mediated control of Helicobacter pylori infection and for the induction of chronic Gastritis and Colitis. *J. Immunol.* **187,** 3578–86 (2011).

266. O'Connell, R. M. *et al.* MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development. *Immunity* **33,** 607–19 (2010).

267. Thai, T.-H. *et al.* Regulation of the germinal center response by microRNA-155. *Science* **316,** 604–8 (2007).

268. Vigorito, E. *et al.* microRNA-155 Regulates the Generation of Immunoglobulin Class-Switched Plasma Cells. *Immunity* **27,** 847–859 (2007).

269. Esquela-Kerscher, A. & Slack, F. J. Oncomirs - microRNAs with a role in cancer. *Nat. Rev. Cancer* **6,** 259–69 (2006).

270. Faraoni, I., Antonetti, F. R., Cardone, J. & Bonmassar, E. miR-155 gene: a typical multifunctional microRNA. *Biochim. Biophys. Acta* **1792,** 497–505 (2009).

271. Costinean, S. *et al.* Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 7024–9 (2006).

272. O'Connell, R. M. *et al.* Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J. Exp. Med.* **205,** 585–94 (2008).

273. Metcalf, D. *et al.* Inactivation of PU.1 in adult mice leads to the development of myeloid leukemia. *Proc. Natl. Acad. Sci.* **103,** 1486–1491 (2006).

274. Jiang, S. *et al.* MicroRNA-155 Functions as an OncomiR in Breast Cancer by Targeting the Suppressor of Cytokine Signaling 1 Gene. *Cancer Res.* **70,** 3119–3127 (2010).

275. Jiang, S. *et al.* A novel miR-155/miR-143 cascade controls glycolysis by regulating hexokinase 2 in breast cancer cells. *EMBO J.* **31,** 1985–98 (2012).

276. Ovcharenko, D., Kelnar, K., Johnson, C., Leng, N. & Brown, D. Genome-scale microRNA and small interfering RNA screens identify small RNA modulators of TRAIL-induced apoptosis pathway. *Cancer Res.* **67,** 10782–8 (2007).

277. Kong, W. *et al.* MicroRNA-155 regulates cell survival, growth, and chemosensitivity by targeting FOXO3a in breast cancer. *J. Biol. Chem.* **285,** 17869–79 (2010).

278. Yang, L. *et al.* MicroRNA-155 inhibits angiotensin II-induced vascular smooth muscle cell proliferation. *J. Renin. Angiotensin. Aldosterone. Syst.* **15,** 109–16 (2014).

279. Martin, M. M. *et al.* The human angiotensin II type 1 receptor +1166 A/C polymorphism attenuates microRNA-155 binding. *J. Biol. Chem.* **282,** 24262–9 (2007).

280. Cheng, W. *et al.* microRNA-155 regulates angiotensin II type 1 receptor expression in umbilical vein endothelial cells from severely pre-eclamptic pregnant women. *Int. J. Mol. Med.* **27,** 393–399 (2011).

281. Sun, H.-X. *et al.* Essential role of microRNA-155 in regulating endothelium-dependent vasorelaxation by targeting endothelial nitric oxide synthase. *Hypertension* **60,** 1407–14 (2012).

282. Lu, F. *et al.* Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence. *J. Virol.* **82,** 10436–43 (2008).

283. Jiang, J., Lee, E. J. & Schmittgen, T. D. Increased expression of microRNA-155 in Epstein-Barr virus transformed lymphoblastoid cell lines. *Genes. Chromosomes Cancer* **45,** 103–6 (2006).

284. Cameron, J. E. *et al.* Epstein-Barr virus growth/latency III program alters cellular microRNA expression. *Virology* **382,** 257–66 (2008).

285. Kluiver, J. *et al.* Lack of BIC and microRNA miR-155 expression in primary cases of Burkitt lymphoma. *Genes. Chromosomes Cancer* **45,** 147–53 (2006).

286. Yin, Q. *et al.* MicroRNA-155 is an Epstein-Barr virus-induced gene that modulates Epstein-Barr virus-regulated gene expression pathways. *J. Virol.* **82,** 5295–306 (2008).

287. Du, Z.-M. *et al.* Upregulation of MiR-155 in nasopharyngeal carcinoma is partly driven by LMP1 and LMP2A and downregulates a negative prognostic marker JMJD1A. *PLoS One* **6,** e19137 (2011).

288. Linnstaedt, S. D., Gottwein, E., Skalsky, R. L., Luftig, M. A. & Cullen, B. R. Virally induced cellular microRNA miR-155 plays a key role in B-cell immortalization by Epstein-Barr virus. *J. Virol.* **84,** 11670–8 (2010).

289. Yin, Q. *et al.* MicroRNA miR-155 inhibits bone morphogenetic protein (BMP) signaling and BMP-mediated Epstein-Barr virus reactivation. *J. Virol.* **84,** 6318–27 (2010).

290. Gottwein, E. *et al.* A viral microRNA functions as an orthologue of cellular miR-155. *Nature* **450,** 1096–1099 (2007).

291. Zhao, Y. *et al.* A functional MicroRNA-155 ortholog encoded by the oncogenic Marek's disease virus. *J. Virol.* **83,** 489–92 (2009).

292. Kanezaki, R. *et al.* Transcription factor BACH1 is recruited to the nucleus by its novel alternative spliced isoform. *J. Biol. Chem.* **276,** 7278–84 (2001).

293. Yin, Q., Wang, X., McBride, J., Fewell, C. & Flemington, E. B-cell Receptor Activation Induces BIC/miR-155 Expression through a Conserved AP-1 Element. *J. Biol. Chem.* **283,** 2654–2662 (2007).

294. Song, X. *et al.* Regulation of c-Jun/JunB heterodimers mediated by Epstein-Barr virus encoded latent membrane protein 1 on p16. *Chinese Sci. Bull.* **49,** 676–683 (2004).

295. Malumbres, M. & Barbacid, M. Mammalian cyclin-dependent kinases. *Trends Biochem. Sci.* **30,** 630–41 (2005).

296. De Bondt, H. L. *et al.* Crystal structure of cyclin-dependent kinase 2. *Nature* **363,** 595–602 (1993).

297. David-Pfeuty, T. & Nouvian-Dooghe, Y. Human cyclin B1 is targeted to the nucleus in G1 phase prior to its accumulation in the cytoplasm. *Oncogene* **13,** 1447–60 (1996).

298. Desai, D., Wessling, H. C., Fisher, R. P. & Morgan, D. O. Effects of phosphorylation by CAK on cyclin binding by CDC2 and CDK2. *Mol. Cell. Biol.* **15,** 345–50 (1995).

299. Fisher, R. P., Jin, P., Chamberlin, H. M. & Morgan, D. O. Alternative mechanisms of CAK assembly require an assembly factor or an Activating Kinase. *Cell* **83,** 47–57 (1995).

300. Van den Heuvel, S. & Harlow, E. Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* **262,** 2050–4 (1993).

301. Parker, L. L. & Piwnica-Worms, H. Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase. *Science* **257,** 1955–7 (1992).

302. Devault, A. *et al.* Concerted roles of cyclin A, cdc25+ mitotic inducer, and type 2A phosphatase in activating the cyclin B/cdc2 protein kinase at the G2/M phase transition. *Cold Spring Harb. Symp. Quant. Biol.* **56,** 503–13 (1991).

303. Heald, R., McLoughlin, M. & McKeon, F. Human wee1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated Cdc2 kinase. *Cell* **74,** 463–74 (1993).

