The regulation of EBV lytic reactivation in epithelial cells

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Abstract

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Epstein-Barr virus (EBV) is a herpesvirus infecting >90% of the population. An EBV infection can be latent, where few genes are expressed, or lytic, where the entire viral gene complement is expressed to replicate the genome and produce infectious virions**.** EBV infects B cells, which are considered to be the latent reservoir of the virus, and epithelial cells, which are an important site of lytic replication. While lytic reactivation from latency in B cells has been extensively studied, the regulation of this process in epithelial cells is relatively uncharacterized. Furthermore, EBV infection of these two cell types is associated with malignancies such as Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma, and gastric carcinoma. All EBV-associated cancers maintain a latent EBV infection and understanding how the EBV latent-to-lytic switch is regulated and maintained by cellular factors can give an understanding of not only how this event occurs, but also how cancers develop. In this thesis, I will show that three cellular proteins: p63, YAP, and TAZ, have differential effects on the ability of EBV to reactivate from latency.

In Chapter 2 of my thesis, I will show that the p63 isoforms ΔNp63α and TAp63α inhibit EBV from undergoing lytic reactivation in epithelial cells and B cells, respectively. I will also demonstrate that ΔNp63α inhibits Z promoter activity. I found that ΔNp63α expression increases the expression of the lytic repressor c-myc, and decreases activity of the lytic enhancer p38 MAPK, indicating that ΔNp63α and TAp63α can inhibit EBV lytic reactivation through at least two mechanisms. In Chapter 3 of my thesis, I will demonstrate that the Hippo signaling effector

genes YAP and TAZ induce lytic reactivation via the EBV immediate-early BZLF1 promoter and require the co-activator gene family TEADs for this effect. Finally, I show that YAP, TAZ, and TEADs are expressed in EBV-infected epithelial cell lines, but are not highly expressed in EBV-infected B cell lines. Thus, this phenomenon is an epithelial cell-specific mechanism by which the virus reactivates. My work indicates that cellular genes that are commonly upregulated in epithelial cancers regulate EBV lytic reactivation.

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List of Abbreviations

AIDS- Acquired immune deficiency syndrome

ARE- Adenosine/uridine-rich element

ATM- Ataxia-telangiectasia mutated

BARTs- BamH1 A rightward transcript

BCR- B cell receptor

BL- Burkitt Lymphoma

BZLF1- BAMH1 Z Leftward Frame 1

BRLF1- BAMH1 R Leftward Frame 1

BLIMP1- B-lymphocytes induced maturation protein 1

BMRF1- BAMHI-M rightward frame 1

CD- Cluster of differentiation

DLBCL- Diffuse large B-cell lymphoma

DNMT- DNA methyl transferase

EBERs- EBV encoded RNA

EBNA- Epstein-Barr virus nuclear antigen

EBV- Epstein-Barr virus

EEC- Ectrodactyly ectodermal dysplasia cleft syndrome

EMT- Epithelial mesenchymal transition

GC- Gastric carcinoma

GCPR- G-coupled protein receptors

GFP- Green fluorescent protein

HL- Hodgkin's lymphoma

HPV- Human papillomavirus

KSHV- Kaposi's sarcoma-associated herpesvirus

KLF4- Kruppel-like factor 4

IE- Immediate-early

IHC- Immunohistochemistry

IM- Infectious mononucleosis

LMP1- Latent membrane protein 1

LMP2A- Latent membrane protein 2A

LMP2B- Latent membrane protein 2B

LPA- Lysophosphatidic acid

MAPK- Mitogen-activated protein kinase

MAPKAPK2- MAP kinase-activated protein kinase 2

NOKs- Normal oral keratinocytes

NPC- Nasopharyngeal Carcinoma

ORF- Open reading frame

OHL- Oral hairy leukoplakia

PIAS1- Protein inhibitor of activated STAT1

PKR- Protein kinase R

RB1- Retinoblastoma protein

RIG-I- Retinoic acid-inducible gene I

RRE- R-responsive element

TAZ- Transcriptional co-activator with PDZ-binding motif

TGF-β- Transforming growth factor beta

TTP- Tristetraprolin

TPA- Phorbol-12-myristate-13-acetate

VCA- Viral capsid antigen

YAP- Yes-associated protein

ZRE- Z-responsive element

Chapter 1

Introduction

Epstein-Barr virus overview

Epstein-Barr virus (EBV) is a human gamma-herpesvirus that infects 90% of the population. EBV infects B cells, where it mostly resides as a latent reservoir, and epithelial cells, where EBV actively replicates (1–3). Primary infection with EBV is mainly spread by saliva and usually occurs in childhood or early adolescence (though EBV can be acquired at any stage of life). From adolescence into adulthood, primary EBV infection is the cause of infectious mononucleosis (IM) (4). Primary EBV infection becomes controlled, but not cleared, once CD8+ T cell surveillance is established against EBV lytic proteins and some latent proteins (5). The virus subsequently retreats into a restrictive gene expression pattern known as latency, where few genes are expressed. Latent infection enables the virus to evade the immune system, ensure the survival of infected cells, and maintain the viral genome. During cellular processes such as differentiation or B-cell receptor (BCR) stimulation, EBV switches from latency to lytic replication, where the virus produces infectious virions that can infect new cells or new hosts. With this persistent and evasive evolutionary strategy, EBV is arguably one of the more successful (if not most widespread) human pathogens ever.

The health impact of EBV

The health impact of EBV infections is notable as EBV is not only associated with IM, a condition that can significantly impact the quality of life during formative years, but also several malignancies that account for approximately 1.5% of all new cancer cases each year (6). EBV infection is present in a number of cancers, including Burkitt lymphoma (BL), Hodgkin lymphoma (HL), post-transplant immunoproliferative disease, AIDS-related lymphomas, nasopharyngeal carcinomas (NPC), some NK/T lymphomas, and 10% of all gastric carcinomas (GC) (1). As a result of the persistent EBV infection that is thought to be spurring the development of these cancers, about 200,000 deaths a year are attributed to EBV-associated malignancies (7).

Discovery of Epstein-Barr virus

Epstein-Barr virus was initially discovered in 1964 by Anthony Epstein and Yvonne Barr. However, the story of this tumor virus precedes its identification (8). In 1954, Denis Burkitt was a missionary surgeon in Uganda and encountered children under his care presenting large tumors along the jaw that turned out to be aggressive lymphomas. Eventually, these childrens' lymphomas would be described as Burkitt lymphoma, and during his study of this malignancy, Denis Burkitt noticed that these tumor cases were only occurring in regions where malaria was also endemic (9). This aroused his suspicion that an infectious agent was at the root of these malignancies. Burkitt reported these findings on a university lecture circuit, where he established a collaboration with Epstein who had a background in electron microscopy and an interest in viruses being as cause of cancer. When Epstein and Barr acquired tumor biopsies from Burkitt, they examined them with electron microscopy and were able to identify viral particles of a novel herpesvirus, thus demonstrating the first instance of a human tumor being associated with a virus.

