**Establishment of Oncogene-Induced Senescence by the Host**

**DNA Damage Response After EBV Infection**

by

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Thomas Petes

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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# Abstract

Epstein-Barr virus (EBV) is an oncogenic gamma-herpesvirus that infects over 90% of adults worldwide. Typically, EBV establishes a benign latent infection that is controlled by a strong cytotoxic T cell immune response. However, EBV infection in immunocompromised patients has been associated with development of several lymphoid and epithelial cell malignancies, including Burkitt's lymphoma, Hodgkin's lymphoma, post-transplant lymphoproliferative disease, and nasopharyngeal carcinoma. In primary human B cells, EBV infection has been shown to induce a transient period of hyper-proliferation, but many of these infected cells succumb to a DNA damage response (DDR)-mediated growth arrest. We hypothesize that EBV infection triggers replicative stress early after infection and facilitates persistent activation of the DDR establishing oncogene-induced senescence. To test this hypothesis, we infected primary human B cells with EBV and examined cellular proliferation and host DNA damage response pathways at early and late stages post infection. We found that early after EBV infection, rapidly proliferating B cells exhibited signs of replication stress and reduced levels of purine dNTP nucleotide pools that are necessary for sustained proliferation. These findings suggest that purine dNTP biosynthesis plays a critical role in the early stages of EBV-mediated B cell immortalization. Furthermore, we observed the formation of persistent DDR foci in arrested B cells and identified key regulators of long-term outgrowth of EBV-infected B cells. Ultimately, this work has shown that early after EBV infection, cells that experience aberrant proliferation establish oncogene-induced senescence by chronically activating the DDR in the context of reduced dNTP nucleotide pools.

# Dedication

To my parents for teaching me the importance of being persistent.

# Contents

# List of Figures

# Acknowledgements

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# 1. Introduction

*Parts of the introduction for this dissertation were adapted from a book chapter, titled "DNA tumor viruses and the host DNA damage response." This book chapter is in press and will be published by Amy Y. Hafez and Micah A. Luftig in DNA Tumour Viruses: Virology, Pathogenesis and Vaccines. Roberts, S (Ed.) Caister Academic Press, UK. (2018).*

## *1.1 Human tumor virus*

While the earliest evidence of human cancer was observed in ancient Egyptian mummies dating back to 3000 BCE, it wasn't until the 20$^{th}$ century that cancer was considered to have an infectious etiology (Javier and Butel 2008). Today it is known that about 20% of all human cancers are caused by infectious agents. More specifically, 12% of human cancers worldwide are causally linked to viruses, known as human tumor viruses (White, Pagano et al. 2014). Seven human tumor viruses have been discovered, including Epstein-Barr virus, hepatitis B virus, human papillomavirus, human T-cell lymphotropic virus, hepatitis C virus, Kaposi's sarcoma herpesvirus, and Merkel cell polyomavirus (White, Pagano et al. 2014). These tumor viruses have been shown to be linked to a number of human malignancies, including lymphoma, cervical cancer, and liver cancer. Human tumor viruses have served an important role in cancer research by not only improving our understanding of how infectious agents cause cancer, but also by serving as a research model for cellular transformation. In this dissertation, I will focus on Epstein-Barr virus (EBV), the first human tumor virus to be discovered (Epstein, Achong et al. 1964), and investigate the mechanisms that suppress EBV-mediated cell transformation.

## *1.2 Epstein-Barr virus*

EBV is an oncogenic gamma herpesvirus with a large DNA genome consisting of approximately 172 kilobases. Over 90% of the adult human population is infected with EBV. The virus typically spreads through saliva, but can also be found in blood or genital secretions. Individuals are usually infected with EBV in the first few years of life; however, in developed countries infection can be delayed until adolescence (Crawford 2001). EBV typically infects human B cells where it establishes a lifelong asymptomatic latent infection. The virus can also infect epithelial cells, where it usually undergoes the lytic cycle allowing the virus to propagate and be transmitted to uninfected hosts. The majority of people infected with EBV remain asymptomatic for life due to protection by a strong cytotoxic T cell immune response that controls the viral infection (Longnecker, Kieff et al. 2013). However, in the context of immune suppression due to HIV infection, malaria exposure, or organ transplant, EBV is causally linked to a number of B cell malignancies, including Burkitt's lymphoma, Hodgkin's lymphoma, post-transplant lymphoproliferative disorders, HIV-associated lymphoproliferative disorders, and NK/T cell lymphomas. EBV has also been associated with epithelial cancers, including EBV-associated gastric cancers and nasopharyngeal carcinoma (NPC) (Kieff and Rickinson 2007). Furthermore, infection as an adolescent can result in infectious mononucleosis.

### 1.2.1 Establishment of latent infection

EBV-associated malignancies are characterized by latent infections in which a limited subset of viral proteins and small non-coding RNAs are expressed. The viral latency proteins include EBV nuclear antigens (EBNA 1, 2, 3A, 3B, 3C and LP), along with the latent membrane proteins (LMP1, 2A and 2B) (Yates, Warren et al. 1985). EBNA-LP and EBNA2 are the first viral latent proteins to be expressed in infected B cells and are

involved in transcriptional up-regulation of many viral and cellular genes. Viral genes, EBNA1, EBNA 3A, 3B, and 3C, along with cellular proto-oncogenes c-Myc, E2F1, and cyclin D2 are among the numerous genes up-regulated upon infection and their expression allows the infected B cells to enter the cell cycle transitioning from the resting to proliferating state (Sinclair, Palmero et al. 1994, Kaiser, Laux et al. 1999). The EBNA3 proteins are responsible for modulating EBNA2-dependent gene expression and can also regulate a number of other targets including repression of key tumor suppressors (Johannsen, Koh et al. 1995, Zhao, Marshall et al. 1996, Skalska, White et al. 2010, Maruo, Zhao et al. 2011, Skalska, White et al. 2013). LMP1, a constitutively active mimic of the TNF receptor and B cell co-stimulatory molecule CD40, is then activated by EBNA2 and EBNA3C (Cahir McFarland, Izumi et al. 1999, Lin, Johannsen et al. 2002). LMP1 activation can then trigger NFκB, p38, and JNK signaling pathways to promote proliferation and survival. LMP2 proteins act as a mimic of the B cell receptor and suppress endogenous B-cell receptor signaling and constitutively activate downstream tyrosine kinases for PI3K signaling and other pro-survival pathways (Miller, Burkhardt et al. 1995). Expression of all viral latent proteins and non-coding RNAs is referred to as the latency III gene expression program. Latency III is established in EBV-associated AIDS lymphomas, post-transplant lymphoproliferative disorder, and *in vitro* production of LCLs (Rickinson and Kieff 2007). Alternatively, Hodgkin's lymphoma and nasopharyngeal carcinoma express a restricted latency II program, expressing EBNA1, LMP1, LMP2, and viral non-coding RNAs, while Burkitt's lymphoma (BL) establishes a latency I program of EBNA1 and non-coding RNAs (Babcock, Hochberg et al. 2000).

### 1.2.2 EBV-mediated transformation of B cells

Establishment of the Latency III program sustains growth, prevents cell death, and facilitates transformation of primary B cells *in vitro* into indefinitely proliferating lymphoblastoid cell lines (LCLs). This makes LCLs a useful model for investigating EBV-induced tumorigenesis. Interestingly, while B cells can be infected *in vitro* at a high efficiency multiplicity of infection where 100% of the cells become EBV positive, only ~1% of primary human B cells become transformed into lymphoblasts (Figure 1) (Henderson, Miller et al. 1977, Sugden and Mark 1977). This low transformation efficiency suggests that innate tumor suppressor mechanisms prevent transformation of EBV-infected B cells; however, these mechanisms are not well understood.

Early work by Martin Allday and Elliott Kieff's laboratories showed that EBV proteins, EBNA3C and 3A, interact with C-terminal binding protein (CtBP) to promote LCL outgrowth by repressing expression of tumor suppressor molecules $p16^{INK4A}$ and $p14^{ARF}$ (Skalska, White et al. 2010, Maruo, Zhao et al. 2011). Furthermore, the Allday laboratory identified that EBV induces $p16^{INK4A}$ accumulation early after EBV infection of primary human B cells. If EBNA3C is deleted, this accumulation goes unchecked and prevents LCL outgrowth (Skalska, White et al. 2013). While repression of $p16^{INK4A}$ and $p14^{ARF}$ expression is involved in outgrowth of LCLs, it remains unclear as to the major barrier to transformation of B cells. To better understand the innate barriers to transformation, our laboratory focuses on investigating innate tumor suppressor mechanisms, such as the human DNA damage response (DDR), which has been implicated as a tumor suppressor pathway *in vitro* and *in vivo* (DiTullio, Mochan et al. 2002, Bartkova, Horejsi et al. 2005, Gorgoulis, Vassiliou et al. 2005).

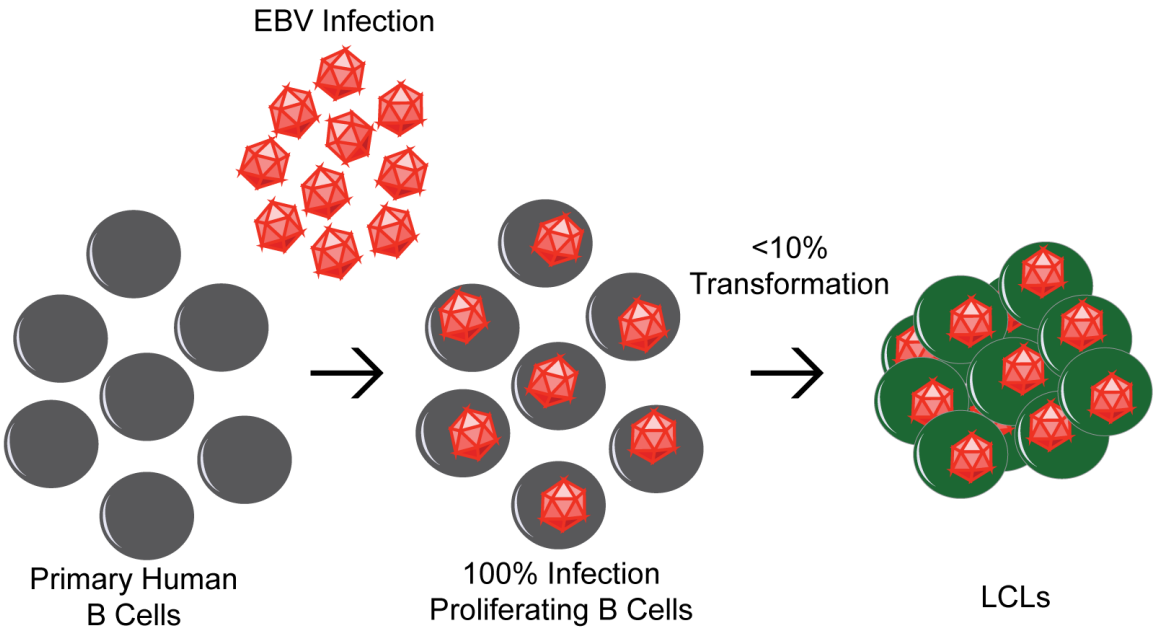


**Figure 1: EBV infection yields low transformation efficiency.** Schematic of EBV-mediated transformation in primary human B cells.

## *1.3 DNA damage response*

As cells grow and divide they experience constant threats to their genetic material. It has been estimated that each cell incurs 10,000 DNA lesions per day in the form of double-strand breaks (DSBs) and single-strand breaks (SSBs) (Lindahl 1993). These lesions occur as a result of exposure to endogenous threats such as reactive oxygen species and exogenous environmental agents, for example ionizing radiation and radiomimetic chemicals that can damage cellular DNA (Figure 2). Furthermore, errors in normal cellular processes such as DNA replication, V(D)J recombination and meiotic recombination may also result in DNA damage and lead to genetic mutations that can be passed down to subsequent generations (Hartlerode and Scully 2009). If these mutations are not dealt with properly they can ultimately give rise to a number of diseases, including cancer. To maintain genetic integrity, cells have developed an integrative DNA damage response pathway that is tasked with sensing damaged DNA, initiating a signaling

cascade to alert necessary effector molecules, and controlling cell cycle checkpoints. Thus, this damage response serves as an important innate tumor suppressor pathway. These DNA damage checkpoints have been found to function in activation of cell cycle arrest, DNA repair, activation of transcriptional programs, telomere length control, and induction of apoptosis (Figure 2) (Clarke, Purdie et al. 1993, Xu and Baltimore 1996, Naito, Matsuura et al. 1998, Cortez, Wang et al. 1999, Martin, Laroche et al. 1999, Mills, Sinclair et al. 1999, Gatei, Young et al. 2000, Lim, Kim et al. 2000, Wu, Ranganathan et al. 2000, Zhao, Weng et al. 2000).

Our understanding of the intricacies of the DNA damage response (DDR) signaling pathway and how viruses interact with this host defense mechanism has greatly increased over the last few decades. Viruses have developed mechanisms to evade recognition by the host DDR, prevent downstream signaling consequences, and, in some cases, use the DDR to facilitate viral replication.

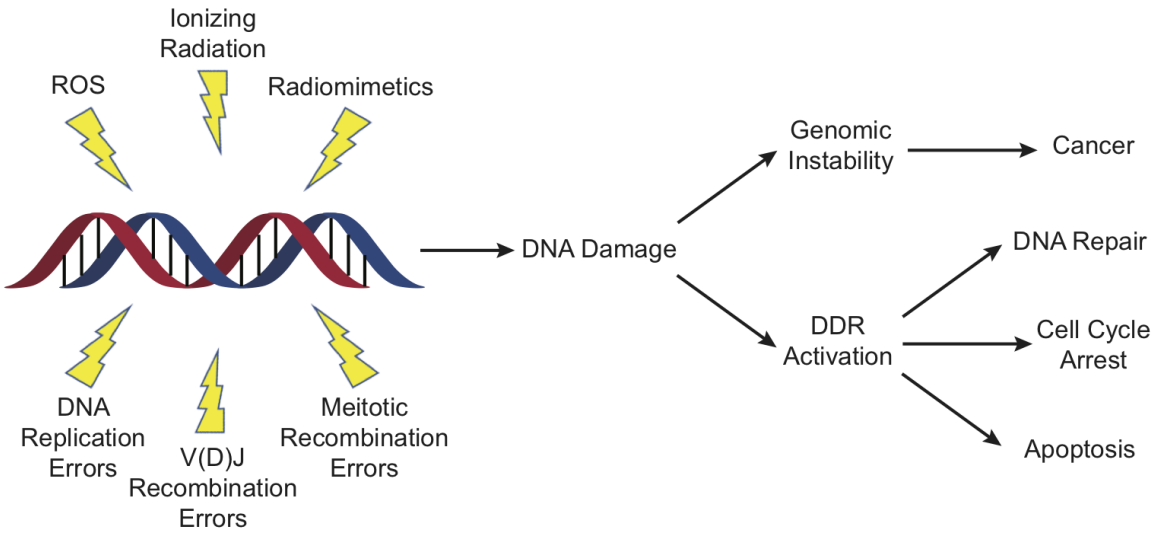


**Figure 2: Sources of DNA damage.** Schematic diagram of potential endogenous and exogenous sources of DNA damage that cause single-stranded DNA breaks and double-strand DNA breaks.

### 1.3.1 Cellular DNA damage response pathways

In humans, initiation of the DDR is controlled by three major phosphoinositide 3-kinase-related protein kinases (PIKKs): ataxia telangiectasia mutated (ATM), ATM-Rad3-related protein (ATR), and DNA-dependent protein kinase (DNA-PK) (Sancar, Lindsey-Boltz et al. 2004, Ciccia and Elledge 2010). ATM is known to primarily respond to DSBs, and has also been implicated in the general response to reactive oxygen species and irradiation (Shiloh 2003, Alexander and Walker 2010, Guo, Deshpande et al. 2010, Guo, Kozlov et al. 2010, Cosentino, Grieco et al. 2011). Like ATM, DNA-PK is also a major kinase involved in DSB recognition primarily caused by genotoxic stresses or non-homologous end joining (NHEJ) during V(D)J recombination (Dobbs, Tainer et al. 2010, Nagasawa, Little et al. 2011). In contrast, ATR is predominantly activated by exposure of single-strand DNA (ssDNA) ends and is crucial for controlling DNA replication (Zou 2007, Shiotani and Zou 2009, Shiotani and Zou 2009, Lopez-Contreras and Fernandez-Capetillo 2010, Flynn and Zou 2011). Activation of these DNA damage protein kinases leads to initiation of a signaling cascade resulting in phosphorylation of a number of downstream effector molecules, which may lead to one of three outcomes: DNA repair, senescence, or apoptosis.

Following DSB formation, the MRE11-RAD50-NBS1 (MRN) complex is one of the first complexes to be recruited where it functions as a DNA damage sensor and assists in the recruitment of ATM to the damaged site (Figure 3A) (Falck, Coates et al. 2005, Derheimer and Kastan 2010). ATM is then activated by autophosphorylation at serine$^{1981}$, which induces its conformational change from a dimer to a monomer (Bakkenist and Kastan 2003, So, Davis et al. 2009). Upon initial activation, ATM then triggers a DDR signaling cascade that involves phosphorylation of histone variant H2AX at serine$^{139}$ within

minutes after DNA damage (Burma, Chen et al. 2001). This signal for H2AX phosphorylation (γH2AX) is amplified and rapidly spreads over 500kb of the chromatin domains flanking the DNA damage (Meier, Fiegler et al. 2007, Savic, Yin et al. 2009). Mdc1 has been shown to facilitate this process by interacting with both ATM through its FHA domain as well as directly binding to γH2AX. This provides a mechanism for ATM to target nucleosomes containing γH2AX and recognize chromatin flanking the DNA damage site (Stewart, Wang et al. 2003, Stucki, Clapperton et al. 2005, Lou, Minter-Dykhouse et al. 2006). In addition to γH2AX phosphorylation, ATM signals to activate downstream effectors including Chk2, p53, BRCA1, 53BP1, and many more. Activation of H2AX triggers ubiquitinylation and SUMOylation cascades to promote recruitment of BRCA1 and 53BP1 and regulate DSB repair by homologous recombination (Huen, Grant et al. 2007, Mailand, Bekker-Jensen et al. 2007, Doil, Mailand et al. 2009, Stewart 2009, Mattiroli, Vissers et al. 2012). Furthermore, ATM also phosphorylates Chk2 at threonine$^{68}$ and p53 at serine$^{15}$ to induce senescence or apoptosis (Figure 3A).

Like ATM, DNA-PK is also recruited to sites of DSBs. The KU70/KU80 heterodimer recruits and stabilizes DNA-PK at the DSB (Figure 3B) (Mahaney, Meek et al. 2009). Artemis-mediated resectioning of the DSB occurs and the KU/DNA-PK complex recruits the XRCC4 adaptor and DNA ligase IV to allow for non-homologous end joining (NHEJ).

During each S phase, ATR is activated in response to ssDNA exposure, regulates firing of replication origins and initiates repair of stalled or collapsed replication forks (Nyberg, Michelson et al. 2002, Shechter, Costanzo et al. 2004). Replication protein A (RPA) coats ssDNA and is important for the localization of ATR to the damage site (Figure 3C). ATR-interacting protein (ATRIP) forms a complex with ATR and binds directly to RPA-ssDNA. While RPA is sufficient for recruitment of ATR, the ATR-ATRIP complex must

interact with the Rad9-Rad1-Hus1 (9-1-1) complex to activate ATR (Michael, Ott et al. 2000, Stokes, Van Hatten et al. 2002, Byun, Pacek et al. 2005, MacDougall, Byun et al. 2007). The 9-1-1 complex can then stimulate ATR signaling by bringing in topoisomerase-binding protein-1 (TOPBP1) to activate ATR (Furuya, Poitelea et al. 2004, Delacroix, Wagner et al. 2007, Lee, Kumagai et al. 2007). Once triggered, ATR goes on to activate downstream effectors, like p53, Chk1, and BRCA1 that are involved in induction of apoptosis, senescence, and DNA repair. Chk1 is activated by phosphorylation at serine$^{317}$ and serine$^{345}$ by means of Claspin, which is an adaptor molecule that brings ATR to Chk1 (Kumagai and Dunphy 2000). Once Chk1 is phosphorylated it can target the cdc25 phosphatase cell cycle regulators that function by removing the inhibitory phosphorylation of cyclin-dependent kinases (Boutros, Dozier et al. 2006). ATR can also activate BRCA1, Werner syndrome ATP-dependent helicase and Bloom syndrome protein, which are all important proteins involved in regulating recombination and thus promote repair of DNA lesions (Figure 3C) (Tibbetts, Cortez et al. 2000, Pichierri, Rosselli et al. 2003, Davies, North et al. 2004, Li, Kim et al. 2004).

While the ATM and ATR pathways are distinct, they do experience cross talk with each other. Most downstream effectors are phosphorylated by both ATM and ATR kinases, including BRCA1 and p53 (Siliciano, Canman et al. 1997, Cortez, Wang et al. 1999, Tibbetts, Brumbaugh et al. 1999, Sarbassov, Ali et al. 2004). Furthermore, ATR has been shown to phosphorylate H2AX in response to replication stress. This may recruit ATM to stressed replication forks (Ward and Chen 2001). Although the ability for cross talk is available, evidence has been shown that ATM and ATR have unique functions and can act independent of each other (Brown and Baltimore 2003, Chanoux, Yin et al. 2009, Balmus, Zhu et al. 2012).

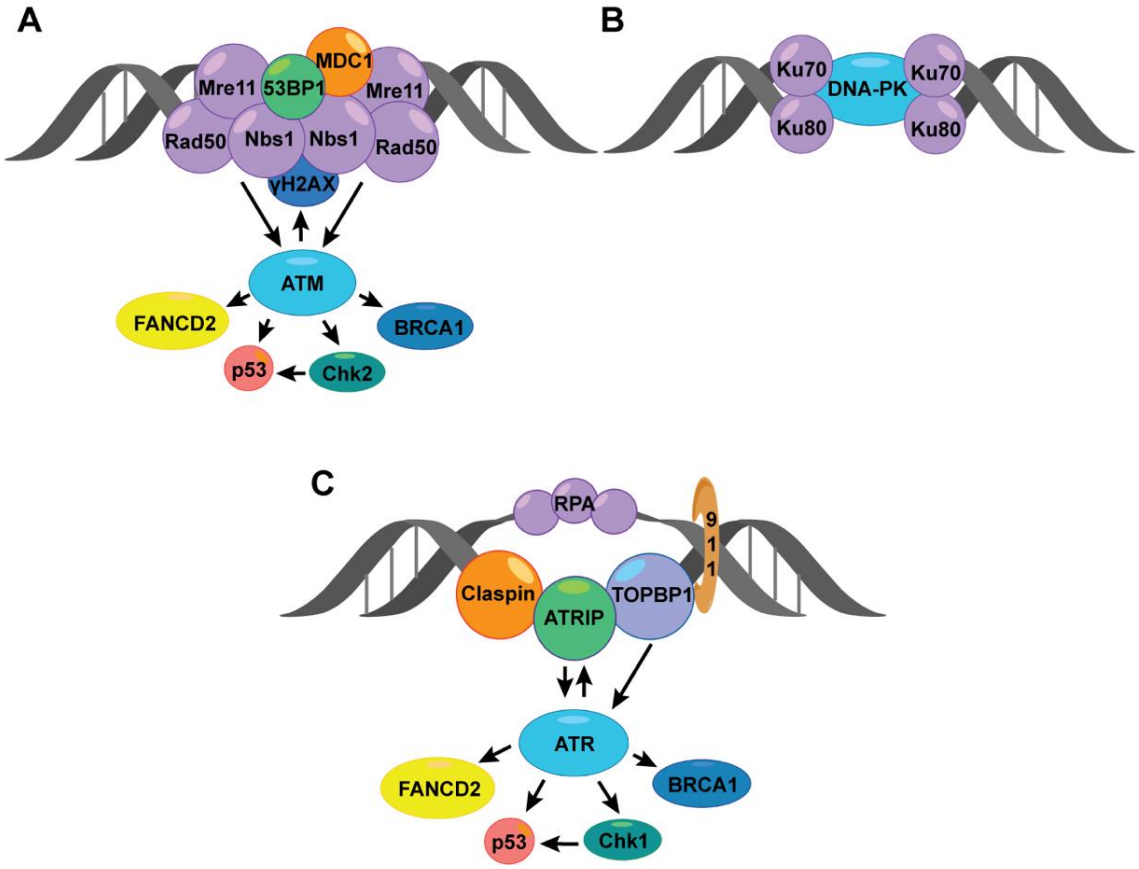


**Figure 3: DNA damage response signaling pathways.** (A) Schematic of the ATM DNA damage response pathway to double-stranded breaks. DNA damage is sensed by the MRN complex, which recruits ATM to the damage site. This allows activation of a downstream signaling cascade, which involves phosphorylation of H2AX, activation of repair factors, including FANCD2, BRCA1 and 53BP1, and cell cycle arrest effectors, including Chk2 and p53. (B) Schematic representation of the DNA-PK DNA damage response pathway to double-stranded breaks. Ku70/80 recruits and stabilizes DNA-PK at the site of DNA damage to facilitate non-homologous end joining. (C) Schematic of the ATR DNA damage response pathway to single-stranded breaks. RPA coats single-stranded DNA and facilitates localization of ATR to DNA damage site. ATRIP forms a complex with ATR, binds RPA and interacts with the 911 complex to activate ATR. ATR goes on to activate downstream effectors, including p53, CHk1, and BRCA1.