304. Honda, R., Ohba, Y., Nagata, A., Okayama, H. & Yasuda, H. Dephosphorylation of human p34cdc2 kinase on both Thr-14 and Tyr-15 by human cdc25B phosphatase. *FEBS Lett.* **318,** 331–4 (1993).

305. Darzynkiewicz, Z., Gong, J., Juan, G., Ardelt, B. & Traganos, F. Cytometry of cyclin proteins. *Cytometry* **25,** 1–13 (1996).

306. Sudakin, V. *et al.* The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. *Mol. Biol. Cell* **6,** 185–97 (1995).

307. Irniger, S., Piatti, S., Michaelis, C. & Nasmyth, K. Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. *Cell* **81,** 269–78 (1995).

308. King, R. W. *et al.* A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* **81,** 279–88 (1995).

309. Meyerson, M. & Harlow, E. Identification of G1 kinase activity for cdk6, a novel cyclin D partner. *Mol. Cell. Biol.* **14,** 2077–86 (1994).

310. Matsushime, H., Roussel, M. F. & Sherr, C. J. Novel mammalian cyclins (CYL genes) expressed during G1. *Cold Spring Harb. Symp. Quant. Biol.* **56,** 69–74 (1991).

311. Hatakeyama, M., Brill, J. A., Fink, G. R. & Weinberg, R. A. Collaboration of G1 cyclins in the functional inactivation of the retinoblastoma protein. *Genes Dev.* **8,** 1759–71 (1994).

312. Koff, A. *et al.* Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. *Science* **257,** 1689–94 (1992).

313. Hwang, H. C. & Clurman, B. E. Cyclin E in normal and neoplastic cell cycles. *Oncogene* **24,** 2776–86 (2005).

314. Won, K. A. & Reed, S. I. Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. *EMBO J.* **15,** 4182–93 (1996).

315. Clurman, B. E., Sheaff, R. J., Thress, K., Groudine, M. & Roberts, J. M. Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation. *Genes Dev.* **10,** 1979–90 (1996).

316. Kitagawa, M. *et al.* The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. *EMBO J.* **15,** 7060–9 (1996).

317. Wang, Y. & Prives, C. Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases. *Nature* **376,** 88–91 (1995).

318. Zhang, T. & Prives, C. Cyclin a-CDK phosphorylation regulates MDM2 protein interactions. *J. Biol. Chem.* **276,** 29702–10 (2001).

319. Fuster, J. J. *et al.* Control of cell proliferation in atherosclerosis: insights from animal models and human studies. *Cardiovasc. Res.* **86,** 254–64 (2010).

320. el-Deiry, W. S. *et al.* WAF1, a potential mediator of p53 tumor suppression. *Cell* **75,** 817–25 (1993).

321. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. & Elledge, S. J. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75,** 805–16 (1993).

322. Dulić, V. *et al.* p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell* **76,** 1013–23 (1994).

323. Chan, F. K., Zhang, J., Cheng, L., Shapiro, D. N. & Winoto, A. Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to p16ink4. *Mol. Cell. Biol.* **15,** 2682–8 (1995).

324. Guan, K. L. *et al.* Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes Dev.* **8,** 2939–52 (1994).

325. Pavletich, N. P. Mechanisms of cyclin-dependent kinase regulation: structures of Cdks, their cyclin activators, and Cip and INK4 inhibitors. *J. Mol. Biol.* **287,** 821–8 (1999).

326. Hannon, G. J. & Beach, D. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* **371,** 257–61 (1994).

327. Hara, E. *et al.* Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. *Mol. Cell. Biol.* **16,** 859–67 (1996).

328. Morse, L., Chen, D., Franklin, D., Xiong, Y. & Chen-Kiang, S. Induction of cell cycle arrest and B cell terminal differentiation by CDK inhibitor p18(INK4c) and IL-6. *Immunity* **6,** 47–56 (1997).

329. Kriwacki, R. W., Hengst, L., Tennant, L., Reed, S. I. & Wright, P. E. Structural studies of p21Waf1/Cip1/Sdi1 in the free and Cdk2-bound state: conformational disorder mediates binding diversity. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 11504–9 (1996).

330. Luo, Y., Hurwitz, J. & Massagué, J. Cell-cycle inhibition by independent CDK and PCNA binding domains in p21Cip1. *Nature* **375,** 159–61 (1995).

331. Saha, P., Eichbaum, Q., Silberman, E. D., Mayer, B. J. & Dutta, A. p21CIP1 and Cdc25A: competition between an inhibitor and an activator of cyclin-dependent kinases. *Mol. Cell. Biol.* **17,** 4338–45 (1997).

332. Chen, J., Jackson, P. K., Kirschner, M. W. & Dutta, A. Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. *Nature* **374,** 386–8 (1995).

333. Moldovan, G.-L., Pfander, B. & Jentsch, S. PCNA, the maestro of the replication fork. *Cell* **129,** 665–79 (2007).

334. Gartel, A. L. & Tyner, A. L. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol. Cancer Ther.* **1,** 639–49 (2002).

335. Soos, T. J. *et al.* Formation of p27-CDK complexes during the human mitotic cell cycle. *Cell Growth Differ.* **7,** 135–46 (1996).

336. Zhang, H., Hannon, G. J. & Beach, D. p21-containing cyclin kinases exist in both active and inactive states. *Genes Dev.* **8,** 1750–8 (1994).

337. Blain, S. W., Montalvo, E. & Massagué, J. Differential interaction of the cyclin-dependent kinase (Cdk) inhibitor p27Kip1 with cyclin A-Cdk2 and cyclin D2-Cdk4. *J. Biol. Chem.* **272,** 25863–72 (1997).

338. Cheng, M. *et al.* The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. *EMBO J.* **18,** 1571–83 (1999).

339. Abbas, T. & Dutta, A. p21 in cancer: intricate networks and multiple activities. *Nat. Rev. Cancer* **9,** 400–14 (2009).

340. Suzuki, A. *et al.* Survivin initiates procaspase 3/p21 complex formation as a result of interaction with Cdk4 to resist Fas-mediated cell death. *Oncogene* **19,** 1346–53 (2000).

341. Suzuki, A., Tsutomi, Y., Yamamoto, N., Shibutani, T. & Akahane, K. Mitochondrial regulation of cell death: mitochondria are essential for procaspase 3-p21 complex formation to resist Fas-mediated cell death. *Mol. Cell. Biol.* **19,** 3842–7 (1999).

342. Zhou, B. P. *et al.* Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat. Cell Biol.* **3,** 245–52 (2001).

343. Child, E. S. & Mann, D. J. The intricacies of p21 phosphorylation: Protein/protein interactions, subcellular localization and stability. *Cell Cycle* **5,** 1313–1319 (2006).

344. Warbrick, E. The puzzle of PCNA's many partners. *Bioessays* **22,** 997–1006 (2000).

345. Rössig, L. *et al.* Akt-dependent phosphorylation of p21(Cip1) regulates PCNA binding and proliferation of endothelial cells. *Mol. Cell. Biol.* **21,** 5644–57 (2001).

346. Harreman, M. T. *et al.* Regulation of nuclear import by phosphorylation adjacent to nuclear localization signals. *J. Biol. Chem.* **279,** 20613–21 (2004).

347. Asada, M. *et al.* Apoptosis inhibitory activity of cytoplasmic p21(Cip1/WAF1) in monocytic differentiation. *EMBO J.* **18,** 1223–34 (1999).