EBV Latency

EBV is a gamma-herpesvirus and, like all herpesviruses, has a latent and lytic phase. EBV latency describes a state of viral infection where relatively few genes are expressed; however, these genes are essential for the maintenance of the EBV genome and the survival of the infected cell. During EBV latency, the viral genome is maintained as a circular episome, and this episome is replicated solely by the cellular DNA polymerase during cell division. There are three patterns of latency with distinct gene expression profiles defined as type I, type II, and type III latency as depicted in **Table 1**.

Type III Latency

Type III latency is characterized by the expression of nine different gene products, the BART and BHRF1 microRNAs, and the EBV non-coding RNAs (EBERs). These genes are EBNA1, LMP1, LMP2A/B, EBNA2, EBNA-LP, EBNA3A, EBNA3B, and EBNA3C. Of the genes specific to type III latency, the ones essential for the ability of the virus to immortalize B cells *in vitro* are EBNA2, EBNA-LP, EBNA3A, and EBNA3C (in addition to EBNA1 and LMP1, which are expressed in other latency patterns) (1,10). In the context of the human host, type III latency can occur when the patient's immune system is compromised, such as AIDS-related lymphomas, post-transplantation lymphomas, and during initial B cell infection. Importantly, type III latency does not occur in epithelial cells at least in part because epithelial cells do not express the transcription factor PAX5, which induces activation of the Wp promoter and the expression of EBNA2 (11). EBNA-LP and EBNA2 are the first viral genes to be expressed during primary viral infection in B cells, initially from the W promoter and, subsequently, the C promoter as the infection progresses. EBNA2 is a mimic of an activated Notch receptor and recruits RBPJ-K to induce c-myc and cyclin D2 expression (12,13) as well as activate the promoters of all type III latency genes (with the exception of the BARTs and EBERs) (14,15).

EBNA3A and EBNA3C are both essential genes for EBV immortalization of B cells *in vitro* (1). Both EBNA3A and EBNA3C prevent the expression of the tumor suppressor proteins p16 and p14ARF through epigenetic modifications that silence their expression, which will cease EBV-infected B cell proliferation if unchecked (16–19). Additionally, both genes can inhibit apoptosis by epigenetically repressing BIM expression (20–22). Recently, the Kenney lab has shown that both EBNA3A and EBNA3C are not required for lymphomagenesis *in vivo*, although the presence of these genes allows for more lymphomas to efficiently develop (23,24). The role of EBNA3B is relatively uncharacterized and is dispensable for the generation of lymphoblastoid cell lines (LCLs) (25). Interestingly, the Allday group has demonstrated that

EBNA3B acts as a tumor suppressor by recruiting T cells to the infected cells, with a mutant virus lacking EBNA3B having enhanced lymphomagenesis in humanized mice (26). Finally, there is a set of EBV-encoded microRNAs specific to type III latency, the BHRF1 group. These three microRNAs are important, but not essential, for the transformation of B cells (27,28). BHRF1 also encodes an anti-apoptotic protein that mimics BCL-2 (29).

Type II Latency

In Type II latency, the BART microRNAs, EBERs, EBNA1, and the latent membrane proteins LMP1, LMP2A, and LMP2B are all expressed. This latency pattern is observed in Hodgkin lymphoma and nasopharyngeal carcinoma. Latent membrane protein 1 (LMP1) was initially discovered in 1985 by the Kieff group, who determined that this viral gene was capable of transforming Rat1 cells and enabled these cells to grow in soft agar (30). As the name suggests, LMP1 is a membrane protein. It is most notable for being a mimic of the CD40 ligand, enabling the infected B cell to survive (31,32). Unsurprisingly, LMP1 is essential for EBV to transform B cells *in vitro* (33). However, LMP1 expression is not necessarily essential *in vivo* in the context of a lymphoma since tumor-infiltrating T cells can provide CD40 ligand required for these cells to survive (34). In epithelial cells, LMP1 expression manipulates a number of cellular signaling pathways, notably activating both the canonical and non-canonical NF-κB signaling pathways as well as the ERK/MAPK, PI-3K/AKT, and PKC pathways (35). LMP1 can also inhibit the function of p53, in part by altering the activities of the A20 protein which is upstream of p53 (36). In light of all of these known properties of LMP1, it has been considered an oncogene of EBV. While LMP1 is only expressed in a minority of NPC cases, LMP1 still may be pivotal for the development of these tumors as it has been detected in premalignant lesions, and NPC tumors lacking LMP1 expression have mutations that increase NF-κB activity (37–39).

While LMP1 is a latency gene, LMP1 expression also increases during lytic replication when R induces activation of both LMP1 promoters (40,41). The function of LMP1 during lytic

reactivation may be different depending on the cellular context in which it is expressed. In EBV-infected B cells, LMP1 has been reported by several groups to inhibit lytic replication (42–45). However, in epithelial cells, work from our laboratory and others has shown that when LMP1 expression is dramatically increased during epithelial cell differentiation, and can facilitate efficient EBV lytic reactivation (41,46). This increase in LMP1 expression is mediated by the cellular differentiation factors KLF4 and BLIMP1 (41) and, interestingly, LMP1 expression precedes that of the immediate-early (IE) genes. Thus, LMP1 has an essential role in mediating viral re-entry into replication in a cell-type and context-specific manner.

LMP2A is another membrane protein expressed during type II latency. LMP2A is known for mimicking certain components of the signaling pathway of the B-cell receptor, delivering essential survival signals to infected B cells (47). A recent phosphoproteomics report adds nuance to this statement, by demonstrating that LMP2A signaling in B cells alters the phosphorylation of over 600 proteins distinct from the BCR signaling pathway such as the RB1 protein (which LMP2A induced degradation of) (48). Despite LMP2A contributing to B cell survival, LMP2A itself is not critical to transforming B cells *in vitro*, except for instances where germinal center cells have a non-functional BCR arrangement (49). Crosslinking of the BCR reactivates EBV, and LMP2A can enforce viral latency by dampening these signals (50,51). LMP2A can also act as a positive inducer of lytic reactivation depending on the expression level of LMP2A (52,53). In the context of epithelial cells, LMP2A can decrease differentiation, induce AKT signaling, and inhibit anoikis (54–56). LMP2B is a splice variant of LMP2A that interferes with LMP2A BCR signaling repression and can inhibit interferon signaling by degrading the interferon receptor (57,58).

Type I Latency

Infected cells in type I latency are characterized by the expression of EBV microRNAs BARTs, the non-coding RNAs EBERs, and EBNA1. This latency is the most restrictive but

allows the virus to evade immune surveillance. Examples of type I latent infections include infected resting B cells circulating in the blood, Burkitt's lymphoma, and gastric carcinoma.