### 1.3.2 Viral interactions with the DDR

When viruses infect human cells, they must successfully resist host defenses to faithfully replicate their genetic material. To do so, viruses interface with a number of cellular processes including the host DNA damage response. DNA viruses have a

particularly precarious situation; in that they must infect cells without alerting the host DNA damage response pathway to recognize and launch a response against the viral DNA genome. This challenge has required DNA viruses to develop unique methods to allow the virus to interact with and evade the host DNA damage response. These viruses modulate the DNA damage response by activating or deactivating sensors of DNA damage as well as key downstream effector molecules.

The DDR can serve as an important cellular defense against viral pathogens. DNA viruses experience a significant challenge in preventing the DDR from recognizing replicating viral DNA genomes as damaged DNA. However, depending on the nature of viral interaction with the DDR, this can be beneficial or deleterious for viral replication. In the case of adenovirus, the DDR recognizes the DNA viral genome, preventing the virus from carrying out DNA cleavage and packaging. To overcome this barrier, adenovirus protein complex E4orf3/E1b55k degrades the hosts DDR sensing complex, MRN, to allow for DNA processing and prevent concatemerization (Stracker, Carson et al. 2002). This is a classic example of the complex relationship between DNA viruses and the host DDR.

Human DNA tumor viruses interface with the host DDR through a few distinct mechanisms. One mechanism is that viral oncoproteins can drive host cell proliferation to induce replicative stress thereby activating the DDR. As viruses are typically dependent on host cell machinery to replicate, triggering the host cell to proliferate boosts viral replication and diverts the DDR to the host DNA. During both latent and lytic phases of virus replication, a second mechanism of DDR engagement involves direct interaction of viral proteins with DDR machinery. This interaction can modulate the activity of the DDR such that it activates or inactivates effectors involved in the DDR to prevent senescence or apoptosis from occurring, which would ultimately suppress viral replication and promote

DNA repair. Finally, human DNA tumor viruses can form viral DNA structures during replication that can be recognized by the DDR. During latency, DDR factors are crucial for maintaining the episomal DNA of large DNA tumor viruses. Furthermore, during lytic replication these viruses take advantage of DDR-mediated recombination and repair to process viral DNA and prepare it for packaging. Since the DDR can have positive and negative effects on viral DNA replication, the interaction between the virus and the DDR must be properly regulated.

## 1.3.3 Latent EBV infection and the DDR

### 1.3.3.1 Viral gene expression induces a growth suppressive DDR

Many tumor viruses utilize the DDR to alter the host cellular environment to promote long-term proliferation, oncogenic transformation, and ultimately survival. B cell transformation efficiency when infected with EBV is low where only approximately 1% of infected B cells are immortalized (Henderson, Miller et al. 1977, Sugden and Mark 1977). This suggests that there must be host cell innate barriers at play to restrict virally induced proliferation. The DDR was identified by Nikitin *et al.* to induce a growth suppressive barrier responsible for ~10-fold restriction (Nikitin, Yan et al. 2010). EBV infection of primary human B cells induced an ATM-mediated DDR, as indicated by phosphorylation of H2AX, ATM, Chk2, and formation of 53BP1 nuclear foci. These DDR marks did not co-localize with the viral genome, thus signifying that host DNA damage induced the DDR and not viral DNA damage. Furthermore, through fluorescent dye proliferation tracking studies it was reported that EBV induced a transient period of hyper-proliferation followed by a period of attenuated proliferation. Interestingly, this hyper-proliferative period was associated with activation of the DDR. Importantly, inhibition of ATM and Chk2 enhanced viral-mediated transformation, which suggests a mechanism where the DDR is an innate

host tumor suppressor pathway responsible for restricting viral-mediated cell proliferation (Nikitin, Yan et al. 2010).

The host gene expression of the hyper-proliferative B cells was studied in comparison to resting B cells and transformed LCLs to determine what distinct host factors may be responsible for inhibiting transformation. Microarray analysis revealed distinct transcriptomic changes among these cell populations, including enrichment for genes associated with cell cycle progression and the DDR in early proliferation (Nikitin, Yan et al. 2010, Forte, Salinas et al. 2012, Price, Tourigny et al. 2012). The rapidly proliferating EBV-infected B cells transitioned from expressing increased levels of EBNA-LP and EBNA2, to accumulating EBNA3 proteins and attenuated proliferation. As EBNA3 proteins begin accumulating they can act to attenuate EBNA2-regulated targets such as CD23 and c-Myc (Nikitin, Yan et al. 2010). Overall, these studies support a model of heightened proliferative capacity due to c-Myc promoting replicative stress and DDR-mediated growth arrest. As cells progress beyond this phase, attenuated growth rates and c-Myc no longer activated the growth suppressive DDR.

Recently, McFadden *et al.* reported that growth arrest of hyper-proliferating EBV-infected B cells was associated with reduced level of mitochondrial respiration and decreased expression of genes involved in the TCA cycle and oxidative phosphorylation (McFadden, Hafez et al. 2016). Growth arrest was rescued by supplemented TCA cycle intermediates. Furthermore, arrested cells exhibited an increase in expression of p53 pathway gene targets, including sestrins leading to activation of AMPK, a reduction in mTOR signaling, and elevated autophagy, which was found to be important for maintaining hyper-proliferation, and ultimately survival during metabolic stress. Interestingly, glucose import and surface glucose transporter 1 (GLUT1) levels increased

with long-term outgrowth, leading to increased metabolic processes, including glycolysis, oxidative phosphorylation, and suppression of basal autophagy (McFadden, Hafez et al. 2016). This study identified metabolic imbalance as a key instigator of DDR-mediated growth arrest during the hyper-proliferative period and subsequent barrier to B cell immortalization by EBV.

#### 1.3.3.2 DDR activation generates genomic instability

Expression of viral latent proteins from DNA tumor viruses can induce cell proliferation and promote growth transformation. However, this is often accompanied by persistent DDR due to irreparable DNA damage. Unrepaired DNA damage can lead to genomic instability and cancer development. Tumor viruses promote genomic instability and initiate tumorigenesis through diverse mechanisms including telomere dysfunction and induction of reactive oxygen species (ROS).

Chromosomal aberrations are a hallmark of malignant transformation and while transformed LCLs are known to be karyotypically stable (Lacoste, Wiechec et al. 2009), many EBV-associated malignancies have been observed to carry signs of chromosomal instability. The Masucci group has explored the link between EBV-associated malignancies and genomic instability in depth and has reported that EBV promotes genomic instability in Burkitt's lymphoma (BL). This group conducted cytogenetic analyses in EBV negative and EBV positive BL lines and found that carrying EBV resulted in a significant increase in dicentric chromosomes, chromosome fragments and chromatid gaps. Furthermore, by examining telomeres using fluorescence *in situ* hybridization, EBV positive BL cells exhibited an overall increase in telomere size and telomeric fusion. Virally infected cells also revealed an increase in presence of phosphorylated H2AX (Kamranvar, Gruhne et al. 2007). Expression of EBV nuclear antigen EBNA1, an EBV latency protein,

was determined to be a mechanistic link between EBV viral proteins and chromosomal abnormalities. Stable or conditional expression of EBNA1 induced telomere fusion and heterogeneous length of telomeres (Kamranvar and Masucci 2011). Interestingly, when telomere dysfunction was examined in primary human B cells infected with EBV, activation of the alternative lengthening of telomeres (ALT) pathway was found to be the preferred mechanism for lengthening telomeres (Kamranvar, Chen et al. 2013). While most tumors use telomerase to extend telomeres, 10-15% of tumors employ ALT, a telomere recombination-based mechanism (Bryan, Englezou et al. 1995, Bryan, Englezou et al. 1997, Yeager, Neumann et al. 1999, Dunham, Neumann et al. 2000). Newly infected B cells exhibited hallmark signs of ALT activation, including telomere-associated promyelocytic leukemia nuclear bodies, telomeric-sister chromatid exchange, extra-chromosomal telomeres, telomere fusion, and telomere length heterogeneity. Importantly, early EBV infected B cells expressed low levels of TRF2, TRF1, POT1 and ATRX, shelterin complex proteins responsible for telomere protection (Kamranvar, Chen et al. 2013).

#### 1.3.3.3 Reactive oxygen species facilitates DDR activation

Oxidative stress caused by the up-regulation of reactive oxygen species (ROS) is among the many factors known to activate the DDR pathway. ROS activation upon oncogenic viral infection can cause oxidative DNA damage and results in increased mutagenesis and chromosomal alterations, ultimately promoting tumorigenesis. A number of human tumor viruses have been shown to activate ROS. Recent work from the Masucci laboratory has found that early after EBV infection, B cells exhibited an increased accumulation of ROS, which correlated with high levels of DNA damage (Chen, Kamranvar et al. 2016). Treatment with NACA, a ROS scavenger used to diminish the

levels of ROS, resulted in a decrease in DNA damage along with down-regulation of the viral LMP1 latency protein, which is crucial for viral-mediated transformation. Furthermore, previous work has shown that the EBV protein EBNA1 can induce ROS levels leading to ATM-dependent DDR activation, thus suggesting a molecular source of damage for inducing telomere dysfunction (Gruhne, Sompallae et al. 2009, Kamranvar and Masucci 2011). It is suggested that increasing intracellular ROS levels may create a balance between activation of cellular antioxidant machinery and viral gene expression that inhibit the DDR to promote proliferation of infected cells. (Chen, Kamranvar et al. 2016).

### 1.3.4 Diversity of viral and host DDR

DNA damage present in both viral and cellular genomes triggers DDR activation in infected cells. However, the nature of DDR signaling downstream of these two sources of damage may differ. Recently, a breakthrough study by the O'Shea laboratory provided evidence that viral and cellular genomes activate distinct DDR pathways in infected cells. They focused their investigation on the MRE11-RAD50-NBS1 (MRN) complex, which senses DNA damage and activates a global ATM-mediated DDR following adenovirus infection. In their recent report, they find that MRN binds to the adenoviral genome activating a localized ATM-mediated DDR. Typically, MRN recognition of cellular DNA damage initiates a global DDR where H2AX is phosphorylated over megabases of the genome flanking the damage site. In contrast to this signal amplification observed in cellular DDR pathways, damage to the viral genome did not induce H2AX phosphorylation across the cellular genome (Shah and O'Shea 2015). Furthermore, they used wild type Adenovirus 5, which encodes early viral proteins, to infect human small airway epithelial cells and found that viral oncoproteins E1B-55K and E4-ORF3 inactivate MRN and allow viral replication, consistent with that found by Weitzman and colleagues (Stracker, Carson

et al. 2002). The assembly of viral genomes within the nucleus triggered a unique global MRN-independent ATM response, but this global response was not found to impair viral replication. Lastly, they found that the localized MRN-ATM anti-viral DDR initiated at viral genomes affects viral replication but does not affect cellular DNA replication, thus providing evidence that the MRN-ATM anti-viral DDR selectively inhibits viral replication and maintains cellular proliferation (Shah and O'Shea 2015). This discovery that viral and cellular genomes elicit distinct DDRs is particularly important as it begins to provide a mechanism for how the DDR could selectively target viral genomes while maintaining cellular viability.

### 1.3.5 Viral oncoproteins and the DDR

DNA tumor viruses extensively engage the host DNA damage response. Many strategies are used by these viruses to activate, modulate, or suppress the DDR during latent and lytic stages, which allow the virus to establish a long-term infection and evade the host defense response. As discussed here, tumor viruses express oncoproteins to drive proliferation, which can lead to replicative stress and DNA damage, thus triggering the DDR. This activated DDR signaling cascade can result in one of three outcomes: DNA repair, apoptosis, or senescence. During latency, activation of the DDR mediated by viral oncoprotein expression typically results in strong cell cycle arrest.

During latency, viral oncoprotein activation led to aberrant proliferation, which can induce replicative stress and result in activation of the host DDR and cell cycle arrest. This DDR activation upon latent EBV and KSHV infection was observed to cause a growth suppressive barrier to viral-mediated transformation. Viral oncoproteins have developed methods to regulate the DDR by triggering autophagy. Recent advances in this field have brought the importance of metabolism to the forefront of latent infection. Aberrant

proliferation in EBV-infected B cells was found to induce metabolic stress characterized by an imbalance of glycolysis and oxidative phosphorylation and subsequently triggering cellular senescence.

Our understanding of the interplay between DNA tumor viruses and the host DDR has expanded rapidly. It is clear that viruses have developed a number of strategies to interact with the ATM pathway to promote viral replication, development of progeny virion and induce senescence. Furthermore, oncogenic stress has been shown to increase the incidence of downstream chromosomal abnormalities, including telomere dysfunction. However, to determine how and why the DDR is activated, we must define the upstream molecular sources of the damage and develop a better understanding of the genetic mechanism the DDR pathway employs to induce downstream telomere fusions, irreparable damage, and facilitate or inhibit virus replication. By developing a more specific understanding of the particular DDR factors involved and how they become activated, novel therapeutic strategies can be designed to target these factors and prevent tumorigenesis.

## *1.4 Oncogene-induced senescence*

Cellular senescence is defined as a permanent growth arrest that can be triggered by exogenous or endogenous stresses (Campisi and d'Adda di Fagagna 2007, Collado, Blasco et al. 2007, Adams 2009). While senescent cells maintain proliferative arrest, these cells continue to sustain metabolic activity. Unlike replicative senescence, which is typically induced by critically shortened telomeres that result after many population doublings, oncogene expression can induce cellular senescence early after only a few population doublings.

Oncogene-induced senescence (OIS) is a tumor suppressive mechanism that has been observed in a number of premalignant lesions (Braig, Lee et al. 2005, Chen, Trotman et al. 2005, Collado, Gil et al. 2005, Michaloglou, Vredeveld et al. 2005, Bartkova, Rezaei et al. 2006, Denchi and de Lange 2007, Halazonetis, Gorgoulis et al. 2008, Collado and Serrano 2010). OIS has been shown to induce global heterochromatin by formation of senescence-associated heterochromatic foci (SAHF), which depends on anti-silencing function 1 (ASF1), histone cell cycle regulation defective homologue A (HIRA), and $p16^{INK4a}$ (Narita, Nunez et al. 2003, Narita, Narita et al. 2006, Zhang, Liu et al. 2007, Di Micco, Sulli et al. 2011). Formation of SAHF is proposed to suppress the transcription of genes that are involved in promoting proliferation thereby enforcing OIS (Narita, Nunez et al. 2003, Zhang, Chen et al. 2007) (Funayama and Ishikawa 2007). Importantly, OIS has been characterized by DNA replication stress leading to persistent DNA damage signaling, upregulation of p53/p21 and p16/pRb pathways and induction of irreversible growth arrest (Serrano, Lin et al. 1997, Bartkova, Rezaei et al. 2006, Di Micco, Fumagalli et al. 2006). Recent evidence suggests that nucleotide metabolism and specifically an imbalance in nucleotide pools may contribute to replication stress and activation of the DDR that is necessary to maintain OIS (Bester, Roniger et al. 2011, Aird, Zhang et al. 2013, Aird, Worth et al. 2015).

### 1.4.1 Role of nucleotide metabolism in OIS

Nucleotides are required for many cell processes, including DNA replication and repair. Improper nucleotide metabolism has been shown to cause an imbalance in nucleotide levels, which can contribute to inefficient DNA replication and consequently human diseases, such as cancer (Weinberg, Ullman et al. 1981, Meuth 1989, Bester, Roniger et al. 2011, Chabosseau, Buhagiar-Labarchede et al. 2011, Chang, Guo et al.

2013). Interestingly, recent work has shown that deoxyribonucleotide (dNTP) pools also play an important role in replication stress during establishment of OIS. It has been long established that reduction of dNTP pools by treatment with hydroxyurea leads to an S-phase cell cycle growth arrest (Bianchi, Pontis et al. 1986, Matsumoto, Rey et al. 1990). Recently, the Zhang laboratory provided extensive evidence suggesting that replication stress during OIS is specifically due to a reduction of dNTP pool levels (Aird, Zhang et al. 2013). They found that cells undergoing OIS exhibited a transcriptional decrease in RRM2, a regulatory subunit responsible for synthesis of dNTPs during S phase, and that ectopic expression of RRM2 was sufficient to restore dNTP cellular levels. Importantly, supplementation of exogenous nucleosides was able to rescue the replication stress phenotype (Aird, Zhang et al. 2013). Consistent with these findings, deficient dNTP pools were also found to contribute to replication stress in yeast (Weinberger, Feng et al. 2007).

Furthermore, depletion of dNTP pools has also contributed to accumulation of DNA damage and activation of the DDR during OIS. The Zhang and Kerem groups have both shown that deficient dNTP pools are a key factor in accumulation of DNA damage and that supplementation of exogenous nucleosides can suppress the DDR during OIS triggered by oncogenic RAS or c-myc (Mannava, Moparthy et al. 2012, Aird, Zhang et al. 2013, Mannava, Moparthy et al. 2013). These findings indicate that dNTP pools play an important role in replication stress during OIS and RRM2 downregulation contributes to deficient nucleotide metabolism and establishment of OIS. Currently, clinical trials are underway to determine potential therapeutics that function by specifically inhibiting nucleotide metabolism as a therapeutic strategy for cancer.

## *1.5 Persistent DNA damage response*

OIS has been shown to induce senescence by chronically activating the DDR

pathway, and establishing the formation of persistent DNA damage foci. These foci have been observed in primary cells expressing oncogenes as well as early pre-malignant lesions (DiTullio, Mochan et al. 2002, Bartkova, Horejsi et al. 2005, Gorgoulis, Vassiliou et al. 2005, Bartkova, Rezaei et al. 2006, Di Micco, Fumagalli et al. 2006, Mallette, Gaumont-Leclerc et al. 2007). Persistent DNA damage foci were originally differentiated from transient DNA damage foci based on their resistance to DNA repair. DNA lesions that are repairable often resolve within 24 hours and undergo growth arrest for only a transient period of time. Depending on the phase the cell cycle is in when the damage is sustained, DNA lesions may be repaired by non-homologous end joining or homologous recombination. Alternatively, irreparable DNA damage typically results in constitutive DDR signaling and chronic p53 activation, and ultimately functions to maintain a senescent cellular phenotype (Beausejour, Krtolica et al. 2003, d'Adda di Fagagna, Reaper et al. 2003, Takai, Smogorzewska et al. 2003, Herbig, Jobling et al. 2004, Rodier, Munoz et al. 2011).

Chronic activation of the DDR signaling pathway has been shown to facilitate senescence by triggering the secretion of factors, including IL-6, that allow stressed and damaged cells to communicate with the microenvironment. The Campisi group found that after severe genotoxic stress causing telomere damage, persistent DDR signaling was produced, maintaining a p53-dependent senescence and importantly, inducing inflammatory cytokine secretion. At a lower dose of irradiation causing mild genotoxic stress, DDR was triggered resulting in a transient cell cycle arrest, DNA repair, but no induction of IL-6 secretion (Rodier, Coppe et al. 2009). This suggests that persistent DNA damage foci maintain senescence by induction of IL-6.

Importantly, persistent DNA damage foci were observed specifically at damaged sites on DNA that were determined to be irreparable. Until recently, it was not well understood what kind of DNA damage was irreparable; however, telomeric DNA has been identified and described to be a specific target of persistent DNA damage response in cells that undergo OIS (Hewitt, Jurk et al. 2012). The Passos laboratory observed that by immuno-fluorescent *in situ* hybridization and ChIP, up to half of the DNA damage foci in OIS cells are located at telomeres and all persistent DNA damage foci were located specifically at telomeric DNA (Hewitt, Jurk et al. 2012). Furthermore, d'Adda di Fagagna's laboratory found that in yeast, chromosomal DNA double-strand breaks next to a telomeric sequence resisted repair and observed impaired DNA ligase IV recruitment. Consistently, in mammalian cells, ectopic localization of telomere shelterin complex protein TRF2 next to a double strand DNA break induced persistent DNA damage (Fumagalli, Rossiello et al. 2012, Fumagalli, Rossiello et al. 2014). These studies were among the first to establish that persistent DNA damage targeted telomeric DNA and that once damaged is irreparable.

Irreparable DNA damage is resistant to repair due to a number of challenges associated with the structure of telomeric DNA. Telomeres are composed of repetitive sequences that have a tendency to form secondary structures known as G quadraplexes, which impede access to replication machinery. Additionally, telomeric DNA is highly sensitive to oxidative damage. These increase the risk of DNA replication stress and replication fork stalling contributing to formation of DDR foci localized to telomeres (Suram, Kaplunov et al. 2012). Along with telomeres, chromosomal common fragile sites, are highly targeted by double-strand DNA breaks upon replication stress. These chromosomal sites, like telomeric DNA are composed of repetitive sequences that are prone to forming

secondary structures known to impede replication fork progression (Durkin and Glover 2007, Martinez, Thanasoula et al. 2009, Sfeir, Kosiyatrakul et al. 2009, Letessier, Millot et al. 2011).

Establishment of OIS by persistent DNA damage signaling can lead to the formation of nuclear DDR foci. Three forms of nuclear DDR foci have been described, including DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS), telomere dysfunction-induced foci (TIFs), and alternative lengthening of telomeres (ALT)-associated promyelocytic leukemia nuclear bodies (APBs) (Figure 4) (Takai, Smogorzewska et al. 2003, Rodier, Munoz et al. 2011, Kamranvar, Chen et al. 2013).

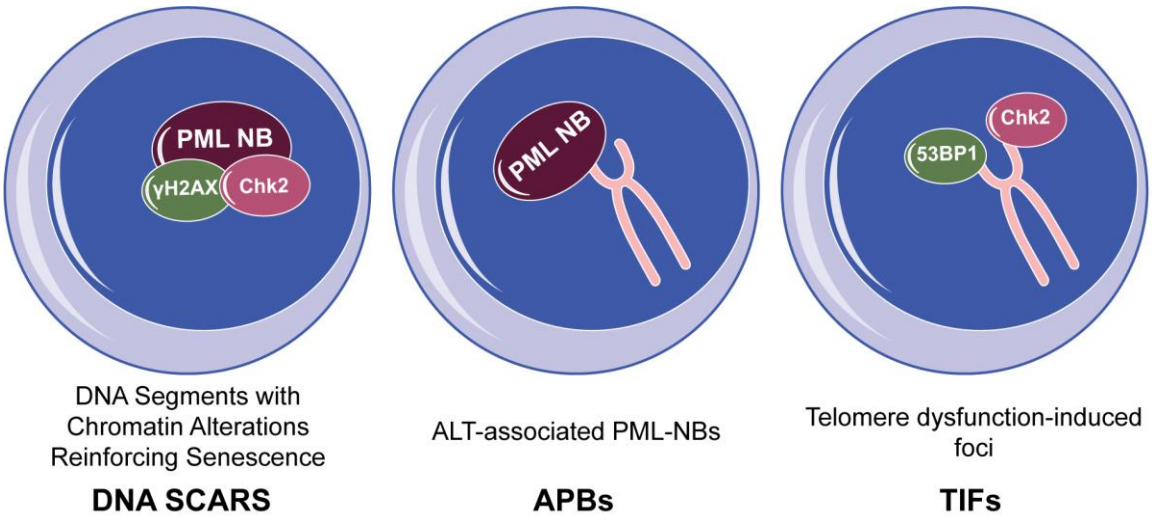


**Figure 4: Persistent DNA damage response.** Schematic of three characterized forms of persistent DNA damage foci, including DNA segments with chromatin alterations reinforcing senescence (DNA SCARS), ALT-associated PML-NBs (APBs), and telomere dysfunction-induced foci (TIFs).

### 1.5.1 DNA segments with chromatin alterations reinforcing senescence

DNA SCARS are considered to be relatively stable structures distinct from the formation of transient DNA damage foci. Identification of the events leading to formation

of DNA-SCARS were originally established by the Campisi group to distinguish the difference between persistent and transient DNA foci. DNA-SCARS were found to associate with PML nuclear bodies, lack RPA and RAD51 DNA repair proteins, and lack single-strand DNA and DNA synthesis (Figure 4) (Rodier, Munoz et al. 2011). DNA-SCARS formation was accelerated in cells deficient in DNA repair proteins, suggesting that these persistent DNA damage foci are triggered by ineffective or defective repair strategies (Rodier, Munoz et al. 2011).

Importantly, DNA-SCARS were found to function in maintaining cellular senescence, a phenomenon observed in cell lines and primary mouse and human endothelial tissues. The Campisi group observed that when the integrity of DNA-SCARS was compromised by depleting H2AX to create H2AX-deficient cells this disruption resulted in suppression of IL-6 secretion after ionizing radiation. These structures were functionally important for IL-6 secretion, which is a key characteristic of the senescence-associated secretory phenotype (SASP) (Rodier, Munoz et al. 2011). Furthermore, formation of these persistent DNA damage foci resulted in accumulation of activated DDR mediators, including phosphorylated ATM, CHK2, and p53. This suggests that DNA-SCARS serve as a reservoir for active DDR signaling, which is required to maintain p53-dependent growth arrest and inflammatory cytokine secretion contributing to senescence (Gire and Wynford-Thomas 1998, Beausejour, Krtolica et al. 2003, d'Adda di Fagagna, Reaper et al. 2003, Gire, Roux et al. 2004, Herbig, Jobling et al. 2004, Rodier, Coppe et al. 2009, Rodier, Munoz et al. 2011).