348. Skomedal, H., Kristensen, G. B., Lie, A. K. & Holm, R. Aberrant expression of the cell cycle associated proteins TP53, MDM2, p21, p27, cdk4, cyclin D1, RB, and EGFR in cervical carcinomas. *Gynecol. Oncol.* **73,** 223–8 (1999).

349. Krolewski, B. & Little, J. B. Overexpression of p21 protein in radiation-transformed mouse 10T(1/2) cell clones. *Mol. Carcinog.* **27,** 141–8 (2000).

350. Wong, S. C., Chan, J. K., Lee, K. C. & Hsiao, W. L. Differential expression of p16/p21/p27 and cyclin D1/D3, and their relationships to cell proliferation, apoptosis, and tumour progression in invasive ductal carcinoma of the breast. *J. Pathol.* **194,** 35–42 (2001).

351. McKenzie, P. P., Danks, M. K., Kriwacki, R. W. & Harris, L. C. P21Waf1/Cip1 dysfunction in neuroblastoma: a novel mechanism of attenuating G0-G1 cell cycle arrest. *Cancer Res.* **63,** 3840–4 (2003).

352. Jung, Y. S., Qian, Y. & Chen, X. Examination of the expanding pathways for the regulation of p21 expression and activity. *Cell. Signal.* **22,** 1003–1012 (2010).

353. Nozell, S. & Chen, X. p21B, a variant of p21(Waf1/Cip1), is induced by the p53 family. *Oncogene* **21,** 1285–94 (2002).

354. Radhakrishnan, S. K., Gierut, J. & Gartel, a L. Multiple alternate p21 transcripts are regulated by p53 in human cells. *Oncogene* **25,** 1812–1815 (2006).

355. Harms, K., Nozell, S. & Chen, X. The common and distinct target genes of the p53 family transcription factors. *Cell. Mol. Life Sci.* **61,** 822–42 (2004).

356. Decesse, J., Medjkane, S., Datto, M. & Cremisi, C. RB regulates transcription of the p21/WAF1/CIP1 gene. *Oncogene* **20,** 962–71 (2001).

357. Gartel, A. L. *et al.* Myc represses the p21(WAF1/CIP1) promoter and interacts with Sp1/Sp3. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4510–5 (2001).

358. Kim, Y.-K. *et al.* Functional links between clustered microRNAs: suppression of cell-cycle inhibitors by microRNA clusters in gastric cancer. *Nucleic Acids Res.* **37,** 1672–81 (2009).

359. Ivanovska, I. *et al.* MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. *Mol. Cell. Biol.* **28,** 2167–74 (2008).

360. Petrocca, F., Vecchione, A. & Croce, C. M. Emerging role of miR-106b-25/miR-17-92 clusters in the control of transforming growth factor beta signaling. *Cancer Res.* **68,** 8191–4 (2008).

361. Petrocca, F. *et al.* E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer. *Cancer Cell* **13,** 272–86 (2008).

362. Fontana, L. *et al.* Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM. *PLoS One* **3,** e2236 (2008).

363. Le, M. T. N. *et al.* MicroRNA-125b is a novel negative regulator of p53. *Genes Dev.* **23,** 862–76 (2009).

364. Rai, D., Kim, S.-W., McKeller, M. R., Dahia, P. L. M. & Aguiar, R. C. T. Targeting of SMAD5 links microRNA-155 to the TGF-beta pathway and lymphomagenesis. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 3111–6 (2010).

365. Blagosklonny, M. V, Wu, G. S., Omura, S. & el-Deiry, W. S. Proteasome-dependent regulation of p21WAF1/CIP1 expression. *Biochem. Biophys. Res. Commun.* **227,** 564–9 (1996).

366. Cayrol, C. & Ducommun, B. Interaction with cyclin-dependent kinases and PCNA modulates proteasome-dependent degradation of p21. *Oncogene* **17,** 2437–44 (1998).

367. Maki, C. G. & Howley, P. M. Ubiquitination of p53 and p21 is differentially affected by ionizing and UV radiation. *Mol. Cell. Biol.* **17,** 355–63 (1997).

368. Bendjennat, M. *et al.* UV irradiation triggers ubiquitin-dependent degradation of p21(WAF1) to promote DNA repair. *Cell* **114,** 599–610 (2003).

369. Sheaff, R. J. *et al.* Proteasomal turnover of p21Cip1 does not require p21Cip1 ubiquitination. *Mol. Cell* **5,** 403–10 (2000).

370. Touitou, R. *et al.* A degradation signal located in the C-terminus of p21WAF1/CIP1 is a binding site for the C8 alpha-subunit of the 20S proteasome. *EMBO J.* **20,** 2367–75 (2001).

371. Bloom, J., Amador, V., Bartolini, F., DeMartino, G. & Pagano, M. Proteasome-mediated degradation of p21 via N-terminal ubiquitinylation. *Cell* **115,** 71–82 (2003).

372. Coleman, M. L., Marshall, C. J. & Olson, M. F. Ras promotes p21(Waf1/Cip1) protein stability via a cyclin D1-imposed block in proteasome-mediated degradation. *EMBO J.* **22,** 2036–46 (2003).

373. Li, Y., Dowbenko, D. & Lasky, L. A. AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival. *J. Biol. Chem.* **277,** 11352–61 (2002).

374. Coulombe, P., Rodier, G., Bonneil, E., Thibault, P. & Meloche, S. N-Terminal ubiquitination of extracellular signal-regulated kinase 3 and p21 directs their degradation by the proteasome. *Mol. Cell. Biol.* **24,** 6140–50 (2004).

375. Dunn, J. M., Phillips, R. A., Becker, A. J. & Gallie, B. L. Identification of germline and somatic mutations affecting the retinoblastoma gene. *Science* **241,** 1797–800 (1988).

376. Liu, H., Dibling, B., Spike, B., Dirlam, A. & Macleod, K. New roles for the RB tumor suppressor protein. *Curr. Opin. Genet. Dev.* **14,** 55–64 (2004).

377. Kaelin, W. G., Pallas, D. C., DeCaprio, J. A., Kaye, F. J. & Livingston, D. M. Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product. *Cell* **64,** 521–32 (1991).

378. Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M. & Nevins, J. R. The E2F transcription factor is a cellular target for the RB protein. *Cell* **65,** 1053–61 (1991).

379. Bandara, L. R. & La Thangue, N. B. Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. *Nature* **351,** 494–7 (1991).

380. Ezhevsky, S. A. *et al.* Hypo-phosphorylation of the retinoblastoma protein (pRb) by cyclin D:Cdk4/6 complexes results in active pRb. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 10699–704 (1997).

381. Lundberg, A. S. & Weinberg, R. A. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Mol. Cell. Biol.* **18,** 753–61 (1998).

382. Matsushime, H. *et al.* D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.* **14,** 2066–76 (1994).

383. Dyson, N. The regulation of E2F by pRB-family proteins. *Genes Dev.* **12,** 2245–62 (1998).

384. Leone, G. *et al.* E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. *Genes Dev.* **12,** 2120–30 (1998).

385. Attwooll, C., Lazzerini Denchi, E. & Helin, K. The E2F family: specific functions and overlapping interests. *EMBO J.* **23,** 4709–16 (2004).

386. Lammens, T., Li, J., Leone, G. & De Veylder, L. Atypical E2Fs: new players in the E2F transcription factor family. *Trends Cell Biol.* **19,** 111–8 (2009).