The EBV microRNAs, known as the BARTs, are a large family of about 44 microRNAs expressed out of two clusters of the EBV genome referred to as type I and type II clusters. The BARTs were the first viral microRNAs to be discovered (59) and are expressed in all stages of EBV infection (60). However, BARTs are highly expressed in NPCs and gastric carcinomas, suggesting BARTs impart crucial selective advantages to these malignancies with a more stringent latency pattern (61–64). The expression of BARTs results in the modulation of both cellular and viral mRNAs by binding to these mRNAs and directing them to the RISC machinery for degradation. The result of this process is reduced expression of target mRNAs.

The BARTs are known to prevent cellular apoptosis by targeting proteins in the cell death pathway such as PUMA, BIM, caspase-3, and p53 (65–69). Work by Vereide *et al.* demonstrated that the EBV microRNAs are essential for the survival of infected Burkitt lymphoma cells by depleting these microRNAs and quantifying cell proliferation; this anti-apoptotic role occurs in the context of NPC and GC as well (70). In EBV gastric carcinoma and Burkitt lymphoma xenograft animal models, expression of the BARTs increased the growth rate of these tumor cells (71). Despite mitigating cell death, the BARTs are not essential for EBV to immortalize B cells. The EBV microRNAs also target the expression of EBV viral genes, most notably LMP1, the immediate-early genes BZLF1 (Z) and BRLF1 (R), and the viral polymerase BALF5 (72–74). The dampening of expression of these viral genes likely acts as a method for maintaining EBV latency in infected cells and for evading the host immune system responses to EBV-infected cells.

There are two additional EBV non-coding RNAs, termed EBER1 and EBER2. EBERs are expressed at all stages of viral latency, as well as during lytic reactivation, but they are not required for EBV to transform B cells (75). Because of their ubiquitous nature, EBERs are

commonly utilized as a marker for detecting EBV infection in tissue samples (76). Notably, EBERs improve the growth rate of EBV-negative Burkitt's lymphoma cells and have an anti-apoptotic role (77–79). Additionally, EBERs appear to prevent interferon-mediated death by inhibiting both PKR and RIG-I during viral infection (80,81).

The EBNA1 gene encodes a protein responsible for the maintenance of the EBV genome during latency and ensures viral DNA replication using the host DNA polymerase (1,82,83). In a latently infected cell, EBNA1 binds to both the viral genome and cellular chromosomes during cell mitosis to maintain viral infection of daughter cells (83–85). During type I and type II latency, EBNA1 acts as a negative regulator and binds to the Q promoter to inhibit its promoter activity (86). Given all of these functions, EBNA1 expression is required for EBV to transform primary B cells as it maintains the viral genome and has the distinction of being the only viral gene expressed in all EBV-associated cancers (9). EBNA1 also physically interacts with the ubiquitin protease USP7, which degrades the tumor suppressor p53 (87,88). In line with this observation, EBNA1 expression provides EBV-infected cells some degree of protection from apoptosis (89).

The EBV lytic replication cycle

EBV lytic replication is defined by the virus expressing its entire gene complement to produce infectious virions. The EBV lytic cascade is initiated when the immediate-early genes' promoters are activated by cellular transcription factors, and become expressed (1,90). EBV has two IE genes that act as transcription factors: BZLF1, also known as Z, and BRLF1, which has the alias R. Once these IE genes are expressed, they go on to induce additional expression of themselves as well as the other IE gene in a positive feedback loop (90–92). The IE proteins also initiate the next stage of lytic replication by binding to and activating the promoters of the viral early genes to induce their expression (the summary of this pathway is illustrated in **Figure 1**). The EBV early genes encode proteins that enable the replication of the EBV genome via the

virally encoded DNA polymerase. Some examples of this group of genes are BMRF1, which encodes the viral DNA processivity factor for the replication complex, and BALF5, the viral DNA polymerase. Once the complex of early proteins replicates the EBV viral genome, the late viral genes are expressed. Most genes classified as late genes encode either capsid proteins such as VCA-p18, envelope glycoproteins such as gp350, or tegument proteins such as BPLF1 that facilitate the infection of new cells (93).

In the EBV infected B cell latent reservoir, the virus can occasionally reactivate from latency when an infected B cell differentiates into a plasma B cell or undergoes BCR stimulation (94–96). However, EBV virions in the saliva originate from infected epithelial cells (97). In organotypic rafts mimicking stratified epithelium, the infection of stratified normal oral epithelial cells supports robust lytic replication (98). There is an extensive list of biological processes known to induce EBV lytic replication, at least *in vitro*. These processes include, but are not limited to, cellular differentiation in both B cells and epithelial cells, DNA damage, hypoxia, TGF-β signaling, B-cell receptor stimulation, and apoptosis (99–104). Additionally, compounds such as gemcitabine, 12-O-tetradecanoylphorbol-1,3-acetate (TPA), calcium, and HDAC inhibitors (e.g. sodium butyrate) all can induce lytic reactivation through one or more of the aforementioned pathways (90,102,105–107)**.**

Regulation of BZLF1 protein function

Expression of the IE gene Z initiates the switch from latency to lytic reactivation in many EBV infected cell types (108). In addition to the ability to induce the expression of early genes, Z also binds to and activates the origins of lytic (*orilyts*) replication during viral DNA replication in a function distinct from the ability to induce early genes (109,110). The Z protein is homologous to the cellular transcription factors c-Fos and c-Jun, which compose the AP-1 transcription factor complex (111). Interestingly, these two related cellular factors are also capable of binding to the Z promoter (90). Z, however, has specific binding sites known as Z-responsive elements that

contain CpG sites and resemble AP-1-like motifs (112,113). When expressed, Z can induce the expression of the other IE gene, R, and can bind to the R promoter to induce R protein expression (114). Z is unusual as a transcription factor in that it preferentially binds to methylated promoters which allows the methylated EBV genome to emerge from latency (113–115). Z transcriptional functionality is negatively regulated by sumoylation, with the protein becoming transcriptionally inefficient when sumoylated, at lysine 12 (116). Z can broadly induce lytic reactivation in EBV-infected B cell lines and epithelial carcinoma cell lines. Still, both Z and R need to be present for EBV to complete the lytic reactivation cycle, indicating that both IE genes have distinct and essential roles during lytic reactivation (117). However, in the TERT-immortalized NOKs cell line, Z cannot initiate EBV lytic reactivation as the genome is hypomethylated, although Z still synergizes with R to induce the lytic cycle (115)**.**

The regulation of the Z promoter

The Z promoter has been extensively studied and was originally shown to have several *cis*-acting elements in the proximal promoter defined as ZI to ZV. An accompanying illustration of these mapped elements and the transcription factors that bind them is depicted in **Figure 2**. There are a number of biological processes that induce transcription factors to bind to and activate the Z promoter. Plasma cell differentiation can induce lytic reactivation, and two crucial cellular factors that are activated during this process, XBP-1s and BLIMP1, can activate the Z promoter (95,96,101,118). Epithelial cell differentiation also induces BLIMP1, as well as another terminal differentiation factor, KLF4, to activate the Z promoter (100). BCR stimulation is well characterized to induce EBV lytic reactivation (119). This cellular process activates NFATc1 and NFATc2, calcium-sensitive transcription factors that can bind to the ZIIIA motif in the context of the Zp-V3 variant in a complex with AP-1 (52,53). Interestingly, B cells infected with type II EBV strains have increased lytic reactivation due to elevated NFATc1 and NFATc2 activity (53).