### 1.5.2 Telomere dysfunction-induced foci

Telomeres are associated with a nucleoprotein complex, also known as the shelterin complex, that protects telomeric DNA and allows cells to distinguish chromosome

ends from DNA breaks (Takai, Smogorzewska et al. 2003, von Zglinicki, Saretzki et al. 2005). When telomere ends are unprotected, the cell recognizes the chromosome end as a DSB and activates the DDR pathway resulting in senescence, apoptosis, or chromosome end fusions forming dicentric chromosomes. Telomere uncapping may result due to critically shortened telomeres or impairment of factors that protect telomere ends, and is referred to as dysfunctional telomeres. Telomere dysfunction has been extensively characterized, most notably by the de Lange Laboratory, to form a domain of telomeric DNA that associates with DNA damage factors, known as telomere-dysfunction-induced foci (TIFs) (Figure 4). The de Lange group established direct evidence that uncapped telomeres are recognized by the cellular DDR. Uncapped telomeres were modeled by inhibition of TRF2 in hTERT-immortalized BJ human fibroblasts, which resulted in association of DDR factors, such as 53BP1, γH2AX, Rad17, ATM, and Mre11, with the uncapped telomeres (Takai, Smogorzewska et al. 2003). Furthermore, dysfunctional telomeres were shown to activate upstream DDR phosphatidylinositol 3-kinase-like kinases, such as ATM or ATR, and phosphorylates downstream DDR factors, including CHK1, CHK2, and p53 (Takai, Smogorzewska et al. 2003, Gire, Roux et al. 2004, Denchi and de Lange 2007, Guo, Deng et al. 2007, Churikov and Price 2008).

Telomeres are known to adopt a number of structures. First, is the fully capped state where the shelterin proteins inhibit NHEJ and DDR activation. If the telomeric conformation is compromised, telomeres may enter the intermediate state whereby they activate the DDR without leading to chromosome fusions due to the presence of shelterin proteins. The third state is the fully uncapped state where critical telomere shortening occurs, telomeric conformation is compromised and shelterin proteins are lost, causing chromosome fusions by NHEJ (Cesare, Kaul et al. 2009). Telomere dysfunction has long

been described to be a result of the fully uncapped state where telomere shortening yields replicative senescence; however, recent evidence strongly suggests that telomere dysfunction can also occur independent of telomere length (Parrinello, Samper et al. 2003, Fumagalli, Rossiello et al. 2012, Hewitt, Jurk et al. 2012, Jurk, Wilson et al. 2014, Birch, Anderson et al. 2015). Recently, evidence supporting a fourth state has been described suggesting that in the presence of sheltering components and proper telomere conformation, persistent DDR activation at telomeric DNA can occur.

Persistent telomeric DDR signaling has been shown to play a role in oncogene-induced senescence as a mechanism of preventing human cancer progression by maintaining senescence. In a seminal paper from the Herbig laboratory, oncogene activation in human cells was found to affect telomere structure and function by causing telomeric replication stress and resulting in telomere dysfunction in cells lacking telomerase as well as the formation persistent telomeric DDR foci (Suram, Kaplunov et al. 2012). An accumulation of recent evidence has shown that persistent DDR signaling at telomeric DNA is due to the irreparability of telomeres (Fumagalli, Rossiello et al. 2012, Hewitt, Jurk et al. 2012, Suram, Kaplunov et al. 2012, Fumagalli, Rossiello et al. 2014). However, this concept has been challenged by evidence suggesting that DSB repair can occur at telomeres (Doksani and de Lange, 2016; Mao, 2016). It is important to note that this has only been observed in proliferating cells, including BJ fibroblasts and HeLa cells, which exhibit faster repair kinetics, thus suggesting proliferation rate may be a determinant for whether telomeric DNA damage is repairable or not (Mao, Liu et al. 2016, Victorelli and Passos 2017).

### 1.5.3 Alternative lengthening of telomeres (ALT)-associated promyelocytic leukemia nuclear bodies

Due to aberrant proliferation, cancer cells require maintenance of telomeres to bypass the end-replication problem and critical shortening of telomeric DNA. Most cancers upregulate the activity of telomerase, a reverse transcriptase that synthesizes telomeric repeats to achieve proper telomere replication (Shay and Bacchetti 1997). However, 10-15% of cancers use the non-canonical telomere maintenance mechanism known as alternative lengthening of telomeres (ALT) to utilize homologous recombination repair and maintain telomere replication during aberrant proliferation (Bryan, Englezou et al. 1995, Bryan, Englezou et al. 1997, Gire and Wynford-Thomas 1998, Yeager, Neumann et al. 1999, Dunham, Neumann et al. 2000). ALT-positive cancer cells exhibit a number of characteristics, including highly heterogeneous telomere length, extrachromosomal telomeric repeats, telomere sister chromatid exchanges, and ALT-associated promyelocytic leukemia nuclear bodies (PML NBs) (Ogino, Nakabayashi et al. 1998, Tokutake, Matsumoto et al. 1998, Yeager, Neumann et al. 1999, Henson, Neumann et al. 2002, Bechter, Zou et al. 2004, Cesare and Griffith 2004, Londono-Vallejo, Der-Sarkissian et al. 2004, Henson, Cao et al. 2009, Lang, Jegou et al. 2010).

ALT-associated promyelocytic leukemia nuclear bodies (APBs) is a key complex formation found in OIS cells where PML NBs localize to telomeric DNA (Figure 4) (Jiang, Nguyen et al. 2011). These structures are comprised of PML NB components, such as PML, SP100, SUMO, telomere shelterin complex proteins TRF1, TRF2, POT1 and RAP1, as well as factors involved in the DDR and DNA repair (Yeager, Neumann et al. 1999, Stavropoulos, Bradshaw et al. 2002, Nabetani, Yokoyama et al. 2004, Potts and Yu 2007, Jiang, Zhong et al. 2009). Importantly, APBs have been shown to be associated with many

proteins present in PML NBs that are necessary for transcription, replication, DDR, DNA repair, and senescence. However, the function of these structures remains unclear.

Recent studies of telomere integrity in EBV-infected B cells yielded evidence for EBV inducing telomeric DNA damage and subsequently activation of ALT to maintain telomere replication through transformation to LCLs. A key finding to support EBV mediating ALT as opposed to telomerase-dependent replication is due to the low or undetectable levels of telomerase activity observed in newly established LCLs (Sugimoto, Tahara et al. 2004). Furthermore, the bulk population of early EBV-infected B cells exhibited extensive telomere dysfunction, including extra-chromosomal telomeres, telomere fusion, and telomere length heterogeneity (Kamranvar, Chen et al. 2013). Researchers also observed the presence of APBs and sister chromatid exchange in these early EBV-infected cells, suggesting that EBV-infected B cells undergo ALT (Kamranvar, Chen et al. 2013).

## *1.6 Summary of the work presented in this thesis*

As introduced here, prior work in the Luftig laboratory identified a transient period of hyper-proliferation that occurs early after EBV infection of primary human B cells. This hyper-proliferative period is associated with activation of the host DDR pathway, and triggers growth arrest, which suppresses transformation into LCLs. Interestingly, these early arrested EBV-infected B cells fail to upregulate oxidative phosphorylation along with genes associated with mitochondrial biogenesis resulting in metabolic imbalance (Nikitin, Yan et al. 2010, Nikitin, Price et al. 2014, McFadden, Hafez et al. 2016).

The work presented in this thesis focuses on the role the human DDR plays in suppressing EBV-mediated transformation in human B cells. Here, we focus on establishing that EBV promotes oncogene-induced senescence early after infection. We

further investigate the senescent population of early EBV-infected B cells and determine how replicative stress and metabolic imbalance lead to deficiency in dNTP levels necessary to sustain proliferation. Lastly, we identify and characterize the formation of persistent DDR foci that contributes to the establishment of senescence in early EBV-infected B cells. Together, the data generated and discussed in this thesis contributes to our understanding of the factors necessary to regulate viral-mediated transformation during the early stages of infection and induce a tumor suppressive cellular senescence.

# 2. EBV infection establishes oncogene-induced senescence early after infection

*Parts of this chapter were modified based on the research article published by Karyn McFadden, Amy Y. Hafez, Rigel Kishton, Joshua E. Messinger, Pavel A. Nikitin, Jeffrey C. Rathmell, and Micah A. Luftig in PNAS in 2016 (McFadden, Hafez et al. 2016).*

## *2.1 Rationale*

EBV is an oncogenic herpesvirus that establishes a latent B cell infection for the life of the host. Over 90% of the adult world population is infected with EBV. While the virus is typically controlled in healthy individuals by a strong cytotoxic T cell immune response, patients that experience a suppressed immune system through co-infection with malaria or HIV, or transplant patients taking immunosuppressive drugs experience a higher risk of developing EBV-associated malignancies, including African endemic Burkitt's lymphoma, post-transplant lymphoproliferative disease, nasopharyngeal carcinoma, and HIV-associated lymphomas (Kieff and Rickinson 2007). Recent evidence has shown that in addition to the immune system, there are intrinsic responses that may contribute to suppression of EBV-associated malignancies.

Early after EBV infection, primary human B cells exhibit a transient period of hyper-proliferation. EBV latency proteins, EBNA2 and EBNA-LP contribute to cell cycle entry by upregulation of pro-growth gene expression (Alfieri, Birkenbach et al. 1991, Wang, Kikutani et al. 1991, Sinclair, Palmero et al. 1994). Upon induction of hyper-proliferation, infected B cells trigger activation of the DDR, which can signal through p53 to induce cellular senescence or apoptosis (Nikitin, Yan et al. 2010). EBV infected cells were observed to bypass apoptosis and a subset of the infected B cell population trigger a G1/S phase cell cycle arrest (Nikitin, Price et al. 2014). While it is understood that early EBV

infected B cells do not undergo apoptosis, it is not well understood whether the observed growth arrest is the establishment of cellular senescence and which cellular pathways contribute to this growth arrest.

Senescence has long been attributed to replicative senescence, which is a barrier brought on by progressive telomere shortening during propagation of human cells in culture (Hayflick and Moorhead 1961). However, it is now understood that premature senescence may arise in the absence of telomere shortening, including oncogene-induced senescence (OIS). OIS is defined by an irreversible growth arrest after chronic oncogene expression or the inactivation of tumor suppressors (Kuilman, Michaloglou et al. 2010, Rodier and Campisi 2011). This form of premature senescence has been characterized by the presence of chromatin structure alterations, known as senescence-associated heterochromatic foci (SAHF) as well as increased DDR activity and autophagy (Narita, Nunez et al. 2003, Patschan, Chen et al. 2008, Young, Narita et al. 2009, Di Micco, Sulli et al. 2011). Recent evidence suggests that OIS is a consequence of persistent DDR signaling resulting from replicative stress triggered during oncogene-driven hyper-proliferation (Bartkova, Rezaei et al. 2006, Di Micco, Fumagalli et al. 2006, Mallette, Gaumont-Leclerc et al. 2007). Furthermore, OIS has been found to serve as a tumor suppressive mechanism in a wide range of cell types (Kuilman, Michaloglou et al. 2010). Importantly, OIS suppresses proliferation driven by the over-expression of viral proteins or following oncogenic virus infection (Koopal, Furuhjelm et al. 2007, Leidal, Pringle et al. 2012). We hypothesize that infection with oncogenic EBV triggers OIS contributing to the predominant growth arrest phenotype observed early after infection of primary human B cells.

In this study, we describe the use of a unique method to infect primary human B cells with EBV and isolate the cells that experience an initial burst in proliferation and then arrest from the infected cells that continue to proliferate past the hyper-proliferative period. Here, we use this approach to characterize the cellular arrest that the majority of EBV-infected B cells undergo and examine the presence of OIS markers in the arrested B cells population.

## *2.2 Results*

### 2.2.1 EBV infection of primary human B cells induces senescence

Upon *in vitro* infection of primary human B cells with EBV, B cells undergo a burst of hyper-proliferation. This transient hyper-proliferative period induces a G1/S phase cell cycle growth arrest, which suppresses EBV-mediated transformation of the majority of infected B cells (Nikitin, Yan et al. 2010, Nikitin, Price et al. 2014). To further examine the arrested population of infected B cells, we developed a unique protocol to specifically identify and isolate the infected B cells that initially proliferate and then arrest from the cells that continue to proliferate. Peripheral blood mononuclear cells (PBMCs) were isolated from human blood and stained with CellTrace Violet proliferation tracking dye followed by infection with EBV on Day 0 post infection. The cells were then stained on Day 4 post infection with CFSE, a second proliferation tracking dye, to coincide with the initial burst of hyper-proliferation. Infected B cells were then monitored over time by fluorescence-activated cell sorting (FACS). B cells that exhibited diluted CellTrace Violet stain, but high CFSE stain represented cells that initially proliferated and then arrested and were termed proliferated-arrested (PA). Cells that diluted both CellTrace Violet and CFSE stains represented cells that continued proliferating and were designated proliferated-proliferated (PP) (Figures 5*A* and *B*).

The identified G1/S phase growth arrest could be a transient quiescence or senescence. Cells undergoing senescence exhibit a number of distinct characteristics often observed in addition to growth arrest that allow for their identification *in vitro* (Kuilman, Michaloglou et al. 2010). To determine whether PA cells are senescent, we sorted the PA population from three independent donors to purity and examined whether they exhibited key characteristics of senescence. The PA cells exhibited a significant increase in the expression of the DDR marker 53BP1, which was consistent with our previous findings that the early population doublings exhibit an upregulated activation of the DDR (Nikitin, Yan et al. 2010) (Figure 5C). Senescent cells are also characterized by the presence of senescence-associated heterochromatic foci, which is an altered chromatin structure enriched for tri-methylated lysine 9 of histone H3 (H3K9me3) (Kuilman, Michaloglou et al. 2010). Immunofluorescence analysis of PA cells showed that the arrested population demonstrated a significant increase in H3K9me3 foci relative to the PP cells (Figure 5D). This was supported by evidence of an increase in overall heterochromatic DNA lining the inner nuclear membrane as observed by transmission electron microscopy (TEM) (Figure 5E). Together, this data suggests that the PA population of EBV-infected B cells undergoes senescence.

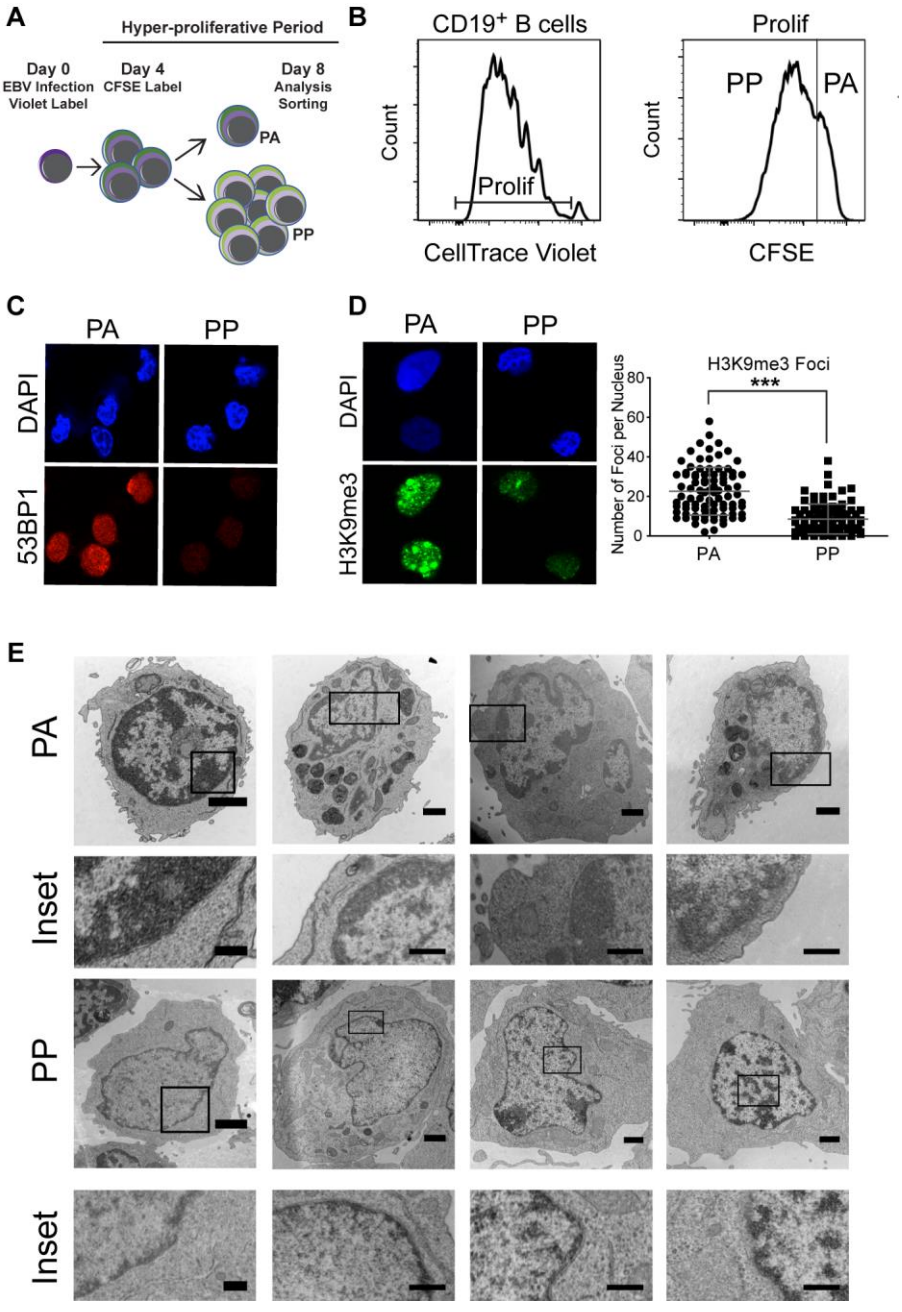


**Figure 5: A subset of EBV induced hyper-proliferating cells exhibit characteristics of OIS.** (*A*) Schematic representing the experimental protocol. (*B*) Histograms showing CD19+ B cell division measured at 8 days post infection. (Left) Proliferation of CD19+ B cells was determined through the dilution of the CellTrace Violet stain. (Right) The cells labeled “Prolif” in the top panel were further analyzed for dilution of the CFSE stain. Cells that dilute the CellTrace Violet stain but not the CFSE stain are considered arrested. (*C*) IF of DAPI (blue) or 53BP1 (red) measured from sorted PA and PP cells (n=3). (*D*, Left) IF of DAPI (blue) or H3K9me3 (green) measured from sorted PA and PP cells (n=3). Error

bars represent SD; ***p<-.001 as determined by a Mann-Whitney test. . n = the number of independent donors tested. (*D*, Right) Quantification of IF. (*E*) Representative TEM images of sorted PA or PP cells (n=2). Magnification for PA and PP ranges from 3,300x to 7,100x. Scale bar is 1 µm. Inset shows heterochromatic DNA (scale bar is 0.5 µm).

### 2.2.2 Senescent EBV-infected B cells cannot be re-stimulated to proliferate

Senescent cells are characterized by inducing an irreversible growth arrest. To further characterize the arrested population of EBV-infected B cells we sorted PA cells to purity and monitored their proliferation over the course of 12 days. Live cell growth was determined by trypan blue exclusion and the number of PA cells were observed to remain constant with no new proliferation or cell death. In contrast, the PP cells continued to proliferate and ultimately transformed into LCLs (Figure 6A). Furthermore, to determine whether the PA population could be re-stimulated to grow after they arrested, we again sorted PA cells to purity and monitored their growth in the presence of the mitogen, CpG DNA as well as mock treatment with DMSO. CpG DNA has been shown to increase cellular proliferation (Aird, Zhang et al. 2013, Nikitin, Price et al. 2014, Aird, Worth et al. 2015). We found that under mitogen and mock treatment, PA cells remained senescent and did not proliferate over nine days, while PP cells continued to proliferate (Figure 6B and C). This data suggests that EBV-infected B cells undergo an irreversible arrest.

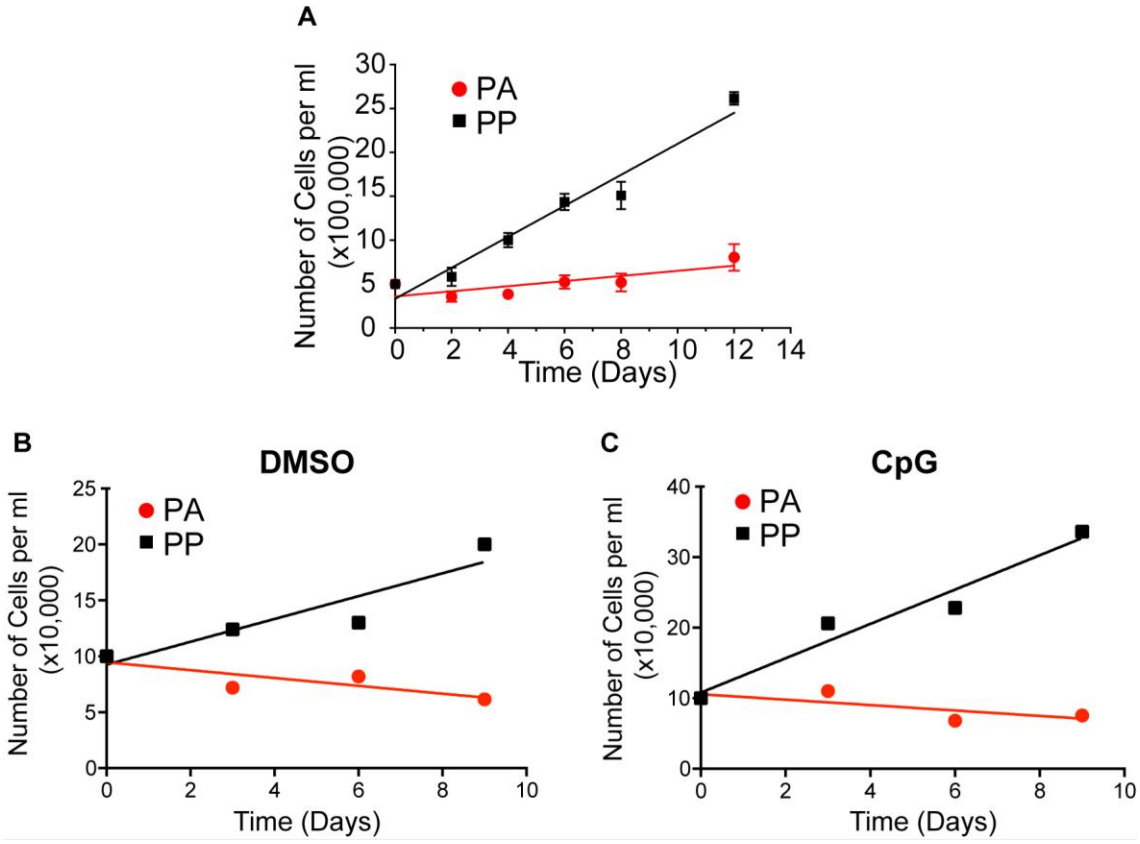


**Figure 6: Arrested EBV-infected B cells demonstrate an irreversible growth arrest and cannot be re-stimulated to grow.** (A) EBV-infected CD19+ B cells were sorted into PA and PP populations and recultured in fresh medium. Samples were counted by trypan blue exclusion every 48 hours. (B) EBV-infected CD19+ B cells were sorted into PA and PP populations and recultured in fresh medium containing 0.1% DMSO. Samples were counted by trypan blue exclusion every 72 hours. (C) EBV-infected CD19+ B cells were sorted into PA and PP populations and recultured in fresh medium containing 2.5 µg/ml CpG. Samples were counted by trypan blue exclusion every 72 hours.

### 2.2.3 Autophagy is elevated during hyper-proliferation

Importantly, induction of autophagy has been linked to the onset of cellular senescence (Young, Narita et al. 2009). To determine if EBV-induced hyper-proliferation was associated with activation of autophagy in arrested cells we assayed for the presence of autophagy, including autophagosomes and phagolysosmes. We observed an increase in the presence of autophagosomes and phagolysosomes by TEM in the PA cells relative

to both the PP cells and LCLs as detected by TEM (Figure 7). This finding was consistent with our positive control that displayed increased autophagosomes and phagolysosomes in LCLs induced to undergo autophagy by bafilomycin treatment (Figure 7). Together, this data suggests that arrested cells induce autophagy upon experiencing hyper-proliferation.

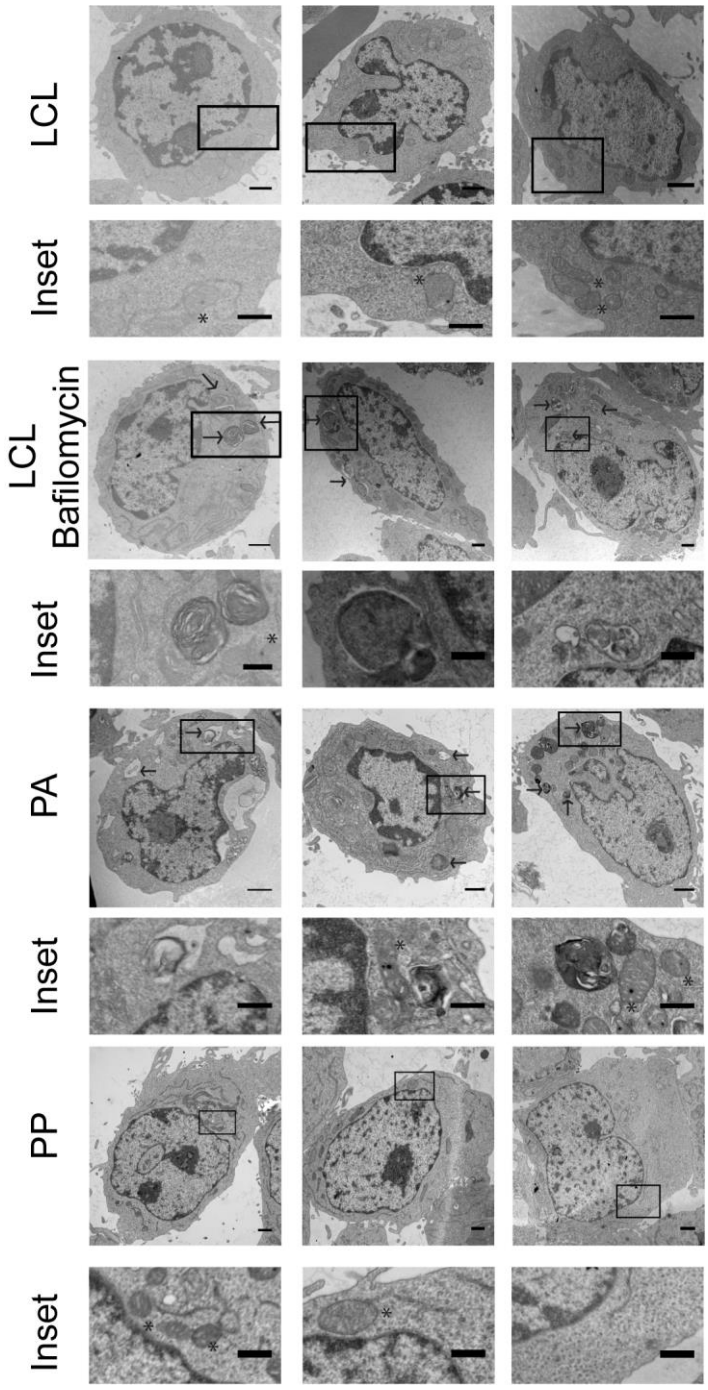


**Figure 7: The arrested cells exhibit increased autophagy.** Representative TEM images of sorted PA and PP cells as well as negative control LCLs and positive control serum starved and bafilomycin-treated LCLs. Arrows indicate autolysosomes and/or phagosomes and stars indicate mitochondria (n=2). (Magnification ranges from 3,400x to 11,500x.) (Scale bar: 1 µm; Inset, 0.5 µm).