387. Blake, M. C. & Azizkhan, J. C. Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. *Mol. Cell. Biol.* **9,** 4994–5002 (1989).

388. Nevins, J. R. Toward an understanding of the functional complexity of the E2F and retinoblastoma families. *Cell Growth Differ.* **9,** 585–93 (1998).

389. Knudsen, K. E., Fribourg, A. F., Strobeck, M. W., Blanchard, J. M. & Knudsen, E. S. Cyclin A is a functional target of retinoblastoma tumor suppressor protein-mediated cell cycle arrest. *J. Biol. Chem.* **274,** 27632–41 (1999).

390. Ohtani, K., DeGregori, J. & Nevins, J. R. Regulation of the cyclin E gene by transcription factor E2F1. *Proc. Natl. Acad. Sci. U. S. A.* **92,** 12146–50 (1995).

391. Krek, W. *et al.* Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. *Cell* **78,** 161–72 (1994).

392. Li, J. *et al.* Synergistic function of E2F7 and E2F8 is essential for cell survival and embryonic development. *Dev. Cell* **14,** 62–75 (2008).

393. Zalmas, L. P. *et al.* DNA-damage response control of E2F7 and E2F8. *EMBO Rep.* **9,** 252–9 (2008).

394. Lin, W. C., Lin, F. T. & Nevins, J. R. Selective induction of E2F1 in response to DNA damage, mediated by ATM-dependent phosphorylation. *Genes Dev.* **15,** 1833–44 (2001).

395. Stevens, C., Smith, L. & La Thangue, N. B. Chk2 activates E2F-1 in response to DNA damage. *Nat. Cell Biol.* **5,** 401–9 (2003).

396. Stevens, C. & La Thangue, N. B. A new role for E2F-1 in checkpoint control. *Cell Cycle* **2,** 435–7

397. Lane, D. P. Cancer. p53, guardian of the genome. *Nature* **358,** 15–6 (1992).

398. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K. & Vogelstein, B. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* **249,** 912–5 (1990).

399. Mercer, W. E. *et al.* Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6166–70 (1990).

400. Michalovitz, D., Halevy, O. & Oren, M. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell* **62,** 671–80 (1990).

401. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88,** 593–602 (1997).

402. Shaw, P. *et al.* Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4495–9 (1992).

403. Yonish-Rouach, E. *et al.* Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature* **352,** 345–347 (1991).

404. Green, D. R. & Kroemer, G. Cytoplasmic functions of the tumour suppressor p53. *Nature* **458,** 1127–30 (2009).

405. Yee, K. S. & Vousden, K. H. Complicating the complexity of p53. *Carcinogenesis* **26,** 1317–22 (2005).

406. Haupt, Y., Maya, R., Kazaz, A. & Oren, M. Mdm2 promotes the rapid degradation of p53. *Nature* **387,** 296–9 (1997).

407. Coutts, A. S., Adams, C. J. & La Thangue, N. B. p53 ubiquitination by Mdm2: a never ending tail? *DNA Repair (Amst).* **8,** 483–90 (2009).

408. Midgley, C. A. & Lane, D. P. p53 protein stability in tumour cells is not determined by mutation but is dependent on Mdm2 binding. *Oncogene* **15,** 1179–89 (1997).

409. Appella, E. & Anderson, C. W. Post-translational modifications and activation of p53 by genotoxic stresses. *Eur. J. Biochem.* **268,** 2764–72 (2001).

410. Kamijo, T. *et al.* Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 8292–7 (1998).

411. Zhang, Y., Xiong, Y. & Yarbrough, W. G. ARF Promotes MDM2 Degradation and Stabilizes p53: ARF-INK4a Locus Deletion Impairs Both the Rb and p53 Tumor Suppression Pathways. *Cell* **92,** 725–734 (1998).

412. Honda, R. & Yasuda, H. Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *EMBO J.* **18,** 22–7 (1999).

413. Llanos, S., Clark, P. A., Rowe, J. & Peters, G. Stabilization of p53 by p14ARF without relocation of MDM2 to the nucleolus. *Nat. Cell Biol.* **3,** 445–52 (2001).

414. el-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W. & Vogelstein, B. Definition of a consensus binding site for p53. *Nat. Genet.* **1,** 45–9 (1992).

415. Hainaut, P. & Hollstein, M. p53 and human cancer: the first ten thousand mutations. *Adv. Cancer Res.* **77,** 81–137 (2000).

416. Vogelstein, B. & Kinzler, K. W. p53 function and dysfunction. *Cell* **70,** 523–6 (1992).

417. McLure, K. G. & Lee, P. W. How p53 binds DNA as a tetramer. *EMBO J.* **17,** 3342–50 (1998).

418. Halazonetis, T. D. & Kandil, A. N. Conformational shifts propagate from the oligomerization domain of p53 to its tetrameric DNA binding domain and restore DNA binding to select p53 mutants. *EMBO J.* **12,** 5057–64 (1993).

419. Vogelstein, B., Lane, D. & Levine, A. J. Surfing the p53 network. *Nature* **408,** 307–10 (2000).

420. Maya, R. *et al.* ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. *Genes Dev.* **15,** 1067–77 (2001).

421. Chehab, N. H., Malikzay, A., Stavridi, E. S. & Halazonetis, T. D. Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 13777–82 (1999).

422. Brown, J. P., Wei, W. & Sedivy, J. M. Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts. *Science* **277,** 831–4 (1997).

423. Hermeking, H. *et al.* 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol. Cell* **1,** 3–11 (1997).

424. Miyashita, T. & Reed, J. C. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80,** 293–9 (1995).

425. Nakano, K. & Vousden, K. H. PUMA, a novel proapoptotic gene, is induced by p53. *Mol. Cell* **7,** 683–94 (2001).

426. Oda, E. *et al.* Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288,** 1053–8 (2000).

427. Chène, P. Inhibiting the p53-MDM2 interaction: an important target for cancer therapy. *Nat. Rev. Cancer* **3,** 102–9 (2003).

428. Garrison, S. P. *et al.* Selection against PUMA gene expression in Myc-driven B-cell lymphomagenesis. *Mol. Cell. Biol.* **28,** 5391–402 (2008).

429. Hemann, M. T. *et al.* Evasion of the p53 tumour surveillance network by tumour-derived MYC mutants. *Nature* **436,** 807–11 (2005).

430. Michalak, E. M. *et al.* Puma and to a lesser extent Noxa are suppressors of Myc-induced lymphomagenesis. *Cell Death Differ.* **16,** 684–96 (2009).

431. Pagano, J. S. Epstein-Barr virus: the first human tumor virus and its role in cancer. *Proc. Assoc. Am. Physicians* **111,** 573–80

432. Scheffner, M., Takahashi, T., Huibregtse, J. M., Minna, J. D. & Howley, P. M. Interaction of the human papillomavirus type 16 E6 oncoprotein with wild-type and mutant human p53 proteins. *J. Virol.* **66,** 5100–5 (1992).

433. Yew, P. R. & Berk, A. J. Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. *Nature* **357,** 82–5 (1992).

434. Dyson, N., Howley, P. M., Münger, K. & Harlow, E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243,** 934–7 (1989).

435. Whyte, P. *et al.* Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature* **334,** 124–9 (1988).

436. Friborg, J., Kong, W., Hottiger, M. O. & Nabel, G. J. p53 inhibition by the LANA protein of KSHV protects against cell death. *Nature* **402,** 889–94

437. Radkov, S. A., Kellam, P. & Boshoff, C. The latent nuclear antigen of Kaposi sarcoma-associated herpesvirus targets the retinoblastoma-E2F pathway and with the oncogene Hras transforms primary rat cells. *Nat. Med.* **6,** 1121–7 (2000).