Multiple groups have reported TGF-β to induce lytic reactivation by inducing the expression of Z through SMADs binding to the dispersed SMAD-binding elements in the Z promoter (120–122). The ZII element can be bound to and activated by numerous cellular transcription factors such as AP-1, ATF1, ATF2/c-Jun, CREB, XBP-1s, as well as inhibited by JDP2 at this location as well (95,123–125). The ZII element is also where R induces Z promoter activation through cellular factors such as p38 MAPK which phosphorylates the downstream ATF2 (123). The activity of this element is heavily influenced by cellular kinases such as PKC, PKD, and p38 MAPK. TPA treatment, which can induce the activity of these kinases, requires this Z promoter region to induce Z promoter activity (90,106). Hypoxia signaling is a process that stabilizes HIF-1α and HIF-2α in response to low oxygen. Both HIF proteins can bind to a HIF-responsive element (HRE) located near ZID on the Z promoter to activate Z expression; they require p53 to do so (99,126).

The cellular kinase ataxia-telangiectasia mutated (ATM) signals through the Z promoter and is required for the ability of EBV to reactivate through multiple stimuli such as the chemotherapy agent paclitaxel, TGF-β, and H_2O_2 (102). The downstream effectors of this kinase that induce the activity of the Z promoter is not entirely clear although, recently, the ATM kinase target KAP1 has been implicated in inducing lytic reactivation (127).

There are several known inhibitors of the Z promoter. MEF2D is mapped to several elements on the Z promoter, where MEF2D can recruit histone deacetylases to inhibit Z promoter activation (128,129). This inhibitory role changes during B-cell receptor engagement, where MEF2D on ZI elements become dephosphorylated and instead becomes an inducer of Z expression (130). PIAS1 can bind to the Z promoter at an unknown site to inhibit Z promoter activity, and PIAS1 repression is lost when caspases 3, 6, and 8 are activated during BCR stimulation (131). The ZV and ZV’ elements of the Z promoter can be occupied by ZEB1 and ZEB2, which are potent inhibitors of the EBV lytic cascade (132–134).

Regulatory mechanisms of R activation of the lytic cycle

The other IE gene of EBV lytic replication is BRLF1 (also known as R or RTA). Unlike Z, R has no cellular homolog, but it is similar to the Kaposi's sarcoma associated herpesvirus (KSHV) RTA which is solely responsible for inducing KSHV's lytic cascade (135,136). R binds to R-responsive elements (RREs) which are defined by the sequence 5'- $GNCCN_9GGNG$ –3' (137,138). R can bind to both methylated and unmethylated EBV promoters, but R only strongly induces promoter activity in unmethylated promoters (115). R can also activate promoters, including the Z promoter, through mechanisms independent of DNA-binding by indirect effects on cellular transcription factors, including ATF2 and OCT1 (123,139). R also interacts with the histone acetylases CBP/p300, which improves R-mediated induction of lytic reactivation by acetylating promoters (115,140). R is notable for inducing lytic reactivation in epithelial cell lines, particularly in the case of the telomerase-immortalized NOKs-Akata epithelial cell line where only R can initiate lytic reactivation (115,141). Viral proteins such as LF2 also impact R functionality which binds to R and re-localizes it to the cytoskeleton to inhibit R-mediated lytic reactivation (142). On the other hand, the early gene Na cooperates with R to induce lytic reactivation (143,144).

The regulation of the R promoter

The R promoter is not nearly as well characterized in terms of regulation as the Z promoter and can be viewed in **Figure 3**. The R promoter contains known ZREs as well as Sp1/KLF4 binding sites, Sp3 binding sites, and an NF1 element that are all known to induce R promoter activity (92,100,101,145). During differentiation of both epithelial cells and B cells, BLIMP1 binds to and activates the R promoter (101). YY1 and the ZEBs can bind to the R promoter and repress lytic activity (132,146). The lytic repressor PIAS1 can bind to the R

promoter to block promoter activity, though the exact site where PIAS1 binds has not been mapped (131). While R does not bind to the R promoter directly, R can indirectly activate its promoter in a positive feedback loop through the Sp1/Sp3 sites (92).

EBV infection of epithelial cells and EBV-associated epithelial cancers

Not long after the initial discovery of EBV, EBV DNA was found to be present in almost all undifferentiated NPCs (147). This established that EBV was not only associated with B cell lymphomas but epithelial carcinomas as well. EBV is also associated with gastric carcinoma (GC), although this was not discovered until 1992 (9). Despite the clinical importance of EBV infection in epithelial cells, EBV infection of B cells has been much better characterized. Progress has been stymied by the noted difficulty of studying EBV infection of epithelial cells. The discovery of EBV infection in normal oral epithelial cells from IM patients did not even occur until the 1980s (2,148). In primary epithelial cell culture models, EBV does not immortalize infected epithelial cells, unlike B cells, and EBV ultimately fails to establish long-term latently infected cell lines from primary epithelial cells (149,150). All EBV-infected epithelial cell lines require an antibiotic selection marker such as G418 to maintain EBV infection as the virus provides no positive selective advantage in this context. Additionally, EBV infection of epithelial cells is very inefficient compared to B cells (150).

While an EBV latent infection is detectable in almost 100% of undifferentiated NPC epithelial cell malignancies, it is difficult to find any evidence of EBV infection (latent or lytic) in normal epithelial tissue of healthy subjects (98,100). An exception to this rule is oral hairy leukoplakia (OHL), the sole known pathology caused by lytic EBV in epithelial cells (151,152). This tongue lesion is associated with patients who are immunocompromised, such as in late-stage AIDS, and biopsies of these lesions show extensive EBV lytic replication occurring in the differentiated epithelial cells (151). Interestingly, while there is scant evidence of latent or lytic EBV infection of the undifferentiated epithelial cells, very sensitive means of detecting EBV such

as qPCR found EBER transcripts in micro-dissected oral epithelia (100). This observation has led investigators to question why normal undifferentiated epithelial tissue is refractory to EBV infection, but tumors such as NPC and some GC contain a latent EBV infection.

Establishment of EBV-infected epithelial cell culture models

While EBV is difficult to find in normal oral tissue, EBV infection is detectable in precancerous lesions of the nasopharynx (38,153). This observation has led to several studies essential for understanding how EBV latent infection could occur in epithelial cells. A study by Tsang *et al.* showed that the tumor suppressor p16 posed a significant barrier to the establishment of EBV latency in epithelial cells and that overexpression of CDK1 can overcome this obstacle (154). This study provided strong evidence that EBV latency can become established in premalignant epithelial tissue.