## *2.3 Materials and Methods*

### 2.3.1 Viruses and Cells

B95-8 virus was produced from the B95-8 Z-HT cell line as previously described (Johannsen, Luftig et al. 2004). Buffy coats were obtained from normal donors through the Gulf Coast Regional Blood Center and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Histopaque-1077 gradient (Sigma, St. Louis, MO, USA #H8889). Primary cells were cultured in RPMI 1640 with 15% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin and streptomycin (1X, Sigma, #G6784) (R15) and 0.5 µg/ml Cyclosporin A, CsA, (Sigma, #30024). All bulk infections were performed by incubating cells with B95-8 Z-HT supernatants (1 mL per $10^6$ B cells calculated from within PBMC population) for 1 hour at 37°C in a $CO_2$ incubator followed by washing in PBS and resuspending in R15 media + CsA. Typical bulk infections were done on 5 x $10^8$ PBMCs. EBV infection was carried out at an MOI of 5 (such that all infected B cells are positive for EBNA-LP). Lymphoblastoid cell lines (LCLs) were generated from normal donors by continuous growth of EBV-infected primary B cells for greater than two months. LCLs were cultured in RPMI with 10% FBS (R10).

### 2.3.2 Antibodies and Chemicals

Mouse anti-human CD19 antibody (clone 33-6-6, kind gift of Dr. Tom Tedder) conjugated with either APC or PE was used as a surface B cell marker in flow cytometry. All surface B cell markers were used at 1uL per $10^6$ cells. H3K9me3 (Millipore, Burlington, MA, USA #07-442) was used as a marker of heterochromatic DNA at 1:100. DNA damage marker, 53BP1 (Cell Signaling, Danvers, MA, USA #4937) was used at 1:50. LC3 (MBL, Woburn, MA, USA #PM036) was used as a marker for autophagy at 1:500 and Glut1

(Abcam, Cambridge, United Kingdom #Ab115730) was used as a marker for glucose import at 1:250.

Bafilomycin A1 (Sigma, #B1793) was resuspended in DMSO. Thioester stabilized CpG ODN 2006 oligonucleotide, a TLR9 ligand, was purchased from IDT and used at 2.5 μg/ml (Hartmann and Krieg 2000).

### 2.3.3 Double Staining to Capture Early Proliferating and Arresting B Cells

PBMCs were isolated from a buffy coat as described above and stained with a proliferation tracking dye, CellTrace Violet (Invitrogen, #C34557), followed by infection with EBV. The cells were grown in R15 media for four days prior to staining with an additional proliferation tracking dye, 6-carboxyfluorescein succinimidyl ester (CFSE, Sigma, # 21888). The samples were resuspended in fresh R15 media and proliferation was monitored and analyzed as described below.

### 2.3.4 Cell Proliferation Analysis and Cell Sorting

PBMCs were infected with EBV and stained with CellTrace Violet and CFSE using the double staining protocol as described above. Proliferation was monitored in CD19+ B cells by the dilution of the CellTrace Violet stain at day 8 post infection on a BD FACS Canto II. The percent arrested population was determined by calculating the percentage of cells that diluted the CellTrace Violet stain but did not dilute the CFSE stain. Data was analyzed using FlowJo 10.0 software.

CD19+ B cells were sorted for the PA and PP populations based on the CellTrace Violet and CFSE profile using either a Beckman Coulter Astrios or Beckman Coulter MoFlo XDP sorter.

### 2.3.5 Immunofluorescence

Samples were pelleted, resuspended in 25μl of PBS, spread on a microscope slide and dried at 37°C for 15 minutes. Cells were fixed in 4% paraformaldehyde for 15 minutes at 4°C, washed in PBS, permeablized in PBS containing 0.5% Triton X-100 for 10 minutes and then blocked in PBS with 0.2% Triton X-100 containing 5% normal goat serum for 1 hour. Primary antibodies were incubated overnight at 4°C followed by secondary antibody incubation with AlexaFluor 488 goat anti-rabbit IgG (Life Technologies, #A11034) for 2 hours. Slides were mounted in Vectashield (Vector Laboratories, H-1200) containing DAPI. All immunofluorescence slides were visualized using Zeiss 780 upright confocal microscope.

### 2.3.6 Electron Microscopy

B cells were pelleted, washed in serum free RPMI media, and 2% glutaraldehyde was overlayed onto the undisturbed cells. The pellets were scraped into 1-1.5mm piles on parafilm and encased in 1% molten agar. The agar-embedded pellets were washed three times with 0.1M phosphate buffer and further fixed and stained in 1% osmium tetroxide in phosphate buffer for 30 minutes by microwave processing or 1-2 hours at room temperature. They were then dehydrated in a graded series of acetone and infiltrated with EmBed 812 epoxy resin using a 50:50 mixture of 100% acetone for 1 hour and two changes of 100% resin for 1 hour each. After baking at 60°C for 48 hours, ultrathin sections were cut on a diamond knife. Sections were post-stained with 2% aqueous uranyl acetate, washed in water, stained with 1% aqueous lead citrate, and washed again in water. Sections were viewed in an FEI CM 12 electron microscope, and micrographs were recorded on an AMT 2.6K digital camera.

## *2.4 Discussion*

While EBV infection is ubiquitous among adults worldwide, the virus is typically well-controlled by the adaptive T cell immune response. It has been well established that EBV mediates a low transformation efficiency where only 1-10% of infected B cells transforms into LCLs (Henderson, Miller et al. 1977, Sugden and Mark 1977). Recent findings suggest that additional intrinsic cellular responses are important for suppressing the transformative properties of EBV. Previously, our laboratory has shown that EBV infection of primary human B cells facilitates entry into a transient period of hyper-proliferation. The majority of infected B cells that undergo rapid proliferation undergo activation of the DDR, which results in the establishment of a G1/S phase growth arrest (Nikitin, Yan et al. 2010, Nikitin, Price et al. 2014). In this study, we sought to better understand the host cell factors that block transformation and promote arrest during the early stages of EBV infection. We developed a double stain proliferation tracking assay to isolate and characterize the population of cells that begin to proliferate upon infection and subsequently undergo growth arrest early after infection. We found that the early arrested EBV-infected B cells exhibited an increase in the presence of factors that are required to establish OIS. Specifically, arrested cells displayed increased activation of the DDR and induction of global heterochromatin formation as seen by the presence of SAHF. Furthermore, arrested B cells could not be re-stimulated to promote proliferation suggesting that these cells endure an irreversible growth arrest. Importantly, the arrested population of B cells demonstrated an increased presence of autophagic vesicles in the form of autophagosomes and phagolysosomes as opposed to proliferating B cells.

Increased autophagic activity has been linked to the production of senescence-associated interleukins and the establishment of OIS (Young, Narita et al. 2009). Cells

may use autophagy to trigger senescence in an effort to prevent aberrant proliferation induced by activation of viral latent oncoproteins. While autophagy can have both negative and positive implications on viral infection, EBV has developed mechanisms to manipulate autophagy to promote virus propagation during lytic replication (Leidal, Pringle et al. 2012, Williams and Taylor 2012, Granato, Santarelli et al. 2014). Autophagy may play an important role in providing the necessary metabolic intermediates required by a cell to sustain growth during hyper-proliferation. Alternatively, if autophagy targets viral proteins for degradation this may be detrimental to the virus (Williams and Taylor 2012). Previously, little was known about the role of autophagy during latent EBV infection. We found that autophagy is increased in specifically cells that undergo hyper-proliferation. Furthermore, in conjunction with the findings from McFadden et al., EBV up-regulates glycolysis and oxidative phosphorylation and suppresses autophagy during the transition from the hyper-proliferative period through transformation into LCLs (McFadden, Hafez et al. 2016). The viral latent protein, LMP1 has been shown to regulate glycolysis and autophagy in LCLs and further, induce autophagy through the unfolded protein response (Lee and Sugden 2008, Lee and Sugden 2008). While LMP1 has been shown to promote autophagy, it has also been observed to reduce autophagy by upregulating glucose import the NFκB signaling (Sommermann, O'Neill et al. 2011). McFadden et al., also observed an increase in expression of Glut1 and glucose uptake in LCLs as compared to early EBV-infected B cells, which interestingly also display lower LMP1 expression levels (Price, Tourigny et al. 2012, McFadden, Hafez et al. 2016).

In summary, early after EBV infection, viral oncogene activation leads primary human B cells to transit through a burst of hyper-proliferation. Aberrant proliferation is known to be prone to errors in DNA replication, such as stalled or collapsed replication

forks that results in replication stress and activate the DDR signaling pathway (Hills and Diffley 2014). Consequently, stress-induced DDR may trigger the establishment of OIS. Our study identifies that distinct characteristics of OIS are present in infected B cells early after infection. This work suggests that EBV infection triggers OIS early after infection of primary human B cells. In the future, it will be important to determine if the metabolic demands that play an important role in DNA replication, such as nucleotide metabolism, are impaired in the senescent cells that succumb to stress-induced DDR.

# 3. Limited nucleotide pools restrict Epstein-Barr virus-mediated B-cell immortalization

*This chapter was modified based on the research article published by Amy Y. Hafez, Joshua E. Messinger, Karyn McFadden, Gyorgy Fenyofalvi, Caitlin N. Shepard, Gina M. Lenzi, Baek Kim, and Micah A. Luftig in Oncogenesis in 2017 (Hafez, Messinger et al. 2017).*

## *3.1 Rationale*

Aberrant cellular proliferation is first recognized by the DNA damage response, an innate tumor suppressor pathway (Lowe, Cepero et al. 2004, Bartkova, Rezaei et al. 2006, Di Micco, Fumagalli et al. 2006, Meek 2009). The activation of oncogenes by mutation or infection with an oncogenic virus triggers this response due to inappropriate entry into the cell cycle and unscheduled initiation of DNA replication. The DDR has thus come to be recognized as an important barrier to tumorigenesis (Bartkova, Horejsi et al. 2005, Gorgoulis, Vassiliou et al. 2005, Bartkova, Rezaei et al. 2006, Di Micco, Fumagalli et al. 2006, Halazonetis, Gorgoulis et al. 2008). Unscheduled replication initiation induced by oncogene over-expression leads to exposed ssDNA/dsDNA junctions recognized by the ATR/Chk1 DDR signaling pathway, which can also be processed to double-stranded breaks recognized by the ATM/Chk2 pathway (Zou and Elledge 2003, MacDougall, Byun et al. 2007, Marechal and Zou 2013). While normal levels of replicative stress experienced in every cell cycle leads to transient cell cycle arrest and DNA repair, the elevated DDR signaling observed following oncogene activation can promote apoptosis or senescence through signaling to the p53 pathway and other regulators of cell fate (Hirao, Kong et al. 2000, Khanna and Jackson 2001, Gorgoulis, Vassiliou et al. 2005, Verdun, Crabbe et al. 2005, Bartkova, Rezaei et al. 2006).

Our model system for the study of innate tumor suppressor responses is the infection of primary human B cells with the oncogenic herpesvirus Epstein-Barr virus (EBV). While EBV latently infects nearly all adults worldwide, the virus causes B-cell lymphomas in immune suppressed individuals such as those following transplant or HIV infection (Rickinson and Kieff 2007, Longnecker, Kieff et al. 2013). *In vitro*, EBV infection of primary human B cells leads to their transformation into indefinitely proliferating lymphoblastoid cell lines, or LCLs. However, only a small percentage of infected cells actually become LCLs suggesting that innate tumor suppressor responses might restrict long-term outgrowth (Henderson, Miller et al. 1977, Sugden and Mark 1977). Indeed, our laboratory and others have found that the DDR is activated early after infection and the ATM/Chk2 pathway limits outgrowth through activation of a senescence-like growth arrest (Gruhne, Sompallae et al. 2009, Nikitin, Yan et al. 2010, Kamranvar, Chen et al. 2013, Koganti, Hui-Yuen et al. 2014, Nikitin, Price et al. 2014).

Upon initial B-cell infection by EBV, the viral latent oncoproteins EBNA2 and EBNA-LP coordinate the up-regulation of cellular gene expression that promotes cell cycle entry and rapid DNA replication and cell division (Alfieri, Birkenbach et al. 1991, Wang, Kikutani et al. 1991, Sinclair, Palmero et al. 1994). At three days post infection, EBV-infected B cells undergo a burst of proliferation in which cells divide up to three or four times within 24 hours. Following this initial period of hyper-proliferation, the infected cells slow their proliferation rate to approximately one division per 24 hours. The proliferative burst correlates with a period of activated DNA damage signaling. We found that the ATM/Chk2 pathway is activated and promotes a senescence-like growth arrest in these infected cells (Nikitin, Yan et al. 2010). Others have found that these cells also display elevated reactive oxygen species and telomere dysfunction that may induce ATM pathway

activation (Kamranvar and Masucci 2011, Kamranvar, Chen et al. 2013, Chen, Kamranvar et al. 2016). Early infected cells also display aberrant karyotypes, but as infected cells grow out over a period of weeks into LCLs the DNA damage signaling wanes and these cells display stable karyotypes (Lacoste, Wiechec et al. 2009).

Given the rapid proliferation of early EBV-infected cells, cellular DNA replicative stress during infection is a likely candidate for the upstream molecular source of the activated DNA damage observed in this system. Indeed, activation of the sensor of replicative stress, ATR, has been reported in early EBV-infected B cells.(Koganti, Hui-Yuen et al. 2014) In other systems both *in vitro* and *in vivo*, the over-expression of oncogenes including c-Myc, H-ras$^{G12V}$, cyclin E, and human papilloma virus E6 and E7 promotes replicative stress and ATR/Chk1 pathway activation (Gilad, Nabet et al. 2010, Bester, Roniger et al. 2011, Jones, Mortusewicz et al. 2013, Srinivasan, Dominguez-Sola et al. 2013). Increased activity of this pathway triggers senescence while loss of one allele of ATR or Chk1 partially overrides senescence leading to increased genomic instability and tumorigenicity (Gilad, Nabet et al. 2010). However, at a higher level of ATR pathway inhibition the growth of tumors harboring oncogenic mutations can be suppressed with minimal impact on highly proliferative normal tissues, highlighting ATR inhibition as a promising therapeutic strategy (Myers, Gagou et al. 2009, Murga, Campaner et al. 2011, Schoppy, Ragland et al. 2012). Inhibitors of this pathway are currently under development for a wide range of tumors that display high proliferative rates and increased markers of replicative stress (Toledo, Murga et al. 2011).

Recent studies linking the metabolic demands of oncogene-induced rapid cell proliferation to the DNA damage response suggest that maintenance of deoxyribonucleotide triphosphate (dNTP) pools is critical to prevent DDR activation and

oncogene-induced senescence (Halazonetis, Gorgoulis et al. 2008, Bester, Roniger et al. 2011, Aird, Zhang et al. 2013, Jones, Mortusewicz et al. 2013, Srinivasan, Dominguez-Sola et al. 2013). Specifically, HPV16 E6 and E7 expression in keratinocytes and H-Ras$^{G12V}$ in fibroblasts leads to dNTP depletion and DNA replicative stress (Bester, Roniger et al. 2011, Aird, Zhang et al. 2013). In both settings, providing exogenous nucleosides rescues replicative stress and overcomes oncogene-induced senescence. Therefore, regulation of dNTP pools is critical to support early steps in oncogenesis.

In the present study, we examine the role of dNTP pools and replicative stress in regulating B-cell immortalization by EBV. Our prior work indicates that during the hyper-proliferative burst following EBV infection, cells that succumb to ATM/Chk2-mediated growth arrest have failed to up-regulate oxidative phosphorylation and genes associated with mitochondrial biogenesis (McFadden, Hafez et al. 2016). We hypothesize that this metabolic imbalance may lead to a deficiency in dNTP levels necessary to sustain hyper-proliferation during the first rounds of B-cell division after infection. In this study, we directly measured DNA damage and replicative stress during early and late times after EBV infection of primary human B cells and assessed the role of the ATR/Chk1 signaling pathway in B-cell outgrowth. We also measured the levels of dNTPs during the course of infection and assessed the role of dNTP pools in B-cell immortalization by EBV.

## *3.2 Results*

### 3.2.1 EBV infection of primary human B cells leads to increased ATR pathway activation early after infection

We previously reported that upon EBV infection, B cells undergo a transient period of hyper-proliferation during which a cell can divide up to four times in one day (Nikitin, Yan et al. 2010). This increased division rate requires that infected cells replicate their

genomes very rapidly, potentially leading to DNA replicative stress and activation of the ATR/Chk1 signaling pathway. To determine whether EBV induces ATR pathway activation, we analyzed infected B cells as they emerged from the resting state using a flow cytometry-based sorting approach and immunofluorescence for phosphorylated/activated ATR (P-ATR Ser428) and phosphorylated RPA32 (P-RPA Ser4/8). We first stained peripheral blood mononuclear cells (PBMCs) with a proliferation tracking dye, Cell Trace Violet (CTV), and then infected with EBV at a multiplicity of infection such that every $CD19^+$ B cell is latently infected with EBV (Nikitin, Yan et al. 2010). At four days post infection, we sorted the majority of infected PBMCs for CD19 positivity and dilution of CTV thereby isolating a purified population of infected, early rapidly proliferating B cells (Figure 8A). We allowed the remainder of the unsorted, infected cells to continue to proliferate for an additional eight days and sorted again on CD19 and dilution of CTV, which had attenuated their proliferation rate as shown previously (Nikitin, Yan et al. 2010). We assayed these two cell populations for P-ATR and P-RPA32 by measuring the number of markers of replicative stress foci per nucleus. We found that early proliferating B cells exhibited significantly more activated P-ATR and P-RPA32 than late proliferating B cells (Figure 8B-E). We also found that EBV-immortalized LCLs displayed low levels of P-ATR and P-RPA32, similar to late proliferating cells, and LCLs induced to undergo replicative stress by treatment with hydroxyurea (HU) displayed significantly elevated levels of P-ATR and P-RPA32 foci (Figure 8B-E). These data suggest that EBV induced hyper-proliferation triggers ATR pathway activation similar to that observed by others (Koganti, Hui-Yuen et al. 2014).

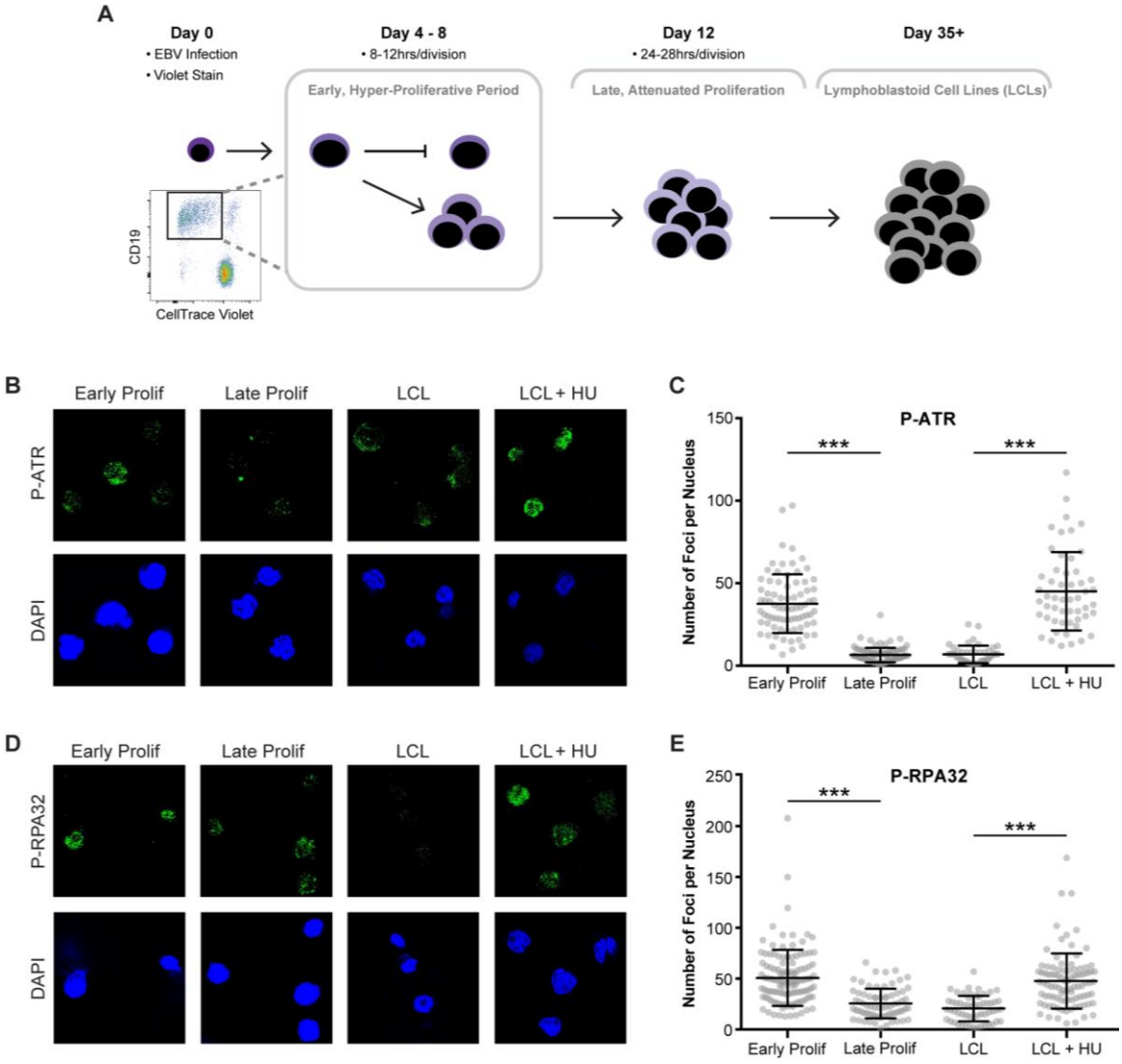


**Figure 8: ATR pathway is activated in early, rapidly proliferating EBV-infected B cells and attenuated following hyper-proliferation.** (A) Schematic demonstrating staining, infection and sorting protocol to separate early, hyper-proliferating populations (PA: proliferating, then arrested and PP: proliferating, then proliferating further), from the later, slower proliferating population, and then LCLs. (B) Immunofluorescence (IF) of phosphorylated ATR S428 (P-ATR) (green) and DAPI (blue) measured from sorted early and late proliferating B cells, untreated LCLs, and 3mM hydroxyurea (HU) treated LCLs. (C) Number of P-ATR S428 foci per nucleus of sorted early and late proliferating B cells, untreated LCLs, and 3mM HU treated LCLs. Error bars represent SD of three independent donors. ***P < 0.001 as determined by a Mann-Whitney test. (D) IF of phosphorylated RPA32 S4/S8 (P-RPA32) (green) and DAPI (blue) measured from sorted early and late proliferating B cells, untreated LCLs, and 3mM HU treated LCLs. (E) Number of P-RPA32 S4/S8 foci per nucleus of sorted early and late proliferating B cells, untreated LCLs, and

3mM HU treated LCLs. Error bars represent SD of three independent donors. ***P < 0.001 as determined by a Mann-Whitney test.

### 3.2.2 Early proliferating EBV-infected B cells are more sensitive to ATR and Chk1 pathway inhibition than late proliferating infected cells

Activation of the ATR signaling pathway in response to oncogene over-expression has been shown to provide a specific vulnerability for cell survival. Several groups have demonstrated that loss of function in the ATR/Chk1 pathway triggers apoptosis in cells with activated oncogene signaling or other inducers of replication stress (Myers, Gagou et al. 2009, Murga, Campaner et al. 2011, Toledo, Murga et al. 2011, Schoppy, Ragland et al. 2012). To further investigate the role of the ATR pathway in EBV-infected B cells, we used a pharmacological approach targeting ATR and Chk1. First, we assessed the level of apoptosis induced by a selective ATR inhibitor (ATRi), VE821 (Charrier, Durrant et al. 2011), during the hyper-proliferative period early after infection. As indicated in Figure 9A, we observed a selective increase in apoptosis as measured by Annexin V positivity in cells undergoing hyper-proliferation following EBV infection (day 4) relative to those that had proceeded beyond the hyper-proliferative period (day 12) or LCLs (>day 35). As a complementary approach, we assessed the relative sensitivity of B cells to EBV transformation in the presence of ATRi at early and late times after infection. We observed that transformation was more potently inhibited by ATRi when administered at early times post infection (day 0) as compared to later times (day 12) (Figure 9B-D). These data collectively suggest that EBV-infected B cells undergoing hyper-proliferation, while ATR is activated due to replicative stress, are also hyper-sensitive to ATR inhibition and depend on ATR for their survival.