438. Chang, Y. *et al.* Cyclin encoded by KS herpesvirus. *Nature* **382,** 410 (1996).

439. Saha, A. *et al.* Epstein-Barr virus nuclear antigen 3C augments Mdm2-mediated p53 ubiquitination and degradation by deubiquitinating Mdm2. *J. Virol.* **83,** 4652–69 (2009).

440. Yi, F. *et al.* Epstein-Barr virus nuclear antigen 3C targets p53 and modulates its transcriptional and apoptotic activities. *Virology* **388,** 236–47 (2009).

441. Buendia, B., Santa-Maria, A. & Courvalin, J. C. Caspase-dependent proteolysis of integral and peripheral proteins of nuclear membranes and nuclear pore complex proteins during apoptosis. *J. Cell Sci.* **112 (Pt 1,** 1743–53 (1999).

442. Oberhammer, F. A., Hochegger, K., Fröschl, G., Tiefenbacher, R. & Pavelka, M. Chromatin condensation during apoptosis is accompanied by degradation of lamin A+B, without enhanced activation of cdc2 kinase. *J. Cell Biol.* **126,** 827–37 (1994).

443. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G. & Earnshaw, W. C. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371,** 346–7 (1994).

444. Xiong, Y. *et al.* p21 is a universal inhibitor of cyclin kinases. *Nature* **366,** 701–4 (1993).

445. Harper, J. W. *et al.* Inhibition of cyclin-dependent kinases by p21. *Mol. Biol. Cell* **6,** 387–400 (1995).

446. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J. & Leder, P. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* **82,** 675–84 (1995).

447. Cayrol, C. & Flemington, E. K. The Epstein-Barr virus bZIP transcription factor Zta causes G0/G1 cell cycle arrest through induction of cyclin-dependent kinase inhibitors. *EMBO J.* **15,** 2748–59 (1996).

448. Jepsen, J. S., Pfundheller, H. M. & Lykkesfeldt, A. E. Downregulation of p21(WAF1/CIP1) and estrogen receptor alpha in MCF-7 cells by antisense oligonucleotides containing locked nucleic acid (LNA). *Oligonucleotides* **14,** 147–56 (2004).

449. Henderson, S. *et al.* Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. *Cell* **65,** 1107–15 (1991).

450. Maruo, S. *et al.* Epstein-Barr virus nuclear protein EBNA3C residues critical for maintaining lymphoblastoid cell growth. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 4419–24 (2009).

451. Zhao, B. & Sample, C. E. Epstein-barr virus nuclear antigen 3C activates the latent membrane protein 1 promoter in the presence of Epstein-Barr virus nuclear antigen 2 through sequences encompassing an spi-1/Spi-B binding site. *J. Virol.* **74,** 5151–60 (2000).

452. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–5 (1970).

453. Kieff, E. & Rickinson, A. in *Knipe D and Howley P (Ed.), Fields Virology* 2626 (Lippincott Williams & Wilkins, 2006).

454. Magrath, I. T. African Burkitt's lymphoma. History, biology, clinical features, and treatment. *Am. J. Pediatr. Hematol. Oncol.* **13,** 222–46 (1991).

455. Rowe, M., Kelly, G. L., Bell, A. I. & Rickinson, A. B. Burkitt's lymphoma: the Rosetta Stone deciphering Epstein-Barr virus biology. *Semin. Cancer Biol.* **19,** 377–88 (2009).

456. Hammond, S. M. MicroRNAs as oncogenes. *Curr. Opin. Genet. Dev.* **16,** 4–9 (2006).

457. Kluiver, J. *et al.* BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. *J. Pathol.* **207,** 243–9 (2005).

458. Yanaihara, N. *et al.* Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* **9,** 189–98 (2006).

459. Iorio, M. V *et al.* MicroRNA gene expression deregulation in human breast cancer. *Cancer Res.* **65,** 7065–70 (2005).

460. Eis, P. S. *et al.* Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 3627–32 (2005).

461. Jiang, D. & Aguiar, R. C. T. MicroRNA-155 controls RB phosphorylation in normal and malignant B lymphocytes via the noncanonical TGF-β1/SMAD5 signaling module. *Blood* **123,** 86–93 (2014).

462. Kluiver, J. *et al.* Regulation of pri-microRNA BIC transcription and processing in Burkitt lymphoma. *Oncogene* **26,** 3769–76 (2007).

463. Zhang, T., Nie, K. & Tam, W. BIC is processed efficiently to microRNA-155 in Burkitt lymphoma cells. *Leukemia* **22,** 1795–7 (2008).

464. Wang, F. *et al.* Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. *J. Virol.* **64,** 2309–18 (1990).

465. Rodriguez, A. *et al.* Requirement of bic/microRNA-155 for normal immune function. *Science* **316,** 608–11 (2007).

466. Mann, K. P., Staunton, D. & Thorley-Lawson, D. A. Epstein-Barr virus-encoded protein found in plasma membranes of transformed cells. *J. Virol.* **55,** 710–20 (1985).

467. Young, L. *et al.* Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. *N. Engl. J. Med.* **321,** 1080–5 (1989).

468. Zindy, F. *et al.* Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev.* **12,** 2424–33 (1998).

469. Pomerantz, J. *et al.* The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* **92,** 713–23 (1998).

470. Stott, F. J. *et al.* The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. *EMBO J.* **17,** 5001–14 (1998).

471. Jacobsen, K. A., Prasad, V. S., Sidman, C. L. & Osmond, D. G. Apoptosis and macrophage-mediated deletion of precursor B cells in the bone marrow of E mu-myc transgenic mice. *Blood* **84,** 2784–94 (1994).

472. Strasser, A., Harris, A. W., Bath, M. L. & Cory, S. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. *Nature* **348,** 331–3 (1990).

473. Kelly, G. L., Milner, A. E., Baldwin, G. S., Bell, A. I. & Rickinson, A. B. Three restricted forms of Epstein-Barr virus latency counteracting apoptosis in c-myc-expressing Burkitt lymphoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 14935–40 (2006).

474. el-Deiry, W. S. *et al.* WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res.* **54,** 1169–74 (1994).

475. Gartel, A. L. & Radhakrishnan, S. K. Lost in transcription: p21 repression, mechanisms, and consequences. *Cancer Res.* **65,** 3980–3985 (2005).

476. Clybouw, C. *et al.* EBV infection of human B lymphocytes leads to down-regulation of Bim expression: relationship to resistance to apoptosis. *J. Immunol.* **175,** 2968–73 (2005).

477. Kaija, H. M. *et al.* Stress-specific responses of p21 expression: Implication of transcript variant p21 alt-a in long-term hypoxia. *J. Cell. Biochem.* **113,** 544–552 (2012).

478. Gartel, A. L. & Tyner, A. L. Transcriptional regulation of the p21((WAF1/CIP1)) gene. *Exp. Cell Res.* **246,** 280–9 (1999).

479. Pouliot, F. & Labrie, C. Role of Smad1 and Smad4 proteins in the induction of p21WAF1,Cip1 during bone morphogenetic protein-induced growth arrest in human breast cancer cells. *J. Endocrinol.* **172,** 187–98 (2002).