When infecting telomerase-immortalized epithelial cell lines such as normal oral keratinocytes (NOKs), we found that EBV could establish a latent infection in these cell lines (albeit under G418 selection) (100,101,115). This cell line presented a novel means for studying EBV in epithelial cells, given it had not been transformed (unlike EBV-infected carcinoma cell lines) and retains the ability to differentiate and stratify similar to non-immortalized epithelial cells (41,100). A collaborative effort between the Kenney, Johannsen, and Lambert laboratories found that the differentiation of NOKs infected by EBV is considerably reduced compared to uninfected controls (100,155,156). The viral gene(s) responsible for this impairment in epithelial cell differentiation is not currently known; it is, however, a pressing question for the field as reduced differentiation is a hallmark of cancer. Recent work by Eichelberg *et al.* demonstrated that when NOKs cells infected with deletion mutants lacking either the lytic Z gene or the EBV microRNAs BARTs, there is still a reduction in the ability of these epithelial cells to differentiate, indicating that perhaps a latency gene such as LMP1 or LMP2A may be inhibiting epithelial cell differentiation in this context (156). If true, this idea would support the hypothesis that the

expression of EBV latent genes could promote further progression of these premalignant cells towards tumorigenesis given LMP1 and LMP2A are both considered to be oncogenes.

Aspects of EBV-infected NPC and GC

While EBV infection of epithelial tissues occurs in nearly all adults, NPC tumors are mostly geographically limited to Southeast Asia, while EBV-associated GC is predominantly observed in a few Asian countries including Korea. Biomarkers of EBV infection such as circulating EBV DNA have been established as a risk factor for NPC development (157). NPC tumors occasionally have mutations in genes that inactivate or delete p53 and activate NF-κB signaling. However, relatively few common mutations are found in NPC tumors compared to HPV-positive head and neck cancers (39,158).

NPC tumors are notable for their hypermethylation, an alternative means to shut off the expression of tumor suppressor genes such as RASSF1A (159,160). This methylation likely begins when EBV initially infects the epithelial cells. Studies conducted by the Scott lab have shown that even transient infection of EBV increased methylation of cellular genes such as TIP30 (161,162). How methylation of the cellular genome occurs during EBV viral infection of epithelial cells has not been fully explored, although the virus may co-opt cellular DNA methyltransferases (DNMTs) for this purpose (162–164). EBV-positive GC is also notable for hypermethylation of tumor suppressor genes such as PTEN (164,165).

Another interesting aspect of EBV infection during the development of NPC tumors is an increase of EBV-specific antibodies against lytic proteins in patients with premalignant NPC (166). This suggests that EBV lytic infection may spur the development of NPC (though EBV infection of these tissues will eventually establish a latent state). Recent work in support of this hypothesis is the finding that the EBV lytic protein BNRF1 can induce chromosomal instability, a hallmark of cancer (167). What circumstances initiate lytic reactivation and tumorigenesis in

these premalignant epithelial cells is not clear, although it may be a failure of host immune surveillance to kill the EBV-infected cells.

An introduction to p63

In Chapter 2 I will demonstrate that ΔNp63α, along with TAp63α, is an inhibitor of EBV lytic reactivation. Tumor protein 63 (TP63, or p63) is a part of the p53 protein family along with p53 and p73. ΔNp63α was initially thought to have roles in both apoptosis and potential antagonism (due to the lack of a transactivating domain) of the functions of the related p53 and p73 proteins in the context of epithelial cells (168). ΔNp63α has since distinguished itself as critical for the epithelial stem cell compartment, development of limbs, and the regulation of epithelial cell differentiation (169–171). As a transcription factor, ΔNp63α can bind to thousands of cellular promoters through its DNA-binding domain and has been reported to bind to the same promoter binding sites as p53 (172,173). This observation has contributed to the hypothesis that ΔNp63α may act as a competitive inhibitor of p53 in some contexts. This DNA binding capability is also essential for ΔNp63α repression of differentiation and maintenance of proliferation, repressing the expression of genes such as KLF4, HES1, PTEN, and p21 by inhibiting their promoter activities (174–177).

p63 isoforms and functions of the C-terminal domain of the α isoform

There are ten different isoforms of p63, they are split into two categories based upon an N-terminal transactivating domain (TA) or lack thereof (ΔN) that are derived from alternative transcriptional start sites. Alternative splicing (or a stop codon in exon 10 in the case of ε) of the C-terminal domain dictates if the isoform is α, β, γ, δ, and ε (178,179). **Figure 4** presents an illustration of the different isoforms and their protein domain structures. The C-terminal domain of both ΔNp63α and TAp63α contains a sterile alpha motif domain (SAM); this SAM domain is not present in the other isoforms (180). The SAM domain has been reported to be important for

protein-protein interactions, mostly with other proteins that also have SAM domains (181). There are also reports of ΔNp63α interacting with histone acetylation protein CBP/p300 within this C-terminal region, and CBP/p300 are known to be crucial for mediating EBV lytic reactivation (140,182). Additionally, the SAM domain increases the expression of c-myc, as this region of ΔNp63α and TAp63α can bind to the c-myc regulator MM1 to induce degradation (183,184). This finding has implications for the EBV latent-lytic switch, given c-myc inhibits the ability of the virus to undergo lytic reactivation by binding to the *orilyts* (185,186).

p63 expression patterns in normal tissue and malignancy

ΔNp63α is the primary isoform expressed in epithelial cells, while TAp63α is expressed in some types of B cell lymphomas such as Burkitt lymphoma and diffuse large B cell lymphoma (DLBCL), as well as in oocytes (168,187). Intriguingly, TAp63α is generally not expressed in non-malignant B cells. Depletion of TAp63α in DLBCLs results in a growth decrease, indicating that TAp63α expression offers a selective advantage in these lymphomas (188).

Genetic studies of ΔNp63α in animal models have provided a foundational understanding of its developmental role. Mice lacking ΔNp63α expression in epithelial cells die shortly after birth (170,189). The epidermis of these mice is only one cell layer thick, and ultimately the cause of death for these mice is dehydration due to a lack of water retention, indicating that ΔNp63α is required for the proliferative potential of the epidermis. Other mouse models with mutated ΔNp63α recapitulate the effects of the developmental disorders found in humans. Mutations in the ΔNp63α DNA-binding domain are commonly associated with developmental disorders such as ectrodactyly–ectodermal dysplasia–cleft syndrome (EEC) (190).

Unlike the related tumor suppressor p53, ΔNp63α is rarely mutated in cancers, although it has been reported to be mutated in some bladder cancers (191). Instead, ΔNp63α likely acts

as an oncogene in the context of some epithelial cancers such as squamous cell carcinoma and head and neck cancers, with ΔNp63α expression allowing the tumor cells to continue a proliferation program and to suppress the cellular death pathways (192–196). These clinical observations are experimentally supported by studies where ΔNp63α over-expression induced cells to grow in soft-agar and to form tumors in mice (197).