To corroborate these findings and assess the role of the downstream ATR effector Chk1, we selectively inhibited this kinase using CHIR124 (Ni, Barsanti et al. 2006).

Consistent with the ATR inhibition results, we observed that EBV-infected cells treated prior to hyper-proliferation were more sensitive to Chk1 inhibition than those treated following the hyper-proliferative period or LCLs (Figure 9E). Furthermore, inhibition of Chk1 during the hyper-proliferative period dramatically suppressed EBV transformation, while treatment at later times during infection had a less pronounced effect (Figure 9F-H). Thus, we conclude that ATR and Chk1 protect early proliferating EBV-infected B cells from apoptosis during the hyper-proliferative period and therefore are critical for long-term outgrowth into LCLs. This ATR and Chk1 protective function is consistent with reports from the replicative stress field (Buisson, Boisvert et al. 2015).

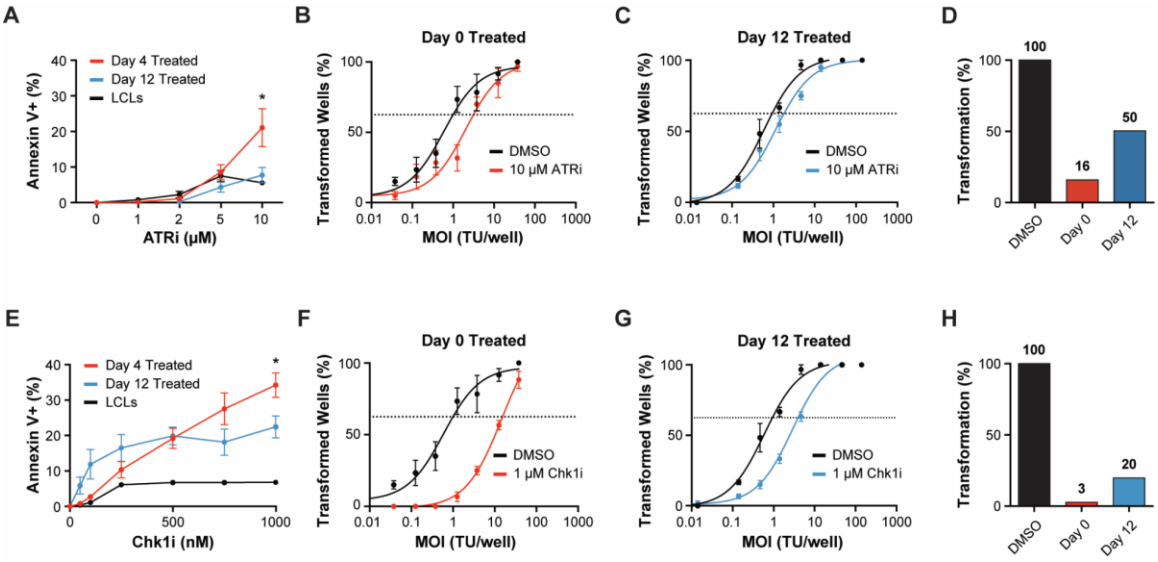


**Figure 9: Early EBV-infected B cells are more sensitive to ATR and Chk1 inhibition than late proliferating B cells.** (A) Percentage of Annexin V positive EBV-infected B cells treated with increasing concentration of ATR inhibitor, VE-821, on days 4 (red) or 12 (blue) post infection. Error bars represent SEM of six independent donors and three LCLs. *P <0.05 as determined by Student's t-test. (B) Quantification of EBV-induced B-cell outgrowth in the presence of either 0.1% DMSO (black) or 10μM VE-821 (red) on day 0 post infection. The percentage of wells positive for LCL outgrowth 5 weeks post infection is shown relative to the transforming units (TU) of EBV B95.8 per well. Error bars represent SEM of three independent donors. (C) Similar experiments were performed as in (B), except that cells were treated with 10μM VE-821 (blue) on day 12 post infection. (D) Quantification of transformation efficiency from (B) and (C). (E) Percentage of Annexin V positive EBV-infected B cells treated with increasing concentration of Chk1 inhibitor, CHIR-124, on the days 4 (red) or 12 (blue) post infection. Error bars represent SEM of six independent donors and three LCLs. *P <0.05 as determined by Student's t-test. (F) Quantification of EBV-induced B-cell outgrowth in the presence of either 0.1% DMSO (black) or 10μM CHIR-124 (red) on day 0 post infection. (G) Similar experiments were performed as in (F), except that cells were treated with 1μM CHIR-124 (blue) on day 12 post infection. (H) Quantification of transformation efficiency from (F) and (G).

### 3.2.3 Early proliferating EBV-infected B cells experience DNA damage and replication stress, which is resolved later in the immortalization process

Here we have reported that early proliferating EBV-infected B cells activate the ATR/Chk1 replicative stress pathway and require this pathway for survival. However, several groups have demonstrated DDR pathway activation in the absence of overt signs

of DNA damage (Soutoglou and Misteli 2008, Toledo, Murga et al. 2008). Therefore, we sought to directly measure the presence of DNA damage and replicative stress in early EBV-infected primary B cells.

To assess DNA damage, we conducted a fluid halo assay, which is capable of detecting DNA single stranded breaks or nicks (Vogelstein, Pardoll et al. 1980, Roti Roti and Wright 1987, Courbet, Gay et al. 2008). EBV-infected, proliferating B cells were sorted early or late after infection. These cells were gently lysed to release the nuclei. Nuclei were de-chromatinized by high salt solution and the resulting nuclear halos, consisting of supercoiled DNA loops periodically attached to the central nuclear matrix, were stained with an over-winding concentration of the DNA intercalating agent, SYBR gold (10X) (Figure 10A). Since an over-winding concentration was used, cells experiencing DNA damage will have longer chromatin loops evidenced as larger nuclear halos, while undamaged DNA would remain tightly compacted and those nuclei would have smaller halos (Vogelstein, Pardoll et al. 1980, Roti Roti and Wright 1987 ). Consistently, control LCLs treated with hydrogen peroxide display larger halo sizes than untreated LCLs when halo assays were performed using over-winding concentrations (Figure 10B and C). We observed that a greater percentage of early proliferating EBV-infected B cells displayed DNA damage as compared to late proliferating cells and LCLs (Figure 10C).

Next, we investigated the presence of replicative stress as a cause of this DNA damage. When replicative stress occurs, latent origins of replication can fire to help compensate for increased DNA replication demands (Courbet, Gay et al. 2008). To detect changes in replication, we performed fluid halo assays, but at relaxation concentrations of SYBR Gold (0.95X) such that latent origins associating with the nuclear matrix generate smaller nuclear halos (Figure 10D) (Buongiorno-Nardelli, Micheli et al. 1982). Firing of the

dormant origins help compensate for the decreased replication speed or increased DNA replication demands (Courbet, Gay et al. 2008). Consistently, triggering of replicative stress by inhibiting DNA replication in LCLs with aphidicolin led to substantially decreased halo sizes relative to untreated LCLs (Figure 10E and F). When we assayed early and later proliferating EBV infected cells, we found that the early hyper-proliferating cells displayed greater levels of replicative stress than those at later times post infection (day 12 or LCLs) (Figure 10F). Taken together, these data suggest that replicative stress occurs in early proliferating EBV-infected B cells, which might lead to the observed DNA damage that will be repaired later in those cells that continue to proliferate past the period of hyper-proliferation.

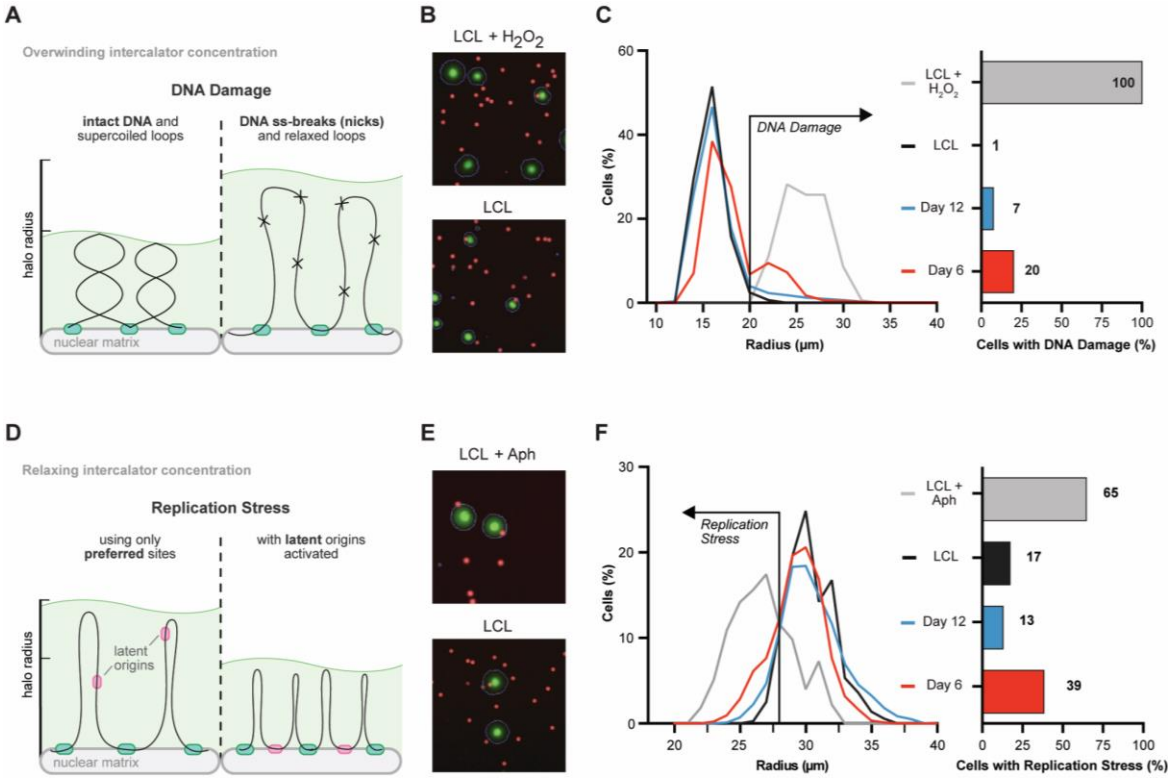


**Figure 10: EBV infected B cells experience DNA damage and replicative stress early after infection.** (A) Schematic of fluid halo assay investigating DNA damage. (B) Representative images of control LCLs that were untreated or treated with hydrogen peroxide to induce DNA damage and subsequently increase halo size. Blue outline indicates halo boundaries as detected by Cellomics ArrayScan. (C) Histogram showing distribution of halo sizes of sorted early (day 5.5) and late proliferating B cells (day 12), untreated LCLs, and hydrogen peroxide treated LCLs. Cells experiencing DNA damage were gated such that all radii greater than untreated LCLs were considered to be experiencing DNA damage. (D) Schematic of fluid halo assay investigating replicative stress. (E) Representative images of control LCLs that were untreated or treated with aphidicolin to induce replicative stress and subsequently reduce halo size. Blue outline indicates halo boundaries as detected by Cellomics ArrayScan. (F) Histogram showing distribution of halo sizes of sorted early (day 5.5) and late proliferating B cells (day 12), untreated LCLs, and aphidicolin treated LCLs. Cells experiencing replicative stress were gated such that all radii smaller than untreated LCLs were considered to be undergoing replicative stress.

### 3.2.4 Limited dNTP pools in early proliferating B cells suppresses EBV-mediated transformation

The presence of DNA damage and replicative stress in EBV-infected early, rapidly proliferating B cells suggests that nucleotide pools may be limiting in these cells. Therefore, we sought to measure the levels of individual deoxyribonucleotide triphosphates (dNTPs) following EBV infection of primary B cells, during early proliferation, and through LCL outgrowth. We found that early proliferating B cells contained much higher levels of dNTPs relative to resting B cells, as expected. However, in the transition from early proliferation through LCL outgrowth, the dNTP levels were further increased, particularly for the purine dNTPs (Figure 11A).

We next sought to determine whether this relative limitation in dNTPs during early proliferation might functionally impede the outgrowth of EBV-immortalized cells. We supplemented the B-cell growth media with adenosine, guanosine, cytosine, uridine, and thymidine (AGCTU) concurrent with EBV infection and this led to an increase in the number of CD19+ proliferating B cells at day 14 post-infection relative to untreated cells (Figure 11B). However, supplementation of LCLs with AGCTU nucleosides had no effect on B cell proliferation (Figure 11B). Furthermore, we observed that nucleoside supplementation overcame a previously defined G1/S phase arrest that occurs prior to OIS in these early-infected cells (Figure 11C) (McFadden, Hafez et al. 2016). Importantly, supplementation with nucleosides rescued replicative stress as observed by a decrease in P-ATR replicative stress foci (Figure 11D and E).

To determine if low levels of dNTP pools contributed to the restriction of EBV-mediated long-term outgrowth, we simultaneously infected PBMCs with EBV and supplemented the growth media with AGCTU nucleosides. We observed a significant

increase in EBV-mediated transformation efficiency with supplementation of nucleosides relative to the DMSO-treated infected PBMCs (Figure 11F). We next assessed whether the time of addition of nucleosides was important in regulating transformation efficiency given our findings of elevated replicative stress markers only during the early hyper-proliferative phase of latent infection. Nucleosides supplemented concurrent with infection markedly increased transformation efficiency of EBV-infected B cells; however, addition of nucleosides on day 12 post infection, after the hyper-proliferative period, had no effect on transformation efficiency (Figure 11G). Collectively, these findings suggest that limited nucleotide pools contribute to replicative stress and arrest of early proliferating B cells and ultimately suppress EBV-mediated transformation.

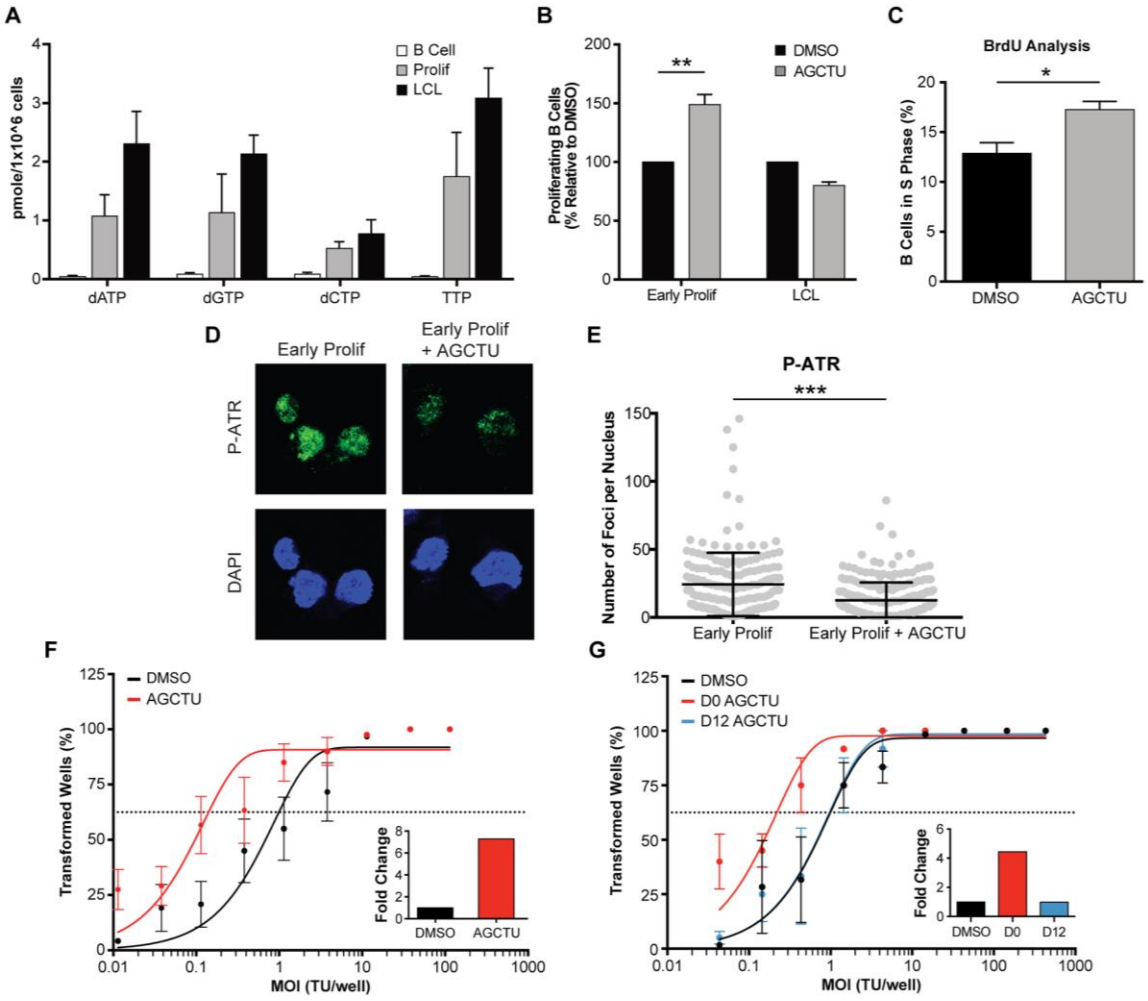


**Figure 11: Nucleoside supplementation rescues EBV-induced growth arrest and presence of replicative stress.** (A) Quantification of individual deoxyribonucleotide pools, including dATP, dGTP, dCTP and TTP measured from sorted uninfected B cells (white), infected early proliferating B cells (grey), and LCLs (black). (B) Percentage of proliferating CD19+ B cells was determined for early proliferating infected B cells and LCLs that were treated with DMSO (black) or supplemented with 30μM nucleosides (AGCTU) (grey) at the time of infection. The data were analyzed by FACS at day 14 post infection. Error bars represent SEM of three independent donors. **P < 0.01 as determined by a Student's t-test. (C) Percentage of B cells in S phase that were treated with DMSO (black) and supplemented with 30μM nucleosides (AGCTU) (grey) at time of infection. The BrdU cell cycle profiles were analyzed by FACS on day 6 post infection. Error bars represent SEM of three independent donors. *P < 0.05 as determined by a Student's t-test. (D) IF of P-ATR (green) and DAPI (blue) measured from early proliferating B cells mock-treated with DMSO (Early Prolif) and supplemented with 30 μM nucleosides on Day 0 (Early Prolif + AGCTU). (E) Number of P-ATR S428 foci per nucleus of sorted early proliferating B cells mock-treated with DMSO (Early Prolif) and 30 μM nucleosides (AGCTU) supplemented on Day 0 (Early Prolif+AGCTU). (F) Quantification of EBV-infected B cell outgrowth

following PBMC infection in the presence of DMSO (black) or 30μM nucleosides (AGCTU) (red) at time of infection. The percentages of wells positive for LCLs at 5 weeks post infection are plotted relative to the transforming units (TU) of B95-8 virus per well. Error bars represent SEM. Dotted line represents 62.5% positive wells, which indicates outgrowth from the virus amount in the x axis of a single LCL per well based on a Poisson's distribution. (F, inset) Fold change of the transformation efficiency. (G) Similar experiments were performed as in (D), except treating with DMSO (black) at the time of infection, 30μM nucleosides (AGCTU) (red) at the time of infection (red) and day 12 post infection (grey). (G, inset) Fold change of the transformation efficiency.

### 3.2.5 Purine dNTP pools are reduced in early proliferating EBV-infected B cells and supplementation with purine nucleosides alone rescues viral-mediated transformation

To further investigate the effect of nucleotide pool depletion on arrest of EBV-infected B cells we specifically measured individual dNTP pools of arrested B cells. Using a previously established double-staining technique to track proliferation of cells using CellTrace Violet and 6-carboxyfluorescein sucinimidyl ester (CFSE), we were able to separate the EBV-infected B cells that proliferate and then arrest (PA) early after infection compared to the proliferating B cells that continue to proliferate (PP) (McFadden, Hafez et al. 2016) (Figure 12A). We measured dNTP levels in these populations and found that the PA population exhibited lower levels of purine dNTP pools (dATP and dGTP) compared with the PP population (Figure 12B).

We next wanted to determine whether limited purine dNTP pools, specifically, influenced cellular arrest and suppression of EBV-mediated transformation. We supplemented the B-cell growth media with only adenosine and guanosine ribonucleosides (AG) on day 0 post infection and conducted FACS on day 14 post-infection to analyze early proliferation of CD19+ B cells. Early proliferating B-cells exhibited a similar increase in B-cell proliferation with supplementation purine nucleosides as that seen in Figure 11B with AGCTU nucleoside addition (Figure 12C). Similar to that observed with AGCTU, LCL proliferation was unaffected by AG supplementation (Figure

12C). To gain a functional understanding of the role of purine nucleosides in long-term EBV outgrowth, we conducted transformation assays by supplementing the growth media with purine nucleosides. Purine nucleosides supplemented on day 0 post infection increased transformation efficiency of EBV-infected B cells by 3-fold over DMSO-treated infected cells (Fig 12D). Together these findings suggest that purine nucleotide pools are a uniquely limiting factor for EBV-mediated hyper-proliferation and transformation.

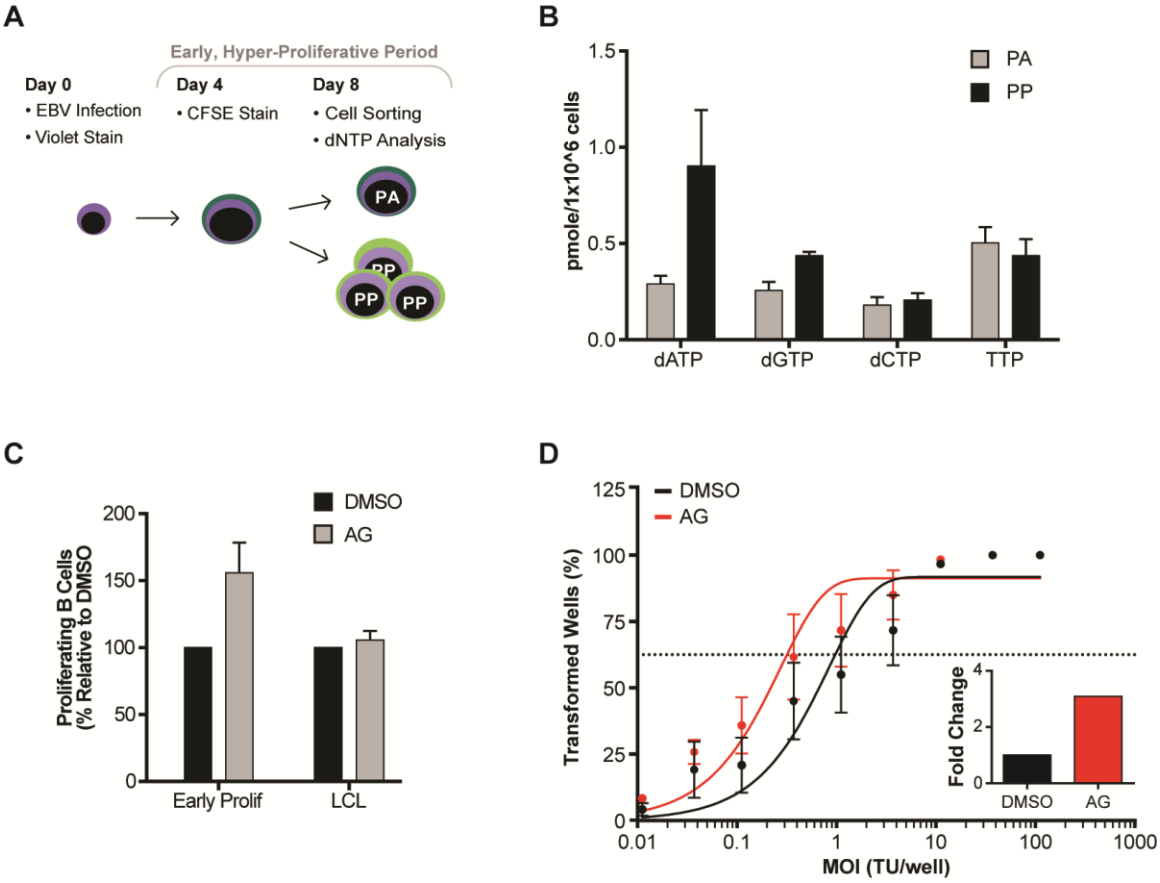


**Figure 12: Supplementation with purine nucleosides alone rescues EBV-induced arrest.** (A) Schematic demonstrating staining, infection and sorting protocol to separate proliferating, then arrested (PA) from proliferating, then proliferating further (PP) cells within the early proliferating period. (B) Quantification of individual deoxyribonucleotide pools, including dATP, dGTP, dCTP and TTP measured from sorted PA cells (grey) and PP cells (black). (C) Percentage of proliferating CD19+ B cells was determined for early proliferating infected B cells and LCLs that were treated with DMSO (black) or supplemented with 30μM purine nucleosides (AG) (grey) at the time of infection. The data were analyzed by FACS at day 14 post infection. Error bars represent SEM of three independent donors. (D) Quantification of EBV-infected B cell outgrowth following PBMC infection in the presence of DMSO (black) or 30μM purine nucleosides (AG) (red) at time of infection similar to experiments performed in (Fig 4. D and E). (E, inset) Fold change of the transformation efficiency.