480. Beckerman, R. *et al.* A role for Chk1 in blocking transcriptional elongation of p21 RNA during the S-phase checkpoint. *Genes Dev.* **23,** 1364–77 (2009).

481. Gartel, A. L., Serfas, M. S. & Tyner, A. L. p21--negative regulator of the cell cycle. *Proc. Soc. Exp. Biol. Med.* **213,** 138–49 (1996).

482. Zambetti, G. P., Bargonetti, J., Walker, K., Prives, C. & Levine, A. J. Wild-type p53 mediates positive regulation of gene expression through a specific DNA sequence element. *Genes Dev.* **6,** 1143–52 (1992).

483. Happo, L. *et al.* Maximal killing of lymphoma cells by DNA damage-inducing therapy requires not only the p53 targets Puma and Noxa, but also Bim. *Blood* **116,** 5256–67 (2010).

484. Artavanis-Tsakonas, S., Rand, M. D. & Lake, R. J. Notch signaling: cell fate control and signal integration in development. *Science* **284,** 770–6 (1999).

485. Hickabottom, M., Parker, G. A., Freemont, P., Crook, T. & Allday, M. J. Two nonconsensus sites in the Epstein-Barr virus oncoprotein EBNA3A cooperate to bind the co-repressor carboxyl-terminal-binding protein (CtBP). *J. Biol. Chem.* **277,** 47197–204 (2002).

486. Kubbutat, M. H., Jones, S. N. & Vousden, K. H. Regulation of p53 stability by Mdm2. *Nature* **387,** 299–303 (1997).

487. Suzuki, A., Tsutomi, Y., Akahane, K., Araki, T. & Miura, M. Resistance to Fas-mediated apoptosis: activation of caspase 3 is regulated by cell cycle regulator p21WAF1 and IAP gene family ILP. *Oncogene* **17,** 931–9 (1998).

488. Dawson, C. W., Tramountanis, G., Eliopoulos, A. G. & Young, L. S. Epstein-Barr virus latent membrane protein 1 (LMP1) activates the phosphatidylinositol 3-kinase/Akt pathway to promote cell survival and induce actin filament remodeling. *J. Biol. Chem.* **278,** 3694–3704 (2003).

489. Gartel, A. L., Radhakrishnan, S. K., Serfas, M. S., Kwon, Y. H. & Tyner, A. L. A novel p21WAF1/CIP1 transcript is highly dependent on p53 for its basal expression in mouse tissues. *Oncogene* **23,** 8154–7 (2004).

490. Claus, R. & Lübbert, M. Epigenetic targets in hematopoietic malignancies. *Oncogene* **22,** 6489–96 (2003).

491. Prives, C. Signaling to p53: breaking the MDM2-p53 circuit. *Cell* **95,** 5–8 (1998).

492. Fiscella, M. *et al.* Mutation of the serine 15 phosphorylation site of human p53 reduces the ability of p53 to inhibit cell cycle progression. *Oncogene* **8,** 1519–28 (1993).

493. Pise-Masison, C. A., Radonovich, M., Sakaguchi, K., Appella, E. & Brady, J. N. Phosphorylation of p53: a novel pathway for p53 inactivation in human T-cell lymphotropic virus type 1-transformed cells. *J. Virol.* **72,** 6348–55 (1998).

494. Shieh, S. Y., Ikeda, M., Taya, Y. & Prives, C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* **91,** 325–34 (1997).

495. Hao, M. *et al.* Mutation of phosphoserine 389 affects p53 function in vivo. *J. Biol. Chem.* **271,** 29380–5 (1996).

496. Ashcroft, M., Kubbutat, M. H. & Vousden, K. H. Regulation of p53 function and stability by phosphorylation. *Mol. Cell. Biol.* **19,** 1751–1758 (1999).

497. Knappskog, S. *et al.* Mutations and polymorphisms of the p21B transcript in breast cancer. *Int. J. Cancer* **121,** 908–10 (2007).

498. Huang, S., Lee, W. H. & Lee, E. Y. A cellular protein that competes with SV40 T antigen for binding to the retinoblastoma gene product. *Nature* **350,** 160–2 (1991).

499. Lane, D. P. & Crawford, L. V. T antigen is bound to a host protein in SV40-transformed cells. *Nature* **278,** 261–3 (1979).

500. Linzer, D. I. & Levine, A. J. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* **17,** 43–52 (1979).

501. Boyer, S. N., Wazer, D. E. & Band, V. E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. *Cancer Res.* **56,** 4620–4 (1996).

502. Scheffner, M., Huibregtse, J. M., Vierstra, R. D. & Howley, P. M. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* **75,** 495–505 (1993).

503. Szekely, L., Selivanova, G., Magnusson, K. P., Klein, G. & Wiman, K. G. EBNA-5, an Epstein-Barr virus-encoded nuclear antigen, binds to the retinoblastoma and p53 proteins. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 5455–9 (1993).

504. Inman, G. J. & Farrell, P. J. Epstein-Barr virus EBNA-LP and transcription regulation properties of pRB, p107 and p53 in transfection assays. *J. Gen. Virol.* **76 (Pt 9),** 2141–9 (1995).

505. Cesarman, E. *et al.* Kaposi's sarcoma-associated herpesvirus contains G protein-coupled receptor and cyclin D homologs which are expressed in Kaposi's sarcoma and malignant lymphoma. *J. Virol.* **70,** 8218–23 (1996).

506. Godden-Kent, D. *et al.* The cyclin encoded by Kaposi's sarcoma-associated herpesvirus stimulates cdk6 to phosphorylate the retinoblastoma protein and histone H1. *J. Virol.* **71,** 4193–8 (1997).

507. Song, X. *et al.* Epstein-Barr virus-encoded latent membrane protein 1 modulates cyclin D1 by c-Jun/Jun B heterodimers. *Sci. China. C. Life Sci.* **48,** 385–93 (2005).

508. Sinclair, A. J., Palmero, I., Holder, A., Peters, G. & Farrell, P. J. Expression of cyclin D2 in Epstein-Barr virus-positive Burkitt's lymphoma cell lines is related to methylation status of the gene. *J. Virol.* **69,** 1292–5 (1995).

509. Sherr, C. J. & Roberts, J. M. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* **13,** 1501–1512 (1999).

510. Broude, E. V *et al.* p21(Waf1/Cip1/Sdi1) mediates retinoblastoma protein degradation. *Oncogene* **26,** 6954–8 (2007).

511. Jänicke, R. U., Walker, P. A., Lin, X. Y. & Porter, A. G. Specific cleavage of the retinoblastoma protein by an ICE-like protease in apoptosis. *EMBO J.* **15,** 6969–78 (1996).

512. Bunz, F. *et al.* Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* **282,** 1497–501 (1998).

513. Cao, S., Liu, J., Song, L. & Ma, X. The protooncogene c-Maf is an essential transcription factor for IL-10 gene expression in macrophages. *J. Immunol.* **174,** 3484–92 (2005).

514. Jayadev, S. *et al.* Transcription factor p53 influences microglial activation phenotype. *Glia* **59,** 1402–13 (2011).

515. Su, W. *et al.* The p53 transcription factor modulates microglia behavior through microRNA-dependent regulation of c-Maf. *J. Immunol.* **192,** 358–66 (2014).

516. Suzuki, H. I. *et al.* Modulation of microRNA processing by p53. *Nature* **460,** 529–33 (2009).

517. Bouamar, H. *et al.* MicroRNA 155 control of p53 activity is context dependent and mediated by Aicda and Socs1. *Mol. Cell. Biol.* **35,** 1329–40 (2015).