Previous studies of ΔNp63α and EBV

ΔNp63α has also been studied in the context of EBV epithelial cancers and EBV latency. ΔNp63α was found by the Allday group to be over-expressed in NPCs, leading them to propose that it may have an inhibitory effect on p53 induction of apoptosis (198). Additionally, ΔNp63α expression and stability is increased by the EBV latency protein LMP2A which is expressed in some NPCs (55). Research conducted by the Middledorp and Kenney groups showed that ΔNp63α binds to the promoter of the EBV early gene BARF1, inducing promoter activity (199). These experiments, however, failed to examine induction of BARF1 mRNA or protein expression in the context of the intact viral genome. Thus, the function of this DNA binding remains unclear. Additionally, ΔNp63α inhibits the expression of KLF4, an inducer of EBV lytic reactivation, by binding to the KLF4 promoter (176).

An important question that I wanted to address in this thesis was what keeps EBV in latency, whether it be in epithelial cells (where the virus has a proclivity to undergo replication, but only in differentiated cells) or in B cells (where EBV maintains a latent reservoir). **In Chapter 2 of this thesis, I will demonstrate that ΔNp63α and TAp63α inhibit EBV lytic replication.**

p38 MAPK is part of a broadly acting kinase cascade

In Chapter 2 of this thesis, I will demonstrate that p38 MAPK is required for R-mediated lytic reactivation, and that ΔNp63α reduces the activity of p38 MAPK to inhibit EBV lytic reactivation. p38 mitogen-activated protein kinase (MAPK) is part of a kinase pathway that responds to

cellular stressors from a diverse range of events such as heat shock, oxidative stress, DNA damage, and inflammation (200). There are four p38 MAPK genes that are highly homologous, designated as α, β, γ, and δ (also known as MAPK14, MAPK11, MAPK12, MAPK13) (201). The p38 MAP kinase pathway is composed of three classes of kinases that phosphorylate the subsequent downstream kinase (this pathway is depicted in **Figure 4**): At the top of the cascade is the MAP kinase kinase kinase (MAP3Ks), MAP kinase kinase (MAP2K), and finally MAP kinase (MAPK). When p38 MAPK is phosphorylated by MAP2Ks (usually MKK3 or MKK6) as part of the stress response, p38 MAPK, in turn, phosphorylates a number of substrates in both the nucleus and cytoplasm that have a diverse array of cellular effects such as MAPKAPK2, c-jun, p53, HIF-1, and ATF2 (200,202). Many of these genes are notable facilitators of EBV lytic reactivation, the Kenney group and others have found p38 MAPK signaling to be essential for the ability of EBV to lytically reactivate with a variety of stimuli such as methotrexate, sodium butyrate, and TGF-β (123,203–207).

In addition to phosphorylation of kinases and transcription factors, p38 MAPK signaling can also regulate gene expression post-transcriptionally through RNA stability. When p38 MAPK phosphorylates MAPKAPK2, this kinase will then phosphorylate tristetraprolin (TTP)/ZFP36. Phosphorylation inactivates TTP, which will otherwise bind to adenosine/uridine-rich elements (AREs) that are common in 3'-UTRs of mRNAs of inflammatory genes such as IL-6 (208). Once bound, TTP will target these transcripts for degradation by recruiting proteins such as CCR4-NOT (208). Given that p38 MAPK phosphorylation targets are often cytotoxic, p38 MAPK phosphorylation is tightly regulated. It is dephosphorylated by phosphatases such PP2A and DUSP6 (202,209), which enables the nuclear export of p38 MAPK via MAPKAPK2 (210). Interestingly, ΔNp63α is reported to dampen p38 MAPK phosphorylation by increasing the expression of DUSP6 (211). **In Chapter 2 of this thesis, I will show that the over-**

expression of ΔNp63α decreases p38 MAPK phosphorylation and that p38 MAPK expression and p38 MAPK function are required for efficient lytic reactivation.

The discovery of Hippo signaling and YAP/TAZ

In Chapter 3 of this thesis, I will show that the Hippo signaling effectors YAP and TAZ induce EBV lytic reactivation via the Z promoter. Hippo signaling was discovered when researchers conducting genetic screens in *Drosophila* mutated the Yorkie regulator *warts (wts),* and the resulting dramatic growth phenotype gave this signaling pathway its eponymous name (212–214). In subsequent studies, the effector of this regulatory pathway, Yorkie (YAP in mammals), was discovered (215). Studies of Hippo signaling further characterized it as a pathway that regulates not only the size of organs but also maintenance of the stem cell niche, wound healing, cell proliferation, and differentiation (216–220). Indicative of the essentiality of this signaling network, the Hippo pathway components have been so highly conserved that wild-type mammalian Hippo proteins can rescue Hippo mutants in *Drosophila* (221).

The regulation of YAP and TAZ function

Hippo signaling refers to the upstream kinase cascade responsible for the negative regulation of the transcriptional co-activator YAP and its paralog TAZ. When Hippo signaling is inactive, these two transcription factors are responsible for the induction of thousands of cellular genes. YAP and TAZ are expressed in a wide range of tissue types (with the striking exception being B cells which only express TAZ to a low degree) and are structurally similar (222). YAP protein structure is comprised of a proline rich region and TEAD-binding region at its N-terminus, two WW domains (TAZ only has the first WW domain), a coiled-coil domain, an SH3 binding site, a transcription activation domain, and at the C terminus of YAP is a PDZ bind motif. TAZ lacks the proline-rich domain and SH3 site, but otherwise remains organized similarly. To

describe the simplified kinase pathway briefly, MST1/2 phosphorylates LATS1/2, which in turn phosphorylates YAP and TAZ. An illustration of this process is shown in **Figure 6** (220). This kinase pathway is a significant determinant (although not the sole one) of YAP and TAZ functionality as phosphorylation can dictate the stability and localization of these proteins. When these two co-activators are phosphorylated by LATS1/2, this post-translational modification prevents YAP/TAZ from going into the nucleus and engaging in transcriptional activity (223). This phosphorylation also enables YAP and TAZ to interact with 14-3-3 proteins for cytoplasmic retention which precedes their degradation by the proteasome (224–226). YAP is phosphorylated by LATS on at least five sites (S61, S109, S127, S164, S381), while TAZ is phosphorylated at four distinct sites (S66, S89, S117, S311) (227,228). The mutation of these sites from serine-to-alanine enables YAP and TAZ to be constitutively active, and these mutants are referred to as YAP5SA and TAZ4SA (229,230). Additionally, it has recently been determined that the phosphorylation of the threonine sites of YAP and TAZ can induce their transcriptional activity. Furthermore, many receptor tyrosine kinases such as FGFR are capable of inducing these post-translational modifications (231).