## *3.3 Materials and Methods*

### 3.3.1 Viruses and Cells

B95-8 virus was produced from the B95-8 Z-HT cell line as previously described (Johannsen, Luftig et al. 2004). Buffy coats were obtained from normal donors through the Gulf Coast Regional Blood Center and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Histopaque-1077 gradient (Sigma, St. Louis, MO, USA #H8889). Primary cells were cultured in RPMI 1640 with 15% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin and streptomycin (1X, Sigma, #G6784) (R15) and 0.5 µg/ml Cyclosporin A, CsA, (Sigma, #30024). All bulk infections were performed by incubating cells with B95-8 Z-HT supernatants (1 mL per $10^6$ B cells calculated from within PBMC population) for 1 hour at 37°C in a $CO_2$ incubator followed by washing in PBS and resuspending in R15 media + CsA. Typical bulk infections were done on 5 x $10^8$ PBMCs. Lymphoblastoid cell lines (LCLs) were generated from normal donors by continuous growth of EBV-infected primary B cells for greater than two months. LCLs were cultured in RPMI with 10% FBS (R10).

### 3.3.2 Chemicals

Hydroxyurea (Sigma, #H8627) was resuspended directly in R15 media at 3 mM. Adenosine (Sigma, #A9251), Cytosine (Sigma, #C3506), Thymidine (Sigma, #T9250), and Uridine (Sigma, #U3750) were resuspended at 3 mM in UltraPure distilled water (Invitrogen, Carlsbad, CA, USA, #10977-015). Guanosine (Sigma, #G6752) was resuspended at 30 mM in DMSO. Both VE-821 and CHIR-124 (Selleckchem, Boston, MA, USA #S8007 and #S2683, respectively) were resuspended in DMSO at 10 mM.

### 3.3.3 Antibodies

Mouse anti-human CD19 antibody (clone 33-6-6, kind gift of Dr. Tom Tedder) conjugated with either APC or PE was used as a surface B cell marker in flow cytometry. Mouse anti-human CD19 PE-Cyanine7 antibody (eBioscience, San Diego, CA, USA #25-0199-42) was used as an additional B cell surface marker. All surface B cell markers were used at 1uL per $10^6$ cells. Phosphorylated ATR (S428) (Santa Cruz Biotechnology, Dallas, TX, USA #sc-109912) and Phosphorylated RPA32 (S4/S8) (Bethyl Laboratories, Montgomery, TX, USA #A300-245A) were used as markers of replicative stress for immunofluorescence at 1:50 and 1:500, respectively.

### 3.3.4 Infections and Cell Sorting

PBMCs were isolated from a buffy coat and stained with CellTrace Violet using the manufacturer's suggested protocol (Invitrogen, #C34557) followed by infection with EBV at an MOI of 5 (such that all infected B cells are positive for EBNA-LP). Proliferation was monitored in CD19+ B cells by the dilution of the CellTrace Violet stain for up to 14 days post infection on a BD FACS Canto II and analyzed using FlowJo 10.0 software (TreeStar). CD19 positive cells were sorted into early and late population doublings based upon their CellTrace Violet profile using either a Beckman Coulter Astrios or Beckman Coulter MoFlo XDP sorter. Sorting to capture early proliferating and late proliferating populations were conducted as follows:

#### 3.3.4.1 Immunofluorescence Analysis

Infected B cells were sorted such that cells were isolated that corresponded to populations of either 1-2 divisions or greater than 5 divisions as determined by CellTrace Violet profile on day 4 and 12, respectively.

#### 3.3.4.2 Fluid Halo Assays

Cell populations that doubled 1-2 times were sorted on day 5.5 and populations that doubled over 5 times were sorted on day 12 for analysis by Fluid Halo assay. Populations positive for propidium iodide were gated out to remove dead cells.

#### 3.3.4.3 dNTP Analysis

Infected cell populations were sorted on day 8 for proliferating B cells that divided over 5 times. Alternatively, to specifically capture early proliferating and arresting B cells infected PBMCs were stained with CellTrace Violet (Invitrogen, #C34557) on Day 0 post infection. The cells were cultured in R15 media for 4 days prior to staining with 6-carboxyfluorescein succinimidyl ester (CFSE, Sigma, #21888). The samples were resuspended in fresh R15 media and cells were sorted into arrested and proliferating populations on day 8 based on both the CellTrace Violet and CFSE fluorescence profile.

### 3.3.5 Immunofluorescence

EBV B95-8-infected B cells were pelleted, resuspended in 25μl of PBS, spread on a microscope slide and dried at 37°C for 15 minutes. Cells were fixed in 4% paraformaldehyde for 15 minutes at 4°C, washed in PBS, permeablized in PBS containing 0.2% Triton X-100 for 10 minutes and then blocked in PBS with 0.2% Triton X-100 containing 5% normal goat serum for 1 hour. Primary antibodies were incubated overnight at 4°C followed by secondary antibody incubation with AlexaFluor 488 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA #A11034) for 2 hours. Slides were mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA #H-1200). All immunofluorescence slides were visualized using Zeiss 780 upright confocal microscope and images were analyzed using ImageJ version 2.0.

### 3.3.6 Fluid Halo Assay

Cells were harvested and resuspended with $10^6$ cells/mL in an isotonic, low ionic strength lysis buffer containing 300 mM sucrose, 20mM Tris pH 7.0, 1 mM spermine, and Triton X-100 0.1% (w/w) on ice for 7 min and infused with APC calibrate beads (BD Biosciences, Franklin Lakes, NJ, USA 340386). Cells were transferred in 100 µL aliquots into the wells of alpha-poly-L-lysine (MW 150 000-300 000, Sigma) coated 96 well plates and centrifuged at 1000xg for 10 min at 4°C. Halos were generated by diluting each well 1:1 with nuclear extraction buffer containing 20 mM Tris pH 7.5, 2.22 M NaCl, 1 mM EGTA, and 5 mM EDTA with either an over-winding (10x) or relaxation (0.95x) concentration of SYBR Gold (Thermo Fisher Scientific, Waltham, MA, USA #S11494) Plates were then sealed and analyzed by Cellomics ArrayScan $V^{TI}$ analyzer for nuclear halo size. Control LCLs were treated with either aphidicolin (Sigma, A0871), which was dissolved in DMSO and stored at 1 mg/mL or hydrogen peroxide (Sigma, #216763) which was stored as a 30% wt stock solution (100 000x) in sterile $H_2O$ to induce replicative stress or DNA damage, respectively. Cells experiencing replicative stress were determined to have smaller halo sizes than LCLs. Cells experiencing DNA damage were determined to have larger halo sizes than LCLs. Doublets and larger clusters were eliminated from the analysis based on object shape, whereas cell debris was excluded by object intensity. To prevent the inclusion of halos in the analysis that could have been damaged by the light source of the scanning microscope, only the first nine vision fields were included in the analysis from each well.

### 3.3.7 Apoptosis Assays

PBMCs were isolated, stained with CellTrace Violet and infected with EBV B95-8 as previously described. On day 4 or day 12, cells were plated into 24-well plates and

treated with 0.1% DMSO or 1, 10, 50, 100, 1000, 2000, 5000, or 10000 nM VE-821. For the CHIR-124 assays, cells were treated either with 0.1% DMSO or 50, 100, 250, 500, 750 or 1000 nM CHIR-124. Two days post treatment cells were stained with CD19-PE and AnnexinV-APC (eBioscience, #17-8007-74) or AnnexinV-FITC (Biolegend, San Diego, CA, USA #640906) for 20 minutes at 4°C before FACS analysis. Proliferating cells were determined by CellTrace Violet profile and this population was gated to determine AnnexinV positive cells. All FACS data was analyzed using FlowJo 10.0 software.

### 3.3.8 Nucleotide Pool Measurement

dNTP extraction and measurement was conducted as previously published (Diamond, Roshal et al. 2004).

### 3.3.9 Cell Cycle Analysis

Isolated PBMCs were infected with EBV B95-8 and supplemented with 0.1% DMSO, 30μM AGCTU, 30μM AG, on Day 0 post infection. BrdU cell cycle analysis was conducted on Day 6 post infection using BD Pharmingen APC Flow Kit (#552598) and analyzed using FlowJo 10.0 software.

### 3.3.10 Transformation Assay

EBV B95-8 infection of human PBMCs was performed in the presence of 0.1% DMSO, 30μM AGCTU, 30μM AG, 1 μM CHIR-124 or 10 μM VE-821 added at varying times post infection. B95-8 Z-HT supernatant was titrated from 300 $\mu L/10^7$ PBMCs to 0.03 $\mu L/10^7$ PBMCs. 7 x $10^6$ infected PBMCs were seeded in 20 wells of a 96-well plate for each infection point. The percentage of wells positive for B cell outgrowth (LCL) at 5 weeks post infection was plotted relative to the MOI per well. The efficiency of transformation was

determined as published where the amount of B95-8 virus necessary to yield 62.5% of positive wells was considered 1 transforming unit (TU) per well.

## *3.4 Discussion*

The recognition of oncogene-mediated aberrant proliferation by the DNA damage response signaling pathway is among the earliest innate tumor suppressor responses. When EBV infects primary B cells, it must drive cell proliferation to establish a reservoir of latently infected cells. We have previously found that EBV induces rapid proliferation such that at approximately three days post infection the first three to four cell divisions occur within a 24-hour period (Nikitin, Yan et al. 2010). The majority of infected cells that begin this hyper-proliferation program ultimately succumb to an ATM/Chk2-dependent senescence-like growth arrest (Nikitin, Yan et al. 2010, McFadden, Hafez et al. 2016). In this study, we sought to determine the upstream molecular source of the DDR. We found that early EBV-infected hyper-proliferating cells display evidence of DNA damage and replicative stress using fluid halo assays and assays for ATR/Chk1 pathway activation. We further observed reduced dNTPs, particularly purine dNTPs, in early-infected cells that led to their inefficient outgrowth. Supplementation of nucleosides rescued activation of replicative stress markers and cellular arrest, further supplementation of specifically purine nucleosides at early times post infection, facilitated EBV-mediated B-cell outgrowth suggesting that purine biosynthesis is a major limiting step in EBV transformation.

Depletion of dNTP pools has been linked to induction of replicative stress. We demonstrated that early, hyper-proliferating EBV-infected cells exhibit increased replicative stress by nuclear halo assays. While depleted dNTPs can generate replication fork collapse randomly throughout the genome, these lesions are typically efficiently repaired (Nam and Cortez 2011). In contrast, replicative stress at genomic sites that are

difficult to repair often lead to persistent DNA damage signaling and senescence or apoptosis (Rodier, Coppe et al. 2009). A primary site of irreparable DNA damage important for triggering senescence is telomeres (Fumagalli, Rossiello et al. 2012). Indeed, the Herbig laboratory has demonstrated that oncogene-induced senescence is mediated by replicative stress and irreparable DDR signaling at telomeres (Suram, Kaplunov et al. 2012). Masucci and colleagues have previously demonstrated that early EBV-infected cells display evidence of telomere-associated DNA damage (Kamranvar, Chen et al. 2013). Our prior work on ATM-mediated growth suppression of early EBV-infected cells together with our demonstration here of replicative stress and telomere dysfunction by the Masucci group is therefore consistent with a model whereby replicative stress at telomeres is the key molecular source of persistent DNA damage triggering senescence in early-infected cells.

An interesting, emerging consequence of hyper-proliferation and replicative stress in pre-neoplastic and neoplastic tissue is a vulnerability to ATR and Chk1 pathway inhibition relative to cells with normal proliferation rates (Myers, Gagou et al. 2009, Murga, Campaner et al. 2011, Schoppy, Ragland et al. 2012). For example, cells expressing oncogenic Ras display substantially increased genomic instability and cell death when ATR levels are depleted genetically or pharmacologically (Gilad, Nabet et al. 2010). Similarly, amplification of Myc or cyclin E, commonly found in many cancers, leads to heightened sensitivity to ATR inhibition (Schoppy, Ragland et al. 2012). In our studies, we find that early EBV-infected, rapidly proliferating cells are more sensitive to ATR and Chk1 inhibition than later, normally proliferating EBV-infected cells. While another group has recently published that ATR-Chk1 pathway facilitates EBV-mediated transformation of tonsillar B cells (Mordasini, Ueda et al. 2017), we have examined this further in a more

rigorous manner by sorting very specific early proliferating EBV-infected B cells towards characterizing the hyper-sensitivity of these cells relative to later proliferating B cells and LCLs. Furthermore, prior work has found that early EBV-infected cells display activated ATR, this group found that Chk1 was not phosphorylated on Ser 345 (Koganti, Hui-Yuen et al. 2014). As cross-talk among downstream phosphorylation targets is common in DDR signaling pathways (Stiff, Walker et al. 2006, Wu and Miyamoto 2008), we hypothesize that Chk1 is phosphorylated on Ser 317 or other sites that might trigger cell cycle arrest downstream of ATR in EBV-induced replicative stress. Ultimately, the DNA damage recognized by the ATR/Chk1 pathway following reduced dNTP pools and replicative stress must be reconciled during early EBV infection to promote the efficient outgrowth of immortalized LCLs.

In summary, EBV infection of primary B cells initially transits through a period of rapid proliferation presenting a high demand for nucleotide synthesis. Viral latency transcription factors must therefore promote activity of E2F complexes to enhance cell cycle progression. The consequences of hyper-replication during these early rapid rounds of proliferation is replicative stress and activation of the DDR. The ATR/Chk1 pathway is initially activated and is important for B-cell outgrowth. However, failure to repair damaged DNA at key sites, such as telomeres, results in a persistent ATM/Chk2-mediated DDR that triggers senescence. Infected cells that overcome this initial challenge to B-cell hyper-proliferation ultimately grow out as LCLs *in vitro*. *In vivo*, T-cell pressure against latent infection pushes these infected cells into true latency where no viral proteins are expressed (Babcock, Decker et al. 1998). However, in immune suppressed individuals these continuously growing latently infected cells are the precursors to B-cell lymphomas.

In the future, it will be important to discern whether EBV-positive lymphomas *in vivo* display hallmarks of overcoming an initial replicative-stress mediated tumor suppressive DDR.

# 4. Characterization of the EBV-induced persistent DNA damage response

*This chapter was modified based on the research article published by Amy Y. Hafez and Micah A. Luftig in Viruses MDPI in 2017 (Hafez and Luftig 2017).*

## *4.1 Rationale*

Epstein-Barr virus (EBV), an oncogenic γ-herpesvirus was the first human tumor virus to be discovered (Rickinson and Kieff 2007). Typically, EBV establishes an asymptomatic latent infection; however, EBV infection is associated with development of several lymphoid and epithelial cell malignancies, including Burkitt's lymphoma, Hodgkin's lymphoma, post-transplant lymphoproliferative disease, and nasopharyngeal carcinoma (Rickinson and Kieff 2007). While the majority of the human population is infected with EBV, malignancies primarily develop in immunocompromised patients as the strong cytotoxic T cell response of the immune system acts to control the infection (Longnecker, Kieff et al. 2013). Additional evidence has been reported to support the idea that other intrinsic responses beyond the immune system serve to further control EBV-mediated transformation.

*In vitro* infection of primary human B cells with EBV leads to transformation of B cells into indefinitely proliferating lymphoblastoid cell lines (LCLs). However, EBV-mediated transformation efficiency is so low that only about 1% of infected B cells become immortalized (Henderson, Miller et al. 1977, Sugden and Mark 1977). It has been show that EBV induces a transient period of hyper-proliferation early after infection by upregulating the viral latency proteins, EBNA2 and EBNA-LP, that induce expression of pro-growth genes, thus allowing for entry into the cell cycle (Alfieri, Birkenbach et al. 1991, Wang, Kikutani et al. 1991, Sinclair, Palmero et al. 1994, Nikitin, Yan et al. 2010). Rapid

cellular proliferation often leads to DNA damage that is recognized by the DNA damage response (DDR). This hyper-proliferative period is associated with increased levels of replicative stress and DNA damage, which triggers activation of the DDR, an innate tumor suppressor pathway (Bartkova, Horejsi et al. 2005, Gorgoulis, Vassiliou et al. 2005, Bartkova, Rezaei et al. 2006, Di Micco, Fumagalli et al. 2006, Halazonetis, Gorgoulis et al. 2008, Nikitin, Yan et al. 2010, Hafez, Messinger et al. 2017). Typically, activation of the DDR leads to DNA repair coupled with a transient cell cycle arrest, or if the damage is too great or irreparable, apoptosis or cellular senescence. We have previously found that EBV-infected B cells suppress apoptosis and trigger a G1/S phase cellular arrest (Nikitin, Price et al. 2014, Price, Dai et al. 2017). Specifically, cellular arrest was observed to occur in approximately 60-80% of infected B cells (Nikitin, Yan et al. 2010).

Cellular senescence was first described by Leonard Hayflick and Paul Moorhead in 1961 to be defined as a stable cell growth arrest (Hayflick and Moorhead 1961). Senescence can be induced by several factors, such as DNA damage, critically shortened telomeres and oncogene activation (Kuilman, Michaloglou et al. 2010, Di Micco, Sulli et al. 2011, Rodier and Campisi 2011). Oncogene-induced senescence (OIS) is a growth arrest initiated upon oncogene overexpression or inactivation of tumor suppressors, which promotes cell cycle exit (Kuilman, Michaloglou et al. 2010, Di Micco, Sulli et al. 2011, Rodier and Campisi 2011). OIS is maintained by certain hallmarks, including DNA replication stress, persistent DDR activation and upregulation of the p53/p21 and p16/pRb pathways, which ultimately facilitate stable growth arrest (Serrano, Lin et al. 1997, Bartkova, Rezaei et al. 2006, Di Micco, Fumagalli et al. 2006, Mooi and Peeper 2006). While OIS is known to trigger irreversible growth arrest, recent evidence suggests that cells arrested in OIS can escape senescence by derepression of *hTERT* expression

(Patel, Suram et al. 2016). Our prior work suggests that EBV infection induces OIS in early proliferating B cells, suppressing transformation of primary B cells into LCLs (McFadden, Hafez et al. 2016). Early after EBV infection, B cells undergo replicative stress and DNA damage as observed by nuclear halo assays and activate ATM/Chk2 and ATR/Chk1 DDR pathways (Nikitin, Yan et al. 2010, Koganti, Hui-Yuen et al. 2014, Hafez, Messinger et al. 2017). Infected cells that arrest after undergoing rapid proliferation exhibit an increase in markers of OIS, including H3K9me3 senescence-associated heterochromatic foci, upregulation of p16 and p21, and enhancement of p53 target gene expression (Nikitin, Price et al. 2014, McFadden, Hafez et al. 2016). Furthermore, early infected B cells have been shown to undergo metabolic stress in the form of depressed oxidative phosphorylation and limited purine nucleotide pools, which may contribute to increased replication stress and establishment of persistent DNA damage foci (McFadden, Hafez et al. 2016, Hafez, Messinger et al. 2017).

Depending on the severity or location of DNA damage, lesions may either induce transient growth arrest to allow time for damage to be repaired, or lesions may establish persistent DNA damage foci, constitutive DDR signaling and chronic p53 activation, which leads to permanent senescence (Beausejour, Krtolica et al. 2003, d'Adda di Fagagna, Reaper et al. 2003, Herbig, Jobling et al. 2004, Coppe, Patil et al. 2008, Rodier, Coppe et al. 2009, Fumagalli, Rossiello et al. 2014). Irreparable persistent DNA damage foci have been shown to associate with complex breaks or uncapped telomeres (Fumagalli, Rossiello et al. 2012, Hewitt, Jurk et al. 2012). Several studies have documented the hallmarks of persistent DNA damage foci, which include accumulation of DDR mediators, association of PML NBs to DDR, localization of DDR to telomeric DNA or telomere dysfunction-induced foci (TIFs), localization of promyelocytic leukemia nuclear bodies

(PML NBs) to telomeric DNA or alternative lengthening of telomeres-associated PML NBs (APBs), and lack of DNA repair proteins (Rodier, Munoz et al. 2011, Fumagalli, Rossiello et al. 2012, Hewitt, Jurk et al. 2012, Kamranvar, Chen et al. 2013). The Masucci group has shown the presence of APBs in pre-immortalized EBV-infected B cells, along with evidence of telomere dysfunction (Kamranvar, Chen et al. 2013). While there is accumulating evidence for EBV-induced OIS, it is unclear the role persistent DDR plays in establishing OIS in early-infected B cells as well as the composition of persistent DDR foci in this population of B cells. Therefore, in this study, we characterize the formation of persistent DDR foci in senescent EBV-infected B cells and the regulators of this response impinging on B-cell transformation (Figure 13).

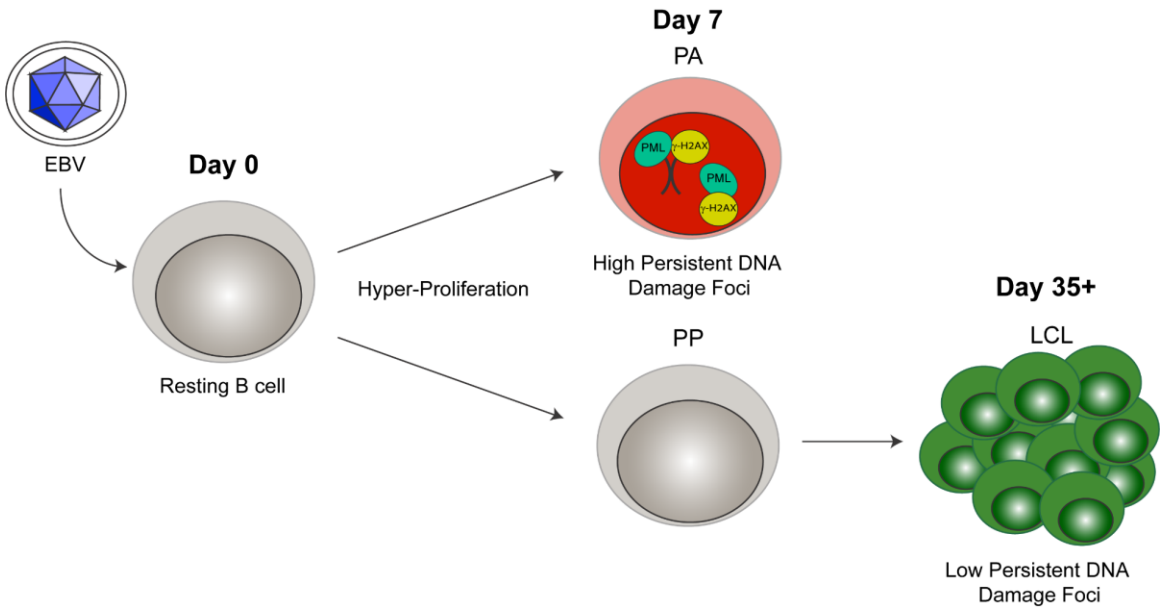


**Figure 13: Graphical abstract.** Schematic of the major findings and overall experimental design for the study presented in this chapter.

## *4.2 Results*

### 4.2.1 EBV infection of primary human B cells induces an increase in PML NBs and association with DDR foci

Cellular senescence often occurs when a cell undergoes DNA damage that cannot be repaired. In the context of irreparable DNA damage, the DDR signaling pathway is persistently activated, which leads to the formation of persistent DNA damage foci and promotes establishment of senescence. To determine whether hallmarks of a persistent DDR are present in EBV-infected B cells that undergo senescence we sought to specifically examine the early subpopulation of B cells that arrest upon hyper-proliferation. We used a double staining technique previously developed by our lab to sort infected B cells that proliferated and then arrested (PA) from cells that become indefinitely proliferating LCLs (McFadden, Hafez et al. 2016). Initially, we stained bulk PBMCs with CellTrace Violet (CTV) and infected the cells with EBV at a multiplicity of infection (MOI) such that every CD19+ B cell is latently infected (Nikitin, Yan et al. 2010). On day 4 post infection, we applied a second proliferation tracking dye, carboxyfluorescein succinimidyl ester (CFSE), to the cells and returned them back to culture. At 8 days post infection, we sorted CD19-positive EBV-infected B cells that arrested based on diluted CTV and high CFSE fluorescence profile (Figure 14A). Our goal in these studies is to characterize the nature of the DDR, which is enriched in the PA population. Therefore, we focused on PA and not PP cells, and we also examined immortalized LCLs as a negative control and LCLs treated with high dose bleomycin sulfate as a positive control for persistent DDR (Rodier, Munoz et al. 2011). We first assayed these cell populations for the major focal component of persistent DDR foci, PML NBs and found that arrested cells exhibited a significantly higher number of PML NB foci per nucleus as compared to LCLs. This similar

increase in PML NBs was also observed in LCLs treated with bleomycin (Figure 14B and C). Furthermore, we investigated whether PML NBs co-localized to markers of DNA damage, a characteristic of persistent DDR foci, and found that arrested cells and LCLs treated with bleomycin exhibited an increase in PML NBs co-localized to DDR markers, including γH2AX and 53BP1 (Figure 14D-G). These data suggest that arrested EBV-infected B cells induce an increase in PML NBs that associate with DNA damage to form a key feature of persistent DDR foci.