518. Huang, X. *et al.* Quantitative proteomics reveals that miR-155 regulates the PI3K-AKT pathway in diffuse large B-cell lymphoma. *Am. J. Pathol.* **181,** 26–33 (2012).

519. Yamanaka, Y. *et al.* Aberrant overexpression of microRNAs activate AKT signaling via down-regulation of tumor suppressors in natural killer-cell lymphoma/leukemia. *Blood* **114,** 3265–75 (2009).

520. White, R. E. *et al.* Extensive co-operation between the Epstein-Barr virus EBNA3 proteins in the manipulation of host gene expression and epigenetic chromatin modification. *PLoS One* **5,** e13979 (2010).

521. Robertson, E. S. *et al.* Epstein-Barr virus nuclear protein 3C modulates transcription through interaction with the sequence-specific DNA-binding protein J kappa. *J. Virol.* **69,** 3108–16 (1995).

522. Cotter, M. A. & Robertson, E. S. Modulation of histone acetyltransferase activity through interaction of epstein-barr nuclear antigen 3C with prothymosin alpha. *Mol. Cell. Biol.* **20,** 5722–35 (2000).

523. Knight, J. S., Lan, K., Subramanian, C. & Robertson, E. S. Epstein-Barr virus nuclear antigen 3C recruits histone deacetylase activity and associates with the corepressors mSin3A and NCoR in human B-cell lines. *J. Virol.* **77,** 4261–72 (2003).

524. McClellan, M. J. *et al.* Modulation of enhancer looping and differential gene targeting by Epstein-Barr virus transcription factors directs cellular reprogramming. *PLoS Pathog.* **9,** e1003636 (2013).

525. Bazot, Q. *et al.* Epstein-Barr Virus Proteins EBNA3A and EBNA3C Together Induce Expression of the Oncogenic MicroRNA Cluster miR-221/miR-222 and Ablate Expression of Its Target p57KIP2. *PLoS Pathog.* **11,** e1005031 (2015).

526. Boccellato, F. *et al.* EBNA2 interferes with the germinal center phenotype by downregulating BCL6 and TCL1 in non-Hodgkin's lymphoma cells. *J. Virol.* **81,** 2274–82 (2007).

527. Martín-Pérez, D. *et al.* Epstein-Barr virus microRNAs repress BCL6 expression in diffuse large B-cell lymphoma. *Leukemia* **26,** 180–3 (2012).

528. Basso, K. *et al.* BCL6 positively regulates AID and germinal center gene expression via repression of miR-155. *J. Exp. Med.* **209,** 2455–65 (2012).

529. Perez-Rosado, A. *et al.* BCL6 represses NFkappaB activity in diffuse large B-cell lymphomas. *J. Pathol.* **214,** 498–507 (2008).

530. Michael, M. Z., O' Connor, S. M., van Holst Pellekaan, N. G., Young, G. P. & James, R. J. Reduced accumulation of specific microRNAs in colorectal neoplasia. *Mol. Cancer Res.* **1,** 882–91 (2003).

531. Akao, Y., Nakagawa, Y., Kitade, Y., Kinoshita, T. & Naoe, T. Downregulation of microRNAs-143 and -145 in B-cell malignancies. *Cancer Sci.* **98,** 1914–20 (2007).

532. Scotto, L. *et al.* Integrative genomics analysis of chromosome 5p gain in cervical cancer reveals target over-expressed genes, including Drosha. *Mol. Cancer* **7,** 58 (2008).

533. Karube, Y. *et al.* Reduced expression of Dicer associated with poor prognosis in lung cancer patients. *Cancer Sci.* **96,** 111–5 (2005).

534. Newman, M. A., Thomson, J. M. & Hammond, S. M. Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing. *RNA* **14,** 1539–49 (2008).

535. Viswanathan, S. R., Daley, G. Q. & Gregory, R. I. Selective blockade of microRNA processing by Lin28. *Science* **320,** 97–100 (2008).

536. Piskounova, E. *et al.* Determinants of microRNA processing inhibition by the developmentally regulated RNA-binding protein Lin28. *J. Biol. Chem.* **283,** 21310–4 (2008).

537. Guil, S. & Cáceres, J. F. The multifunctional RNA-binding protein hnRNP A1 is required for processing of miR-18a. *Nat. Struct. Mol. Biol.* **14,** 591–6 (2007).

538. Davis, B. N., Hilyard, A. C., Lagna, G. & Hata, A. SMAD proteins control DROSHA-mediated microRNA maturation. *Nature* **454,** 56–61 (2008).

539. Rocha, S., Martin, A. M., Meek, D. W. & Perkins, N. D. p53 represses cyclin D1 transcription through down regulation of Bcl-3 and inducing increased association of the p52 NF-kappaB subunit with histone deacetylase 1. *Mol. Cell. Biol.* **23,** 4713–27 (2003).

540. Prives, C. & Hall, P. A. The p53 pathway. *J. Pathol.* **187,** 112–26 (1999).

541. Vousden, K. H. & Prives, C. Blinded by the Light: The Growing Complexity of p53. *Cell* **137,** 413–31 (2009).

542. Beckerman, R. & Prives, C. Transcriptional regulation by p53. *Cold Spring Harb. Perspect. Biol.* **2,** a000935 (2010).

543. Laptenko, O. & Prives, C. Transcriptional regulation by p53: one protein, many possibilities. *Cell Death Differ.* **13,** 951–61 (2006).

544. Samuels-Lev, Y. *et al.* ASPP proteins specifically stimulate the apoptotic function of p53. *Mol. Cell* **8,** 781–94 (2001).

545. Das, S. *et al.* Hzf Determines cell survival upon genotoxic stress by modulating p53 transactivation. *Cell* **130,** 624–37 (2007).

546. Tanaka, T., Ohkubo, S., Tatsuno, I. & Prives, C. hCAS/CSE1L associates with chromatin and regulates expression of select p53 target genes. *Cell* **130,** 638–50 (2007).

547. Donner, A. J., Szostek, S., Hoover, J. M. & Espinosa, J. M. CDK8 is a stimulus-specific positive coregulator of p53 target genes. *Mol. Cell* **27,** 121–33 (2007).

548. Saha, A., Bamidele, A., Murakami, M. & Robertson, E. S. EBNA3C attenuates the function of p53 through interaction with inhibitor of growth family proteins 4 and 5. *J. Virol.* **85,** 2079–88 (2011).

549. Jha, H. C., Yang, K., El-Naccache, D. W., Sun, Z. & Robertson, E. S. EBNA3C regulates p53 through induction of Aurora kinase B. *Oncotarget* **6,** 5788–803 (2015).

550. O'Nions, J. & Allday, M. J. Epstein-Barr virus can inhibit genotoxin-induced G1 arrest downstream of p53 by preventing the inactivation of CDK2. *Oncogene* **22,** 7181–91 (2003).

551. Bartel, F., Taubert, H. & Harris, L. C. Alternative and aberrant splicing of MDM2 mRNA in human cancer. *Cancer Cell* **2,** 9–15 (2002).

552. Cordon-Cardo, C. *et al.* Molecular Abnormalities of mdm2 and p53 Genes in Adult Soft Tissue Sarcomas. *Cancer Res.* **54,** 794–799 (1994).

553. Xiao, Z. X. *et al.* Interaction between the retinoblastoma protein and the oncoprotein MDM2. *Nature* **375,** 694–8 (1995).