YAP and TAZ in development and cancer

While YAP and TAZ have many overlapping functions, they also have distinct cellular roles in contexts such as development. This is most evident in animal models where YAP null mice are nonviable and die early in embryonic development, while TAZ null mice survive into adulthood but go on to develop kidney disease (232,233). Doxycycline-inducible YAP overexpression in mouse models results in liver overgrowth that is reversible if doxycycline is removed and YAP expression is depleted (234,235). Constitutively active YAP expressed in the epidermis of mouse models results in fetal death, epidermal overgrowth of undifferentiated cells, and blocked esophageal passages (218).

YAP and TAZ are overexpressed in a number of epithelial malignancies where expression will stimulate proliferation and dampen apoptotic signaling (236). In Kaposi's sarcoma (which harbor an infection of the herpesvirus KSHV), both YAP and TAZ are localized to the nucleus where they are likely transcriptionally active (221). The viral G-coupled protein receptor (GPCR) of KSHV can induce the expression of YAP, and the HPV oncoprotein E6 stabilizes YAP expression, indicating that other tumor viruses incorporate YAP activation as part of their strategy to ensure infected cell survival (221,237). YAP expression is also known to be increased in gastric carcinomas during infection of *Helicobactor pylori,* and antagonism of YAP function has been proposed as a potential therapeutic mechanism (238,239).

Cellular processes that regulate YAP and TAZ activity

YAP and TAZ are regulated by a variety of cellular stressors and sensors such as the G-protein-coupled receptors (GPCRs). GPCRs represent a broad class of receptors that can be stimulated by ligands specific to particular receptors such as lysophosphatidic acid (LPA) or sphingosine 1-phosphate (240,241). The GPCR ligand and receptor involved can either positively or negatively regulate the functionality of YAP and TAZ. LPA-specific GPCRs dephosphorylate YAP/TAZ by inhibiting LATS1/2 phosphorylation. Conversely, GPCRs induced through ligands such as epinephrine induce phosphorylation of YAP/TAZ by phosphorylating LATS1/2. This mechanism is relevant to health as uveal melanomas containing mutated GPCRs have constitutive activity induce YAP activity. Importantly, treatment with the YAP-TEAD inhibitor verteporfin inhibits the growth of this tumor type (242).

YAP and TAZ functionality is also modulated by mechanotransduction, a process that can be independent of the Hippo kinase cascade (243–245). YAP and TAZ transcriptional activity can be dictated through actin remodeling of the cell in response to environmental factors such as substrate stiffness or cell density. In situations where cells are plated onto a soft substrate or are relatively sparse, YAP/TAZ are transcriptionally active. On the other hand, YAP

and TAZ are inactive on hard surfaces or when the cell population reaches confluency where they are exported to the cytoplasm. The upstream regulators of the mechanotransduction pathway are F-actin remodeling proteins such as Agrin and CapZ which can inhibit YAP and TAZ induction of target genes by phosphorylating Hippo components LATS1/2 (Agrin) or work through other mechanisms independent of Hippo signaling (CapZ) (244,245).

YAP and TAZ require a DNA binding co-activator to induce their transcriptional targets

YAP and TAZ lack a DNA-binding domain and require a cofactor in order to activate gene expression. When YAP and TAZ are active, they bind to DNA binding proteins such as the TEADs and activate gene expression (229,230,246). TEADs are present at approximately 80% of all YAP target binding sites (229). Many of these binding sites are key target genes such as CTGF and CYR61, and knockdown of the TEADs results in the abrogation of much YAP and TAZ transcriptional activity (229,230,247). While the TEADs are one of the better characterized binding partners of YAP and TAZ, there are others such as p73, RUNX1, ErbB4, KLF4, SMADs, and ZEBs (216,248–251).

Several of these YAP/TAZ-interacting transcription factors, notably KLF4, SMADs, and ZEBs, have known roles in the induction of the EBV lytic cycle, presenting a possibility that a YAP or TAZ interaction with these inducers may impact EBV lytic reactivation (90,100,121,132,133). However, the role of YAP or TAZ during lytic reactivation of EBV had not been characterized. **In chapter 3 of this thesis, I demonstrate that YAP and TAZ are positive regulators of EBV lytic reactivation in the context of epithelial cells.**

The TEAD family of DNA binding proteins

Tea domain genes (TEADs, also known as TEF-1) are a family of well-conserved DNA binding genes. *Homo sapiens* express four separate TEAD genes, TEAD1 through TEAD4, that are homologous but are often tissue-specific (252). The study of viruses has long been deeply

informative about important cellular functions, and the discovery of the TEADs was no different. The first studies of the TEADs were in the context of binding to an SV40 enhancer as well as HPV E6 and E7 promoter sequences (253,254). Since then, the role of TEADs in development and oncology has provided the impetus for further study.

TEAD protein structure and role in development

The TEADs do not contain an activation domain and, without a binding partner such as YAP or TAZ, the TEADs have no independent transcriptional activity (252,255,256). The TEAD protein structure consists of a protein-binding motif in the N-terminal domain and a DNA-binding domain in the C-terminal domain. Removal of the protein-interaction domain, where TEADs would interact with YAP or TAZ, results in a protein with a dominant-negative function as the dominant-negative TEAD outcompetes the wild-type TEAD for promoter binding sites but is unable to induce activation of these promoters (229).

The TEAD consensus binding site has been defined as 5'-GGAATG-3', and this consensus site is conserved amongst all the TEAD genes (257). Importantly, the DNA-binding domains of all of the TEADs are 99% homologous with one another. This similarity is reflected in the redundancy of some of the TEAD functions, most notably in knockout models of both TEAD1 and TEAD2 (252,258). This double knockout results in greater developmental deficiencies than single knockouts of TEAD1 or TEAD2 (258). However, each TEAD can have separate roles in developmental contexts as TEAD4 null mice are embryonic lethal during pregnancy as the embryo fails to implant (259). TEAD1 null mice are also lethal because of defects in cardiac development (260). The distinct roles that the TEAD family members have during development remain to be further characterized.

TEAD binding partners

TEADs have three known categories of binding partners that dictate their function: Vestigial-like (VGLL) proteins, YAP and TAZ, and the p160s (252,256). There are four VGLL genes, which derive their name from *Drosophila,* where this gene family is essential for wing development. Like YAP and TAZ, the VGLL proteins can only access DNA through interactions with a co-activator such as the TEADs. Each VGLL protein contains a 25-amino acid long motif that enables binding to TEADs. VGLL4 is of particular interest as VGLL4 binds to TEAD and antagonizes YAP gene activation (239,261). This interaction has recently been hypothesized as a potential therapy for YAP-dependent gastric carcinomas (239,261,262). The p160 family are also transcriptional co-activators of TEAD. However, the biological significance of this interaction has not been studied extensively (252,263).