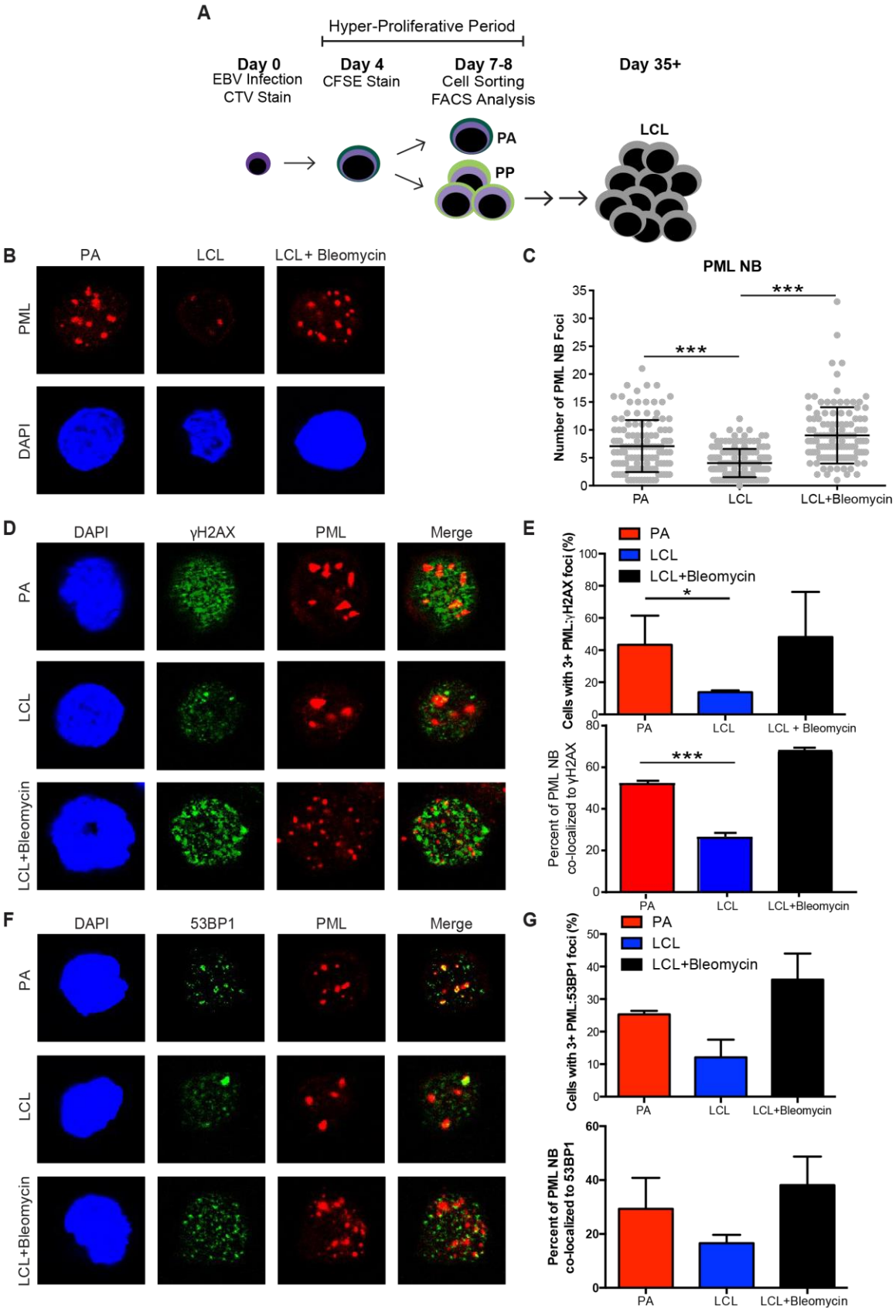
A
Hyper-Proliferative Period
Day 0
EBV Infection
CTV Stain
Day 4
CFSE Stain
Day 7-8
Cell Sorting
FACS Analysis
Day 35+
PA
PP
LCL
B
PA
LCL
LCL + Bleomycin
PML
DAPI
C
PML NB
Number of PML NB Foci
***
***
PA
LCL
LCL+Bleomycin
D
DAPI
γH2AX
PML
Merge
PA
LCL
LCL+Bleomycin
E
PA
LCL
LCL+Bleomycin
Cells with 3+ PML:γH2AX foci (%)
*
PA
LCL
LCL + Bleomycin
Percent of PML NB co-localized to γH2AX
***
PA
LCL
LCL+Bleomycin
F
DAPI
53BP1
PML
Merge
PA
LCL
LCL+Bleomycin
G
PA
LCL
LCL+Bleomycin
Cells with 3+ PML::53BP1 foci (%)
PA
LCL
LCL+Bleomycin
Percent of PML NB co-localized to 53BP1
PA
LCL
LCL+Bleomycin

**Figure 14: Persistent DNA damage foci increase in arrested EBV-infected B cells.** (A) Schematic demonstrating infection, staining and sorting protocol to separate early, hyper-proliferating and then arrested (PA) cell populations from proliferating populations (PP), along with generation of immortalized LCLs. (B) Immunofluorescence (IF) of PML NBs (red) and DAPI (blue) measured from sorted arrested B cells (PA), LCLs and bleomycin-treated LCLs. (C) Quantification of PML NB foci per nucleus from (A). Error bars represent SD of three independent donors. ***P<0.001 as determined by a Mann-Whitney test. (D) IF of γH2AX (green) and PML NBs (red) measured from sorted arrested B cells, LCLs, and bleomycin-treated LCLs. Co-localization of γH2AX-PML is shown in Merge. (E) Upper, quantification of cells with three or more PML NBs co-localized with γH2AX per nucleus from (D). Lower, quantification of percent H2AX co-localization with PML per cell from (D). Error bars represent S.E.M of three independent donors for PA and LCL and two donors for LCL plus bleomycin. *P<0.05, ***P<0.001 as determined by a Student's t test. (F) IF of 53BP1 (green) and PML NBs (red) measured from sorted arrested B cells, LCLs, and bleomycin-treated LCLs. Co-localization of 53BP1-PML is shown in Merge. Note: the single, large 53BP1 focus co-localizing with PML is likely an Oct-1, PTF, transcription (OPT) domain (Harrigan, Belotserkovskaya et al. 2011). (G) Upper, quantification of cells with three or more PML NBs co-localized with 53BP1 per nucleus from (F). Lower, quantification of percent 53BP1 co-localized with PML per cell from (F). Error bars represent S.E.M. of two independent donors.

### 4.2.2 Persistent DNA damage response is localized to telomeric DNA in early arrested EBV-infected B cells

Persistent DDR foci have been shown by a number of groups to preferentially target sites of irreparable DNA damage including telomeres (Fumagalli, Rossiello et al. 2012, Hewitt, Jurk et al. 2012). We have observed characteristics of persistent DNA damage foci in the arrested population of EBV-infected B cells and sought to further investigate whether these foci specifically form at sites of telomeric DNA damage upon infection. We conducted immunofluorescence-telomere fluorescence in situ hybridization (IF-Telomere FISH) to examine the localization of DDR markers to telomeric DNA, also known as telomere dysfunction-induced foci (TIF). To label telomeres we used a TelC PNA FISH probe that is a C-rich probe, which recognizes the leading strand TAACCC repeats. We found that arrested infected B cells exhibited an increase in TIF positive cells as measured by the co-localization of TelC to γH2AX, while LCLs exhibited reduced levels of TIFs (Figure 15A and B).

Next we wanted to determine whether telomeres also localized to PML NBs, known as ALT-associated PML NBs (APBs), a primary characteristic of persistent DDR foci. Recently, the Masucci group showed that bulk early-infected B cells activated the non-canonical telomere maintenance pathway, alternative lengthening of telomeres (ALT). In doing so, they examined the state of telomere dysfunction in infected B cells and reported an increase in the presence of APBs (Kamranvar, Chen et al. 2013). Here we specifically study the arrested subpopulation of early EBV-infected B cells and consistent with their findings, we observed a significant increase in the presence of PML NBs co-localized to telomeric DNA as compared to LCLs (Figure 15C and D). Together, these findings suggest that arrested EBV-infected B cells exhibit characteristic markers of persistent DDR foci that accumulate at telomeric DNA suggesting that telomere dysfunction contributes to the establishment of OIS mediated by EBV infection.

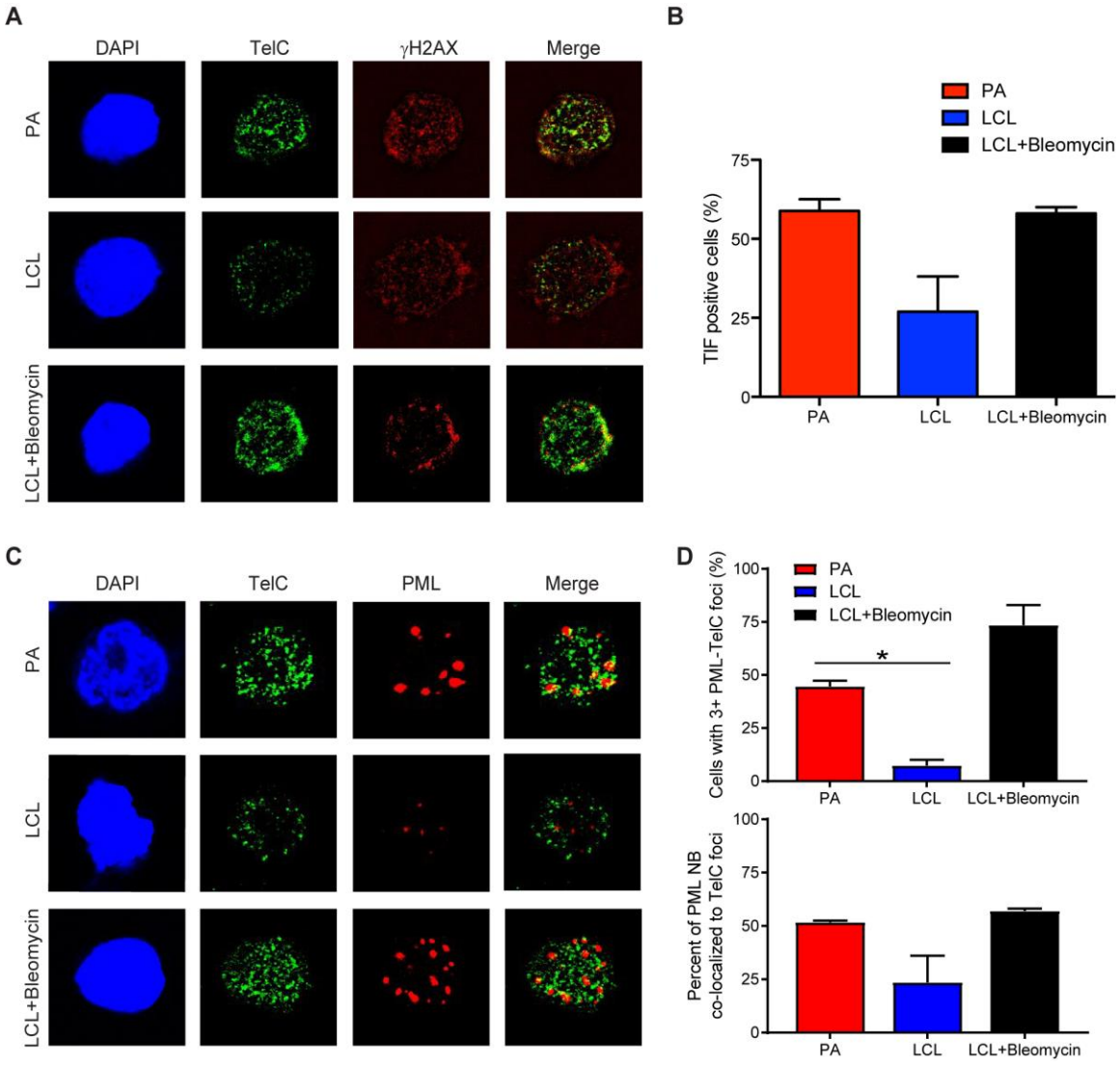


**Figure 15: Persistent DNA damage localizes to telomeres in arrested EBV-infected B cells.** (A) IF-Telomere FISH of γH2AX (red) and TelC (green) measured from sorted arrested B cells, LCLs, and bleomycin-treated LCLs. Co-localization of γH2AX-TelC is shown in Merge. (B) Quantification of TIF positive cells as defined by the co-localization of TelC with γH2AX from (A). (C) IF-Telomere FISH of PML NBs (red) and TelC (green) measured from sorted arrested B cells, LCLs, and bleomycin-treated LCLs. Co-localization of PML-TelC is shown in Merge. (D) Upper, quantification of cells with greater than three PML NBs co-localized with TelC per nucleus from (C). Lower, quantification of the percentage of PML NBs co-localized to TelC foci per cell. Error bars represent S.E.M of three independent donors. *P<0.05 as determined by a Student's t test. For lower graph, PA vs LCL p = 0.0576.

### 4.2.3 Increased hTERT expression enhances EBV-mediated transformation of early-infected B cells

Oncogenic signaling has been shown to play a major role in senescence by inducing telomeric replication stress and telomere dysfunction in cells that lack sufficient hTERT activity (Suram, Kaplunov et al. 2012). Importantly, while telomeric repeats are hypersensitive to DNA replication stress it has been reported that hTERT expression can mitigate telomere dysfunction (Suram, Kaplunov et al. 2012). Since primary human B cells are intractable for heterologous over-expression studies, we sought to use a pharmacological approach to determine if increased hTERT expression can allow early-infected B cells to overcome TIF-associated growth arrest. Recent evidence suggests that androgen hormones can promote hTERT expression and, in fact, danazol has recently been described as a new therapy for patients with telomere diseases (Calado, Yewdell et al. 2009, Townsley, Dumitriu et al. 2016). Addition of danazol to bulk EBV-infected early, proliferating B cells (population doubling 1-4) and LCLs increased the mRNA level of hTERT (Figure 16A). We therefore assessed whether hTERT up-regulation would impact transformation as early-infected cells displayed increased TIFs. Treatment of PBMCs with 3 μM danazol concurrent with EBV infection led to an increase in the number of CD19+ proliferating B cells at day 7 post-infection relative to untreated cells (Figure 16B). However, treatment of LCLs with danazol had no effect on cell proliferation, thus suggesting that danazol acts on a process only relevant early after infection (Figure 16B). Furthermore, we observed an increase in EBV-mediated transformation efficiency with danazol treatment relative to DMSO-treated infected PBMCs (Figure 16C). Collectively, these findings support a model whereby defective telomere maintenance contributes to

the arrest of early proliferating B cells and ultimately suppresses EBV-mediated transformation (Figure 16D).

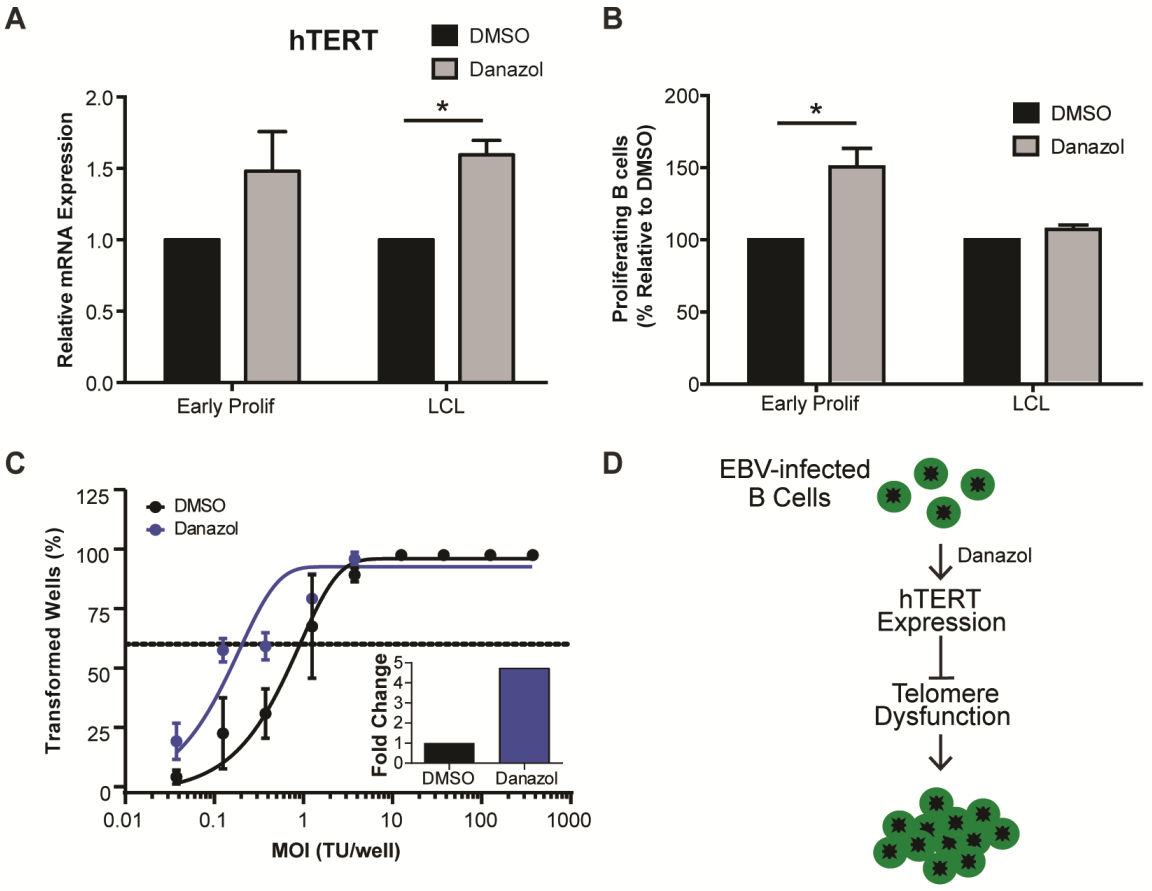


**Figure 16: Danazol treatment enhances EBV-mediated B-cell transformation.** (A) The expression level of hTERT mRNA was measured from sorted early proliferating CD19+ infected B cells on Day 7 and LCLs. Relative mRNA abundance was normalized to SETDB1. Data are represented as fold change relative to DMSO treatment. Error bars represent S.E.M. of three independent donors. *P<0.05 as determined by a Student's t test. (B) Percentage of proliferating CD19+ B cells was determined for early proliferating infected B cells and LCLs that were treated with DMSO (black) or with 3μM Danazol (grey) at the time of infection. The data were analyzed by FACS at day 7 post infection. Error bars represent S.E.M. of three independent donors. *P < 0.05 as determined by a Student's t-test. (C) Quantification of EBV-infected B cell outgrowth following PBMC infection in the presence of DMSO (black) or 3μM Danazol (blue) at time of infection. The percentages of wells positive for LCLs at 5 weeks post infection are plotted relative to the transforming units (TU) of B95-8 virus per well. Error bars represent S.E.M of three independent donors. Dotted line represents 62.5% positive wells, which indicates outgrowth from the virus amount in the x axis of a single LCL per well based on a Poisson's distribution. (C, inset) Fold change of the transformation efficiency. (D) Schematic of danazol mechanism of action during EBV infection.

### *4.2.4 Early EBV-infected B cells are sensitive to inhibition of BLM helicase*

To further investigate the role of telomere maintenance in establishment of a persistent DDR we examined the importance of Bloom syndrome (BLM) helicase in early infected B cells. BLM helicase is a member of the RecQ helicase family, which is involved in homologous recombination and is capable of unwinding G-quadruplex DNA structures formed by telomeric DNA (Rosenthal, Dexheimer et al. 2010, Nguyen, Dexheimer et al. 2013, Chatterjee, Zagelbaum et al. 2014). BLM helicase is specifically important for cells that undergo alternative lengthening of telomeres (ALT) rather than the telomerase-dependent cannonical method of telomere maintenance (Stavropoulos, Bradshaw et al. 2002). In a recent report, it has been shown that EBV-infected B cells maintain telomeres via the ALT pathway (Kamranvar, Chen et al. 2013). To study the importance of BLM helicase in regulating telomere maintenance during early EBV infection we again turned to a pharmacological approach using ML216, a small molecule inhibitor of BLM (Rosenthal, Dexheimer et al. 2010). We found that early proliferating EBV-infected B cells are more sensitive to BLM helicase inhibition by ML216 than LCLs (Figure 17). These data suggest that inhibiting telomere maintenance factors in cells that are already undergoing replicative stress may exacerbate the telomere dysfunction causing cells to become more sensitive to persistent DDR.

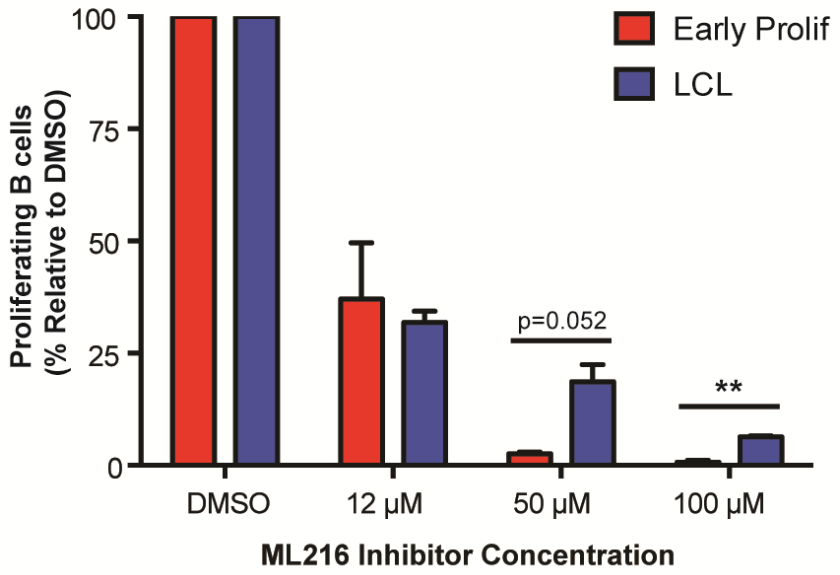


**Figure 17: Early proliferating EBV-infected B cells are more sensitive to BLM helicase inhibition than LCLs.** Early proliferating EBV-infected B cells are more sensitive to BLM helicase inhibition than LCLs. Percentage of proliferating CD19+ B cells was determined for early proliferating EBV-infected B cells (red) and LCLs (blue) that were treated with DMSO or with 12μM, 50μM, or 100μM ML216 BLM helicase inhibitor at the time of infection. The data were analyzed by FACS at day 7 post infection. Error bars represent S.E.M. of three independent donors. **P < 0.01 as determined by a Student's t-test.

## *4.3 Materials and Methods*

### 4.3.1 Viruses and Cells

EBV B95-8 virus was produced from the B95-8 Z-HT cell line as previously described (Johannsen, Luftig et al. 2004). Buffy coats were obtained from normal donors through the Gulf Coast Regional Blood Center and peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll Histopaque-1077 gradient (Sigma, St. Louis, MO, USA #H8889). Primary human B cells were cultured in RPMI 1640 supplemented with 15% fetal bovine serum (FBS), 2mM L-glutamine, penicillin and streptomycin (1X, Sigma, #G6784) (R15)

as well as 0.5 µg/ml Cyclosporin A (CsA) (Sigma, #30024). Bulk infections were performed by incubating cells with B95-8 Z-HT supernatant at 1 mL per $10^6$ B cells calculated from within the PBMC population for 1 hour at 37°C in a $CO_2$ incubator. Incubation was followed by washing in PBS and resuspending in R15 media supplemented with CsA. Bulk infections were conducted on 5 x $10^8$ PBMCs. LCLs were generated from normal donors by continuous outgrowth of EBV-infected primary B cells for greater than 35 days. LCLs were cultured in RPMI media supplemented with 10% FBS (R10). BLM deficient LCLs (GM16377, GM16375, and GM09960) were obtained from Coriell Institute (Coriell Institute, Camden, NJ).

### 4.3.2 Chemicals

Bleomycin sulfate (Selleckchem, Houston, TX, USA #S1214) was resuspended at 100µg/ml in DMSO and used to induce persistent DNA damage in LCLs at 20 µg/ml for two hours. Danazol (Sigma, #D8399) and ML216 BLM helicase inhibitor (Sigma, #SML0661) were resuspended in DMSO at 3mM and 50mM, respectively, and used at dilutions noted in the results section.

### 4.3.3 Antibodies

CD19 mouse anti-human antibody (clone 33-6-6, gift from Dr. Tom Tedder, Duke University) conjugated with either allophycocyanin or phycoerythrin (APC) was used as a surface B cell marker in flow cytometry. Mouse anti-human CD19 PE-Cyanine 7 antibody (eBioscience, San Diego, CA, USA #25-0199-42) was used as an additional marker of the B cell surface. B cell surface markers were used at 1µL per $10^6$ cells. PML antibody (Santa Cruz Biotechnology, Dallas, TX, USA #sc-966) was used at 1:50 to detect PML expression by immunofluorescence. 53BP1 (Cell Signaling Technology, Danvers, MA, USA, #4937)

and Phosphorylated-γH2AX (S139) (Cell Signaling Technology, #9817) were used as markers of DNA damage for immunofluorescence at 1:50. TelC-FITC (PNA Bio, Newbury Park, CA, #F1009) is a telomere PNA probe used as discussed below for immunofluorescence-FISH to detect the C-rich leading strand.

### 4.3.4 Infections and Cell Sorting

PBMCs were isolated from a buffy coat and stained with CellTrace Violet (Invitrogen, #C34557) using the manufacturer's suggested protocol followed by infection with EBV at an MOI of 5 (such that all infected B cells are positive for EBNA-LP). On day 4 post infection, PBMCs were stained with CFSE (Sigma, #21888) using the manufacturer's suggested protocol. Proliferation was monitored in CD19+ B cells by the dilution of the CellTrace Violet and CFSE stains for up to 8 days post infection on a BD FACS Canto II and analyzed using FlowJo 10.0 software (TreeStar) (FlowJo, Ashland, OR, USA). On day 8 the arrested, CD19 positive cells were sorted based upon their diluted CellTrace Violet and high CFSE profile using either a Beckman Coulter Astrios or Beckman Coulter MoFlo XDP sorter.

### 4.3.5 Immunofluorescence

EBV B95-8-infected B cells were pelleted, resuspended in 25μl of PBS, spread on a microscope slide and dried at 37°C for 15 minutes. Cells were fixed in 4% paraformaldehyde for 15 minutes at 4°C, washed in PBS, permeabilized in PBS containing 0.2% Triton X-100 for 10 minutes and then blocked in PBS with 0.2% Triton X-100 containing 5% normal goat serum for 1 hour. Primary antibodies were incubated overnight at 4°C followed by secondary antibody incubation with AlexaFluor 488 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA #A11034) and AlexaFluor 568 goat anti-mouse

IgG (Life Technologies, Carlsbad, CA, USA #A11004) for 2 hours. Slides were mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA #H-1200). All immunofluorescence slides were visualized using the Zeiss 780 upright confocal microscope and images were analyzed using ImageJ version 2.0.