554. Barak, Y., Gottlieb, E., Juven-Gershon, T. & Oren, M. Regulation of mdm2 expression by p53: alternative promoters produce transcripts with nonidentical translation potential. *Genes Dev.* **8,** 1739–49 (1994).

555. Zauberman, A., Flusberg, D., Haupt, Y., Barak, Y. & Oren, M. A functional p53-responsive intronic promoter is contained within the human mdm2 gene. *Nucleic Acids Res.* **23,** 2584–92 (1995).

556. Landers, J. E., Cassel, S. L. & George, D. L. Translational enhancement of mdm2 oncogene expression in human tumor cells containing a stabilized wild-type p53 protein. *Cancer Res.* **57,** 3562–8 (1997).

557. Wu, X., Bayle, J. H., Olson, D. & Levine, A. J. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev.* **7,** 1126–32 (1993).

558. Zauberman, A., Barak, Y., Ragimov, N., Levy, N. & Oren, M. Sequence-specific DNA binding by p53: identification of target sites and lack of binding to p53 - MDM2 complexes. *EMBO J.* **12,** 2799–808 (1993).

559. Ogawara, Y. *et al.* Akt enhances Mdm2-mediated ubiquitination and degradation of p53. *J. Biol. Chem.* **277,** 21843–50 (2002).

560. Feng, J. *et al.* Stabilization of Mdm2 via decreased ubiquitination is mediated by protein kinase B/Akt-dependent phosphorylation. *J. Biol. Chem.* **279,** 35510–7 (2004).

561. Mayo, L. D. & Donner, D. B. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 11598–603 (2001).

562. Li, M., Brooks, C. L., Kon, N. & Gu, W. A dynamic role of HAUSP in the p53-Mdm2 pathway. *Mol. Cell* **13,** 879–86 (2004).

563. Li, M. *et al.* Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* **416,** 648–53 (2002).

564. Meulmeester, E. *et al.* Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2. *Mol. Cell* **18,** 565–76 (2005).

565. Saridakis, V. *et al.* Structure of the p53 binding domain of HAUSP/USP7 bound to Epstein-Barr nuclear antigen 1 implications for EBV-mediated immortalization. *Mol. Cell* **18,** 25–36 (2005).

566. Holowaty, M. N. *et al.* Protein profiling with Epstein-Barr nuclear antigen-1 reveals an interaction with the herpesvirus-associated ubiquitin-specific protease HAUSP/USP7. *J. Biol. Chem.* **278,** 29987–94 (2003).

567. Gerdes, J., Schwab, U., Lemke, H. & Stein, H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int. J. Cancer* **31,** 13–20 (1983).

568. Gerdes, J. *et al.* Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J. Immunol.* **133,** 1710–5 (1984).

569. Van Oijen, M. G., Medema, R. H., Slootweg, P. J. & Rijksen, G. Positivity of the proliferation marker Ki-67 in noncycling cells. *Am. J. Clin. Pathol.* **110,** 24–31 (1998).

570. Kim, G.-Y. *et al.* The stress-activated protein kinases p38 alpha and JNK1 stabilize p21(Cip1) by phosphorylation. *J. Biol. Chem.* **277,** 29792–802 (2002).

571. Jin, Y., Lee, H., Zeng, S. X., Dai, M.-S. & Lu, H. MDM2 promotes p21waf1/cip1 proteasomal turnover independently of ubiquitylation. *EMBO J.* **22,** 6365–77 (2003).

572. Massagué, J., Blain, S. W. & Lo, R. S. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* **103,** 295–309 (2000).

573. Letterio, J. J. & Roberts, A. B. Regulation of immune responses by TGF-beta. *Annu. Rev. Immunol.* **16,** 137–61 (1998).

574. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F. & Massagué, J. Mechanism of activation of the TGF-beta receptor. *Nature* **370,** 341–7 (1994).

575. Gold, L. I. The role for transforming growth factor-beta (TGF-beta) in human cancer. *Crit. Rev. Oncog.* **10,** 303–60 (1999).

576. Zhang, Y., Feng, X., We, R. & Derynck, R. Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. *Nature* **383,** 168–72 (1996).

577. Derynck, R. & Zhang, Y. E. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425,** 577–84 (2003).

578. Fukuda, M., Kurosaki, H. & Sairenji, T. Loss of functional transforming growth factor (TGF)-beta type II receptor results in insensitivity to TGF-beta1-mediated apoptosis and Epstein-Barr virus reactivation. *J. Med. Virol.* **78,** 1456–64 (2006).

579. Prokova, V., Mosialos, G. & Kardassis, D. Inhibition of transforming growth factor beta signaling and Smad-dependent activation of transcription by the Latent Membrane Protein 1 of Epstein-Barr virus. *J. Biol. Chem.* **277,** 9342–50 (2002).

580. Fukuda, M., Kurosaki, W., Yanagihara, K., Kuratsune, H. & Sairenji, T. A Mechanism in Epstein–Barr Virus Oncogenesis: Inhibition of Transforming Growth Factor-β1-mediated Induction of MAPK/p21 by LMP1. *Virology* **302,** 310–320 (2002).

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SELECT PUBLICATIONS

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Bowman ER, Sample CE. Epstein-Barr virus nuclear antigen 3A represses p53-mediated expression of the cyclin-dependent kinase inhibitor p21WAF1/CIP1. *Manuscript in preparation.*

Tursiella ML, **Bowman ER**, Wanzeck KC, Throm RE, Liao J, Zhu J, Sample CE. Esptein-Barr virus antigen 3A promotes cellular proliferation by repression of the cyclin-dependent kinase inhibitor p21WAF1/CIP1. PLoS Pathog. 2014 Oct 2;10(10):e1004415.

Wang Q, Miller DJ, **Bowman ER**, Nagarkar NR, Schneider D, Zhao Y, Linn MJ, Goldsmith AM, Bentley JK, Sajjan US, Hershenson MB. TLR3 and MDA5 signaling initiate pro-inflammatory signaling pathways leading to rhinovirus-induced airways inflammation and hyperresponsiveness. PLoS Pathog. 2011 May;7(5):e1002070.

Nagarkar DR, **Bowman ER**, Schneider D, Wang Q, Shim J, Zhao Y, Linn MJ, McHenry CL, Gosangi B, Bentley JK, Tsai WC, Sajjan US, Lukacs NW and Hershenson MB. Rhinovirus infection of allergen-sensitized and -challenged mice induces eotaxin release from functionally polarized macrophages. J Immunol. 2010 Aug 15;185(4):2525-35.

SELECT ABSTRACTS

Bowman ER, Sample CE. Epstein-Barr virus EBNA3A represses transcription of the cell cycle inhibitor $p21^{WAF1/CIP1}$. Oral presentation, Herpesviruses: Pathogenesis and Cancer Symposium. Philadelphia, PA, June 2, 2015.

Bowman ER, Tursiella ML, Sample CE. Epstein-Barr virus EBNA-3A promotes cellular proliferation by repressing p53-mediated expression of p21WAF1/CIP1. Oral presentation, Pennsylvania State University College of Medicine Graduate Student Research Forum. Hershey, PA, March 2014.

Bowman ER, Schneider D, Nagarkar DR, Hershenson MB. Th2 cytokines alter the response of lung macrophages to RV infection. Am J Respir Crit Care Med 181;2010:A4227. Thematic poster session, American Thoracic Society International Conference. New Orleans, LA, May 15-20, 2010.