### 4.3.6 Immunofluorescence-Fluorescence in situ Hybridization

EBV B95-8-infected B cells were pelleted, resuspended in 25μl of PBS, spread on a microscope slide and dried at 37°C for 15 minutes. Cells were fixed in 4% paraformaldehyde for 15 minutes at 4°C, washed in PBS and permeabilized in PBS containing 0.2% Triton X-100 for 10 minutes. Slides were denatured at 77.8°C for 5 minutes in a hybridization mix composed of 10mM Tris-HCl (pH 7.5), 70% formamide, 0.5% blocking reagent (Roche, Basel, Switzerland, #11096176001), and 0.2-0.5 μg/ml PNA-TelC probe, in ddH20. After denaturation, slides were incubated overnight at 37°C. Slides were washed first in wash buffer containing 70% formamide, 10mM Tris pH7.5 and 0.1% BSA two times for 15 minutes followed by a wash in 4X SSC containing 0.05% Tween-20 for 5 minutes and blocked for 15 minutes in 1X Roche blocking reagent diluted in 4X SSC. Primary antibodies were incubated overnight at 4°C followed by secondary antibody incubation with AlexaFluor 488 goat anti-rabbit IgG (Life Technologies, #A11034) and AlexaFluor 568 goat anti-mouse IgG (Life Technologies, #A11004) for 2 hours. Slides were mounted in Vectashield containing DAPI (Vector Laboratories, #H-1200). All slides were visualized using the Zeiss 780 upright confocal microscope and images were analyzed using ImageJ version 2.0. Co-localization of TelC with γH2AX was determined by calculating the Pearson's coefficient using JACoP plugin for ImageJ. Based on average Pearson's coefficient values for controls, including LCLs and bleomycin-treated LCLs, we defined TIF positive cells as cells with a Pearson's coefficient above 0.35.

### 4.3.7 Cell Proliferation Assays

PBMCs were infected on Day 0, stained with CellTrace Violet, and treated with either 3μM of Danazol or 12μM, 50μM, or 100μM of ML216 inhibitor. Cells were put back into culture and proliferation was monitored for CD19+ B cells by the dilution of the CellTrace Violet stain at Day 8 post infection using a BD FACS Canto II. Early proliferating B cells, which include both the PA and PP populations in bulk, are comprised of population doublings (PD) 1-4.

### 4.3.8 Transformation Assay

PBMC infection was conducted in the presence of 0.1% DMSO and 3μM danazol added at Day 0 post infection. Supernatant from B95-8 Z-HT cells was titrated from 300 $\mu L/10^7$ PBMCs to 0.03 $\mu L/10^7$ PBMCs. Each infection point consisted of $7 \times 10^6$ infected PBMCs seeded in 20 wells of a 96-well plate. The percentage of wells positive for B cell outgrowth into LCLs at 5 weeks post infection was plotted relative to the multiplicity of infection (MOI) per well. The efficiency of transformation was determined as published where 1 transforming unit per well was considered the amount of B95-8 virus necessary to yield 62.5% of positive wells (Henderson, Miller et al. 1977).

### 4.3.9 Real-Time qPCR

Total RNA was isolated by using RNeasy (Qiagen, Hilden, Germany, #74106) and reverse-transcribed by using the High Capacity cDNA Reverse Transcription kit (Life Technologies, #4368814) according to the manufacturer's instructions. Relative mRNA abundance was measured by using SYBR green-based RT-qPCR assay with 5ng of cDNA per reaction. All primers (IDT, Coralville, IA, USA) were used at 1μM per reaction. qRT-PCR was performed using the StepOnePlus Real-Time PCR light-cycler (Applied

Biosystems, Foster City, CA, USA) and analyzed by using the StepOne software. All samples were analyzed in triplicate and expression levels were normalized first to SETDB1 and then to DMSO treated controls. Primer sequences for RT-qPCR were as follows: hTERT: forward primer, 5'-CCGATTGTGAACATGGACTACG-3', and reverse primer, 5'- CACGCTGAACAGTGCCTTC -3' and SETDB1: forward primer, 5'-TCCATGGCATGCTGGAGCGG-3', and reverse primer, 5'-GAGAGGGTTCTTGCCCCGG -3'.

## *4.4 Discussion*

The DDR signaling pathway is known to be an important innate tumor suppressor pathway involved in repairing damaged DNA, inducing apoptosis or arresting the cell cycle. DNA damage at irreparable sites can facilitate entry into an irreversible growth arrest. Importantly, persistent activation of the DDR has been causally linked to the establishment of senescence (Rodier, Munoz et al. 2011, Fumagalli, Rossiello et al. 2012, Hewitt, Jurk et al. 2012, Fumagalli, Rossiello et al. 2014). We have previously found that EBV infection *in vitro* induces a transient period of hyper-proliferation early after infection leading to initiation of DDR-mediated senescence (Nikitin, Yan et al. 2010). Furthermore, we observed metabolic stress and presence of limited nucleotide pools that were insufficient to overcome rapid proliferation contributing to maintenance of OIS in EBV-infected B cells (McFadden, Hafez et al. 2016, Hafez, Messinger et al. 2017). We propose that DNA damage and replicative stress sustained early during hyper-proliferation mediates the formation of persistent DNA damage foci in early EBV-infected B cells to maintain senescence. In this study, we sought to identify the presence of persistent DDR foci and characterize these foci specifically in the arrested subpopulation of EBV-infected B cells. We found that upon EBV infection, the arrested B cells exhibited an increase in

PML NBs as well as PML NB-associated DNA damage foci. We went on to explore the state of telomeres in the arrested infected B cells and observed that persistent DNA damage localized to telomeric DNA forming TIFs. Intriguingly, a drug used to treat patients with telomere maintenance disorders, danazol, was found to increase proliferation of B cells specifically early after infection and enhance transformation of B cells into LCLs. Lastly, we found that early proliferating B cells are more sensitive than LCLs to inhibition of a key telomere replication protein, BLM helicase.

Persistent DDR signaling has been previously shown to form unique persistent DNA damage foci in cells undergoing OIS. The Campisi laboratory has extensively investigated the spatiotemporal dynamics of persistent DNA damage foci and established a role for chronic DDR signaling in maintenance of senescence (Rodier, Munoz et al. 2011). They report that PML NBs are an important staple to the formation of persistent damage foci, which associate with both γH2AX and 53BP1 DDR markers as well as telomeres. Additionally, Bazett-Jones and colleagues have previously shown that PML NBs act as DNA damage sensors and increase in number with the activation of the DDR (Dellaire, Ching et al. 2006). As DNA damage accumulates it alters the chromatin state and causes PML NBs to form microbodies (Dellaire, Ching et al. 2006). The characteristics we describe here of PML-associated DNA damage foci are consistent with these findings, and further support a role for PML NBs serving as a potential scaffold for persistent DNA damage foci responsible for facilitating senescence.

Reparable DNA damage leads to the formation of transient DNA damage foci that are typically resolved in 24 hours. However, in the case of irreparable DNA lesions, persistent DNA damage foci are formed allowing for the maintenance of irreversible senescence. Telomeric DNA has been established by many groups to be a primary site of irreparable

damage and therefore, is a favored target of a persistent DNA damage response (Fumagalli, Rossiello et al. 2012, Hewitt, Jurk et al. 2012). Here we have shown that telomeric DNA is targeted by markers of DNA damage and that persistent DNA damage foci are localized to telomeres in arrested EBV-infected B cells. These findings are consistent with an array of evidence in the senescence field. d'Adda di Fagagna and colleagues have laid much of the foundation for these findings and have specifically shown that persistent DDR is a mechanism for mediating senescence and that telomeric DNA damage is irreparable and associated with persistent DDR signaling (Rodier, Munoz et al. 2011, Fumagalli, Rossiello et al. 2012, Hewitt, Jurk et al. 2012, Fumagalli, Rossiello et al. 2014). Utz Herbig's group has recently demonstrated that replicative stress and irreparable telomeric DNA damage mediate OIS (Suram, Kaplunov et al. 2012). Importantly, Masucci's laboratory has recently shown that early proliferating EBV-infected B cells exhibit a range of telomere dysfunction, including the localization of PML NBs to telomeric DNA. Consistent with these findings, our report goes on to show that the arrested subpopulation of early EBV-infected B cells exhibit telomere dysfunction and telomeric-associated persistent DNA damage foci.

Telomere maintenance is important for proper protection and replication of telomeric DNA. Genetic defects in telomere maintenance and repair have been shown to result in telomere diseases, including bone marrow failure, liver cirrhosis, and increased risk of cancer. Specifically, deficient hTERT activity is known to induce telomere dysfunction and persistent DDR localized to telomeres (Suram, Kaplunov et al. 2012). Telomere dysfunction occurs early, within days, in EBV-infected B cells therefore, it is unlikely that telomere abnormalities exist due to telomere shortening, but rather improper telomere maintenance leading to telomere deprotection. Danazol, an androgen hormone recently

tested in clinical trials for the treatment of telomere diseases has been shown to elongate telomeres in patients with telomere diseases and to have a greater impact on patients with *TERT* mutations (Townsley, Dumitriu et al. 2016). The mechanism behind how danazol elongates telomeres is not well understood; however, evidence has been shown to suggest that androgen therapies have a direct effect on telomerase activity by upregulating hTERT expression (Calado, Yewdell et al. 2009). In line with this mechanism we have shown here that danazol increases hTERT mRNA expression in EBV-infected B cells. As danazol treatment also promotes infected B cells to overcome cellular arrest and enhances transformation efficiency it is possible that hTERT expression is deficient in early infected cells. Furthermore, we found that early EBV-infected B cells displayed elevated sensitivity to ML216, an inhibitor of BLM helicase, which is involved in telomere replication (Stavropoulos, Bradshaw et al. 2002, Rosenthal, Dexheimer et al. 2010, Nguyen, Dexheimer et al. 2013, Chatterjee, Zagelbaum et al. 2014). This further implicates telomere maintenance in the establishment of persistent DDR foci and senescence in early EBV-infected B cells.

In summary, EBV infection induces a period of rapid cell proliferation (~8-12-hours per cell cycle) that presents a challenge for proper DNA replication. In the context of metabolic stress and insufficient nucleotide pools, most infected B cells fail to be immortalized by EBV, but rather undergo OIS due to a persistent DNA damage response. Our group, corroborating initial findings by Masucci, provide data supporting a model whereby irreparable DNA damage at telomeres is the molecular source for the OIS-mediated DDR. Future studies will be aimed at defining the role of telomere dysfunction and the early period of hyper-proliferation in the restriction and development of EBV-associated lymphomas in vivo.

# 5. Future Directions and Conclusions

## *5.1 Future Directions*

The results from my dissertation characterize the initial events that lead to senescence early after EBV infection of primary human B cells. This research raises many questions regarding: i) the viral mechanism required to promote efficient metabolism in order to sustain hyper-proliferation, ii) the importance of persistent DNA damage foci formation to the reinforcement of OIS in EBV-infected B cells, and iii) the analysis of proliferation, DDR activation and establishment of senescence at the single cell level. Due to existing technological barriers in our field, we have not been able to address many of these questions as efficiently as we would like. Currently, efficient introduction of genetic constructs into primary human B has remained elusive making it difficult to conduct genetic analysis of early stage EBV infection. Our lab has made some progress developing techniques to introduce constructs into primary human B cells, but the efficiency remains to be a significant problem. To bypass this barrier we have relied on chemical inhibitors that specifically target molecules involved in many pathways, including the DDR signaling pathway. Despite these barriers, I have used this section to address the questions that require further exploration to elevate our understanding of the early stages of EBV infection.

### 5.1.1 Viral mechanism involved in nucleotide metabolism

EBV-induced hyper-proliferation presents a significant challenge for cells to overcome in order to promote transformation of B cells. During this transient period, it is crucial for cells to meet the metabolic demands that come with rapid proliferation in order to ensure accurate and efficient DNA replication and cell growth. Our lab has presented

strong evidence that the majority of infected primary human B cells succumb to a DNA damage-induced G1/S phase cell cycle arrest (Nikitin, Yan et al. 2010, Nikitin, Price et al. 2014). Upon further investigation of the subpopulation of B cells that arrest, we made the key discovery that EBV induces a metabolic imbalance, which is characterized by low levels of oxidative phosphorylation and limited purine dNTP levels (McFadden, Hafez et al. 2016, Hafez, Messinger et al. 2017). This metabolic deficiency facilitates arrest and suppresses EBV-mediated transformation.

Recently the Vander Heiden and Sabitini groups have shown that oxidative phosphorylation during cell proliferation is necessary to provide a source of electron acceptors for aspartate synthesis, rather than production of ATP (Birsoy, Wang et al. 2015, Sullivan, Gui et al. 2015). Furthermore, purine nucleotide biosynthesis is dependent on consumption of aspartate to facilitate the conversion of IMP to AMP (Sullivan, Gui et al. 2015). Therefore, aspartate deficiency may contribute to the observed reduction in purine dNTP pools and consequently growth arrest. Importantly, aspartate supplementation of growth media has been shown to restore nucleotide levels and cell proliferation in the context of oxidative phosphorylation (Sullivan, Gui et al. 2015). This suggests that oxidative phosphorylation plays an important role in maintenance of purine dNTP pools that are required to sustain EBV-induced hyper-proliferation. However, it remains unclear as to which viral latency proteins contribute to inducing mitochondrial oxidative phosphorylation to subsequently promote generation of purine dNTPs.

Preliminary gene expression analysis from our laboratory indicates that NRF1, a host transcription factor responsible for inducing mitochondrial oxidative phosphorylation by promoting transcription of key mitochondrial biogenesis enzymes, is expressed at reduced levels in the arrested subpopulation of EBV-infected B cells as compared to the

proliferative population. Interestingly, a genome-wide association of viral EBNA latency proteins to NRF1 identified EBNA-LP as the only viral latency protein to have a strong enrichment at NRF target sites (Zhao, Zou et al. 2011, Portal, Zhou et al. 2013, Jiang, Willox et al. 2014, Schmidt, Jiang et al. 2015). Approximately 80% of NRF1 sites coincided with EBNA-LP targets, suggesting that EBNA-LP, an essential gene for EBV-mediated immortalization (Mannick, Cohen et al. 1991), may play a role in regulating metabolic demands. Therefore, it is our hypothesis that EBNA-LP is responsible for inducing NRF1 transcriptional targets necessary to overcome the EBV-induced hyper-proliferative period and promote transformation of B cells. To begin to examine the role of EBNA-LP in up-regulation of NRF1 targets we will perform EBNA-LP and NRF1 ChIP-PCR analysis at NRF1 target genes in EBV-infected early primary B cells analyzing. Additionally, we will conduct co-immunoprecipitation assays to determine if EBNA-LP associates directly with NRF1. These experiments will allow us to begin to understand the viral mechanism behind regulation of nucleotide metabolism during the hyper-proliferative period.

### 5.1.2 Source of telomeric DNA damage

The majority of early EBV-infected B cells are unable to sustain growth during the transient hyper-proliferative period, which is due to a metabolic imbalance caused by low oxidative phosphorylation and insufficient purine dNTP pools (McFadden, Hafez et al. 2016, Hafez, Messinger et al. 2017). My dissertation research has implicated telomeric DNA damage as a crucial contributing factor to establishing OIS (Hafez and Luftig 2017, Hafez, Messinger et al. 2017). To better understand the innate tumor suppressor mechanisms that may alter EBV-driven lymphomas we must determine the molecular source of the EBV-induced telomeric DNA damage.

Recent work from both yeast and human models have identified ribonucleotide incorporation to be an important source of DDR activation and genomic instability (Jinks-Robertson and Klein 2015). In mammalian DNA, typically single ribonucleotides are removed by RNase H2 (Reijns, Rabe et al. 2012). However, the reduction of purine dNTPs in arrested EBV-infected B cells suggests that subsequent ribonucleotide incorporation in DNA may be the source of triggering DDR. Importantly, in yeast, deletion of RNase H2 promotes genomic instability and telomere shortening suggesting a role for ribonucleotide incorporation into telomeric DNA (Askree, Yehuda et al. 2004). Based on this evidence we hypothesize that accumulation of ribonucleotides in telomeric DNA serves as the molecular source of the DDR-induced OIS. To address this hypothesis, we will need to assay the arrested EBV-infected B cells for ribonucleotide accumulation in telomeric DNA. Characterizing ribonucleotide incorporation can be done by alkaline hydrolysis of genomic DNA that is enriched for telomeric repeats (HinfI/RsaI-digested) followed by Southern blotting using a $(CCCRAA)_3$ probe (Counter, Hahn et al. 1998) or by qPCR for telomeric repeats using the modified Cawthon primers (Cawthon 2009). From these approaches, we would expect that the arrested B cells will contain alkaline hydrolysis sensitive telomeres, which will lead to shortened telomeres by Southern or low telomeric qPCR signal. Ultimately, this would suggest a functional role for ribonucleotide incorporation as a source of telomeric DNA damage.

### 5.1.3 Importance of persistent DDR foci formation in regulating EBV-mediated transformation

Formation of persistent DDR foci have been shown by numerous groups to be important for the induction of OIS (Fumagalli, Rossiello et al. 2012). While we have evidence that early EBV-infected B cells induce formation of these foci in senescent cells,

it is unclear as to the function of their presence in the context of EBV infection. To address the importance of persistent DDR formation in EBV-infected B cells, we will need to target the factors responsible for assembling persistent DDR foci at telomeric DNA. PML NBs are the major component involved in the formation of DDR foci at telomeres and it depends on the SUMOylation system, including SUMO1/3, E2 UBC9, and E3 MMS21, to modify PML protein (Osterwald, Deeg et al. 2015). To understand the implications of persistent DDR foci formation in early EBV-infected B cells we will need to introduce shRNA constructs using a pCEP-Tomato backbone to target the aforementioned factors. We expect that PML and SUMOylation and will be required to establish telomeric DDR foci.

My work has begun to address this question by using chemical inhibitors of BLM helicase, an enzyme that is required for the processing of G-quadruplex structures in telomeres for DNA replication. I found that early-infected B cell proliferation is more sensitive to inhibition of BLM than LCLs. However, I have not looked at the impact BLM inhibition has on the formation of DDR foci by IF-telomere FISH. We would expect that this impacts foci formation leading to the inability to establish senescence.

### 5.1.4 Single cell analysis

Through our work describing the barriers to transformation efficiency, including activation of the DDR, metabolic imbalance, deficiency of nucleotide metabolism and establishment of persistent DDR foci, we have extensively described the implications of EBV-induced hyper-proliferation. While this work has served an important role in the field by progressing our understanding of the early stages of EBV infection an important caveat remains. It is essential to note that the data we have collected is based on the bulk population of EBV-infected B cells. Our stringent sorting strategies have facilitated our ability to identify and characterize the specific subpopulation of B cells that arrest;

however, cell populations are not homogenous. It will be important to look at the single cell level to better understand the specific factors required for promoting transformation. More specifically, it will be essential to determine if all EBV-infected B cells undergo hyper-proliferation. It is possible that B cells that arrest early after infection are the only cells that are experiencing hyper-proliferation and further that cells that continue proliferating never induce a burst in proliferation, thus allowing them to bypass this stressful barrier. To address this question, we are developing a single cell array that will allow us to compartmentalize a single infected B cell into a micro well that can be used in conjunction with live cell imaging microscopes to monitor individual cell proliferation kinetics, including time to division and rate of division. Additionally, we can apply single cell analysis toward our understanding of how chemical inhibitors and nutrient supplementation effect transformation at the single cell level. Developing this system will further inform our research of the proliferative barriers and the variation in proliferation that exist.

## *5.2 Conclusion*

The work discussed in this dissertation has primarily focused on understanding the intrinsic human cellular responses that act to suppress EBV-mediated transformation. Here, we have specifically studied the early stages of EBV infection to determine the upstream factors at play that are responsible for inducing DNA damage contributing to the cellular senescence phenotype we observe in the majority of infected human B cells. Furthermore, we sought to understand the OIS manifests itself downstream of the instigation of DNA damage.

Our initial discoveries discussed in this dissertation expanded on previous findings that early after EBV infection, B cells undergo a transient period of hyper-proliferation, which coincides with increased activation of the DDR leading to a G1/S phase growth

arrest (Nikitin, Yan et al. 2010, Nikitin, Price et al. 2014). To better study the predominantly arrested population of EBV-infected B cells, we developed a unique double staining proliferation assay to identify and isolate the arrested cells from those that continue to proliferate and transform into LCLs. We found that the arrested cells exhibited many characteristics of senescence, specifically OIS. The arrested population of B cells exhibited an increase in DDR activation and induced global heterochromatin as evidenced by the presence of SAHF (McFadden, Hafez et al. 2016). Additionally, the arrested cells could not be re-stimulated to proliferate with fresh media or mitogen supplementation. Interestingly, hyper-proliferating B cells that underwent arrest displayed presence of autphagosomes and phagolysosomes suggesting that EBV-infected B cells undergo autophagy (McFadden, Hafez et al. 2016), which has been shown to trigger OIS potentially in an effort to avoid continuation of aberrant cellular proliferation.

Next, we examined the upstream molecular source of the DDR-mediated OIS. We discovered that EBV-infected B cells undergoing hyper-proliferation early after infection displayed upregulation of replication stress as compared to the infected cells that successfully overcame hyper-proliferation. This was evidenced by activation of the ATR/Chk1 pathway. We further probed the presence of SSB and DSB using the unique fluid halo assay technique and identified consistent evidence of increased DNA damage and replicative stress in specifically the early infected B cells (Hafez, Messinger et al. 2017). Importantly, we discovered that B cells undergoing hyper-proliferation exhibit reduced dNTP levels, particularly purine dNTPs, that led to their inefficient transformation. Supplementation of growth media with exogenous nucleosides rescued activation of replicative stress markers and senescence. Further, specifically purine nucleoside supplementation at early times post infection facilitated EBV-mediated B-cell outgrowth

suggesting that purine biosynthesis contributes to the source of DNA damage and is a major limiting step in EBV transformation (Hafez, Messinger et al. 2017).

Lastly, we established that early infected B cells that arrest after hyper-proliferation undergo OIS by the formation of persistent DNA damage foci that function to allow cells to maintain senescence. We characterized the presence of persistent DDR foci specifically in the arrested subpopulation of EBV-infected B cells and discovered that following the burst in proliferation, arrested B cells displayed an increase in PML NBs as well as PML NB-associated DNA damage foci, or APBs (Hafez and Luftig 2017). We further discovered that persistent DNA damage localized to telomeric DNA forming TIFs. Interestingly, danazol, a drug used to treat telomere maintenance disorders increased proliferation of B cells specifically early after infection and enhanced transformation of B cells into LCLs. Lastly, we determined that early proliferating B cells are more sensitive to inhibition of a key telomere replication protein, BLM helicase than LCLs (Hafez and Luftig 2017). This suggests that telomeric DNA damage plays a major role as a transformation barrier upon EBV infection.

Ultimately, this work describes key findings that shape our understanding of how the host controls EBV infection. EBV infection and expression of viral oncogenes drive primary human B cells to enter a transient period of hyper-proliferation. In the context of limited nucleotide pool levels, cells are unequipped to accurately or efficiently replicate their DNA making it difficult to sustain rapid proliferation. These low dNTP levels promote replication stress and DNA damage, which may be a result of stalled or collapsed replication forks. The observed activation of DNA damage specifically occurs at telomeres and are irreparable, which consequently lead to the formation of persistent DNA damage foci and promote the establishment of OIS in the majority of EBV-infected B cells. Taken

together, these results portray a stressful environment imposed upon EBV infection of primary human B cells.

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# Biography

Amy Youssef Hafez was born in Salisbury, Maryland on October 31st, 1988. She attended Salisbury University in Maryland from 2006 to 2010 where she graduated *cum laude* and received her Bachelor of Science in Biology with a concentration in Microbiology. During her undergraduate career, Amy worked in Dr. Mark Frana's laboratory investigating the sources of *Escherichia coli* and *Enterococcus faecalis* found in watersheds on the Eastern Shore of Maryland. While at Salisbury University she was inducted in the Tri Beta Biological Honor Society and Omicron Delta Kappa Leadership Honor Society. Amy then began her graduate career at Johns Hopkins University in Baltimore, Maryland from 2010 to 2012 where she received a Master of Science in Biotechnology. She conducted her Master's thesis research in Dr. Egbert Hoiczyk's laboratory in the Department of Molecular Microbiology and Immunology studying the mechanisms responsible for bacterial osmoprotection by using the soil bacterium *Myxococcus xanthus*.

Amy continued her graduate career at Duke University in Durham, North Carolina in 2012 where she joined the Doctor of Philosophy program in the Department of Molecular Genetics and Microbiology. She conducted her PhD thesis research in Dr. Micah Luftig's laboratory where she investigated the role that the human DNA damage response plays in suppression of Epstein-Barr virus-mediated growth transformation. Amy authored and co-authored several scientific research articles and book chapters, including "Epstein-Barr virus induced B-cell immortalization is suppressed by metabolic imbalance leading to autophagy-associated senescence," "Nucleotide pool depletion limits Epstein-Barr virus-mediated B-cell immortalization," "Characterization of the EBV-induced persistent DNA damage response," and "DNA tumour viruses and the host DNA damage response." Amy

was selected to receive the Viral Oncology Training Grant from 2012 to 2014, was the recipient of numerous travel awards, including the Viral Oncology Travel Award and the Chairman's Meritorious Travel Award, and received Best Poster awards at the Duke-UNC Viral Oncology Symposium in 2014 and 2016 as well as in 2015 at the MGM departmental retreat.