TEAD expression in normal and malignant contexts

TEADs are expressed in differing tissue types, although some tissue types will express all four TEAD genes (229). Interestingly, only TEAD2 is expressed to any degree in most B cells (222). The TEADs have an important role in cancer as the increased expression of TEADs in cancers of epithelial origin (e.g., melanoma and breast cancer) is often considered to be a poor prognostic biomarker (256). In breast cancer, TEAD2 is upregulated during the epithelial to mesenchymal transition (EMT). The upregulation of TEAD2 then enables additional YAP or TAZ nuclear binding to occur (264). This is likely a mechanism common amongst many TEAD overexpressing tumors. The KSHV-associated malignancy Kaposi's Sarcoma overexpresses TEAD1 but, so far, no study has been done to determine if TEADs are upregulated in any EBV+ tumor types (265). Finally, it was not known if TEADs bind to the EBV genome, nor was it known if they mediate any YAP/TAZ effects on the EBV lytic reactivation cascade. **In Chapter 3 of this thesis, I demonstrate that the TEAD genes mediate YAP and TAZ induction of EBV**

lytic reactivation by binding to the EBV genome. Additionally, I also show that limited expression of the TEAD genes in B cells may promote latent infection in this cell type.

Figures

Latency type	Latent gene expression pattern	Malignancies associated with this latency
Type III	**EBNA1, BARTs, EBERS LMP1, LMP2A, LMP2B, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, BHRF1 miRNAs**	**Post-transplant lymphoma, HIV-associated lymphoma**
Type II	**EBNA1, BARTs, EBERS, LMP1, LMP2A, LMP2B**	**Hodgkin lymphoma, Nasopharyngeal carcinoma, T/NK-cell lymphoma**
Type I	**EBNA1, BARTs, EBERS**	**Burkitt lymphoma, Gastric carcinoma**

Table 1: Epstein-Barr virus latency expression pattern. Epstein-Barr virus has three distinct modes of latency. Type I is the most restrictive latency type, where only EBNA1, EBERs, and BART microRNAs are expressed. Type II latency expresses LMP1 and LMP2A/LMP2B proteins in addition to the aforementioned genes. Type III latency allows for the expression of all latency genes, including the full suite of EBNA genes: EBNA1, EBNA2, EBNA-LP, EBNA3A, EBNA3B, EBNA3C, as well as BHRF1 microRNAs. The malignancies associated with each latency type are indicated in the table.

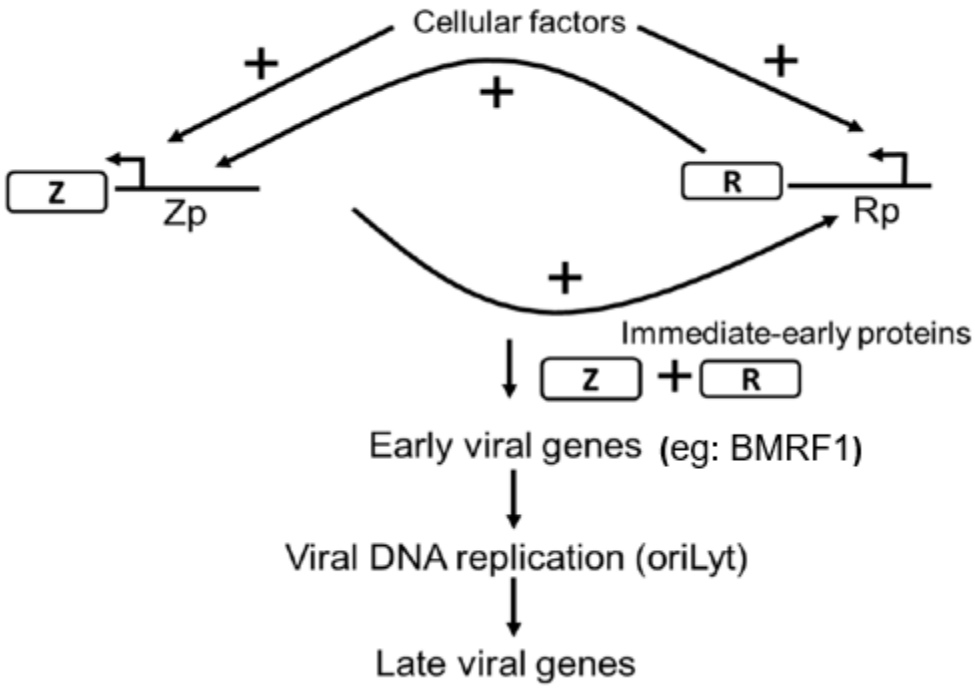

Figure 1: The reactivation cascade of EBV from latency. EBV lytic reactivation is initiated when cellular factors activate the Z and R promoters to induce their expression. Once Z and R are expressed, they will generate a positive feedback loop where they will activate the other IE gene's promoter in addition to their own. Moreover, Z and R will activate the expression of EBV early genes such as BMRF1, which will enable viral DNA replication to occur. After viral DNA replication, late genes expression (e.g., gp350) will occur.

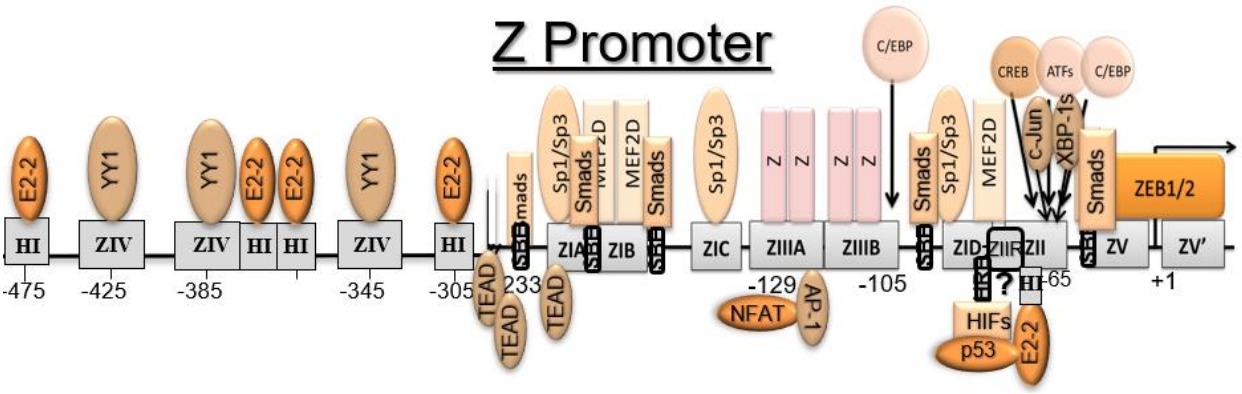

Figure 2: The Z promoter map. The grey squares represent the *cis*-acting elements, with the proteins known to interact with these elements indicated above or below them. (Adapted from Kenney and Mertz, Seminars in Cancer Bio, 2014(90)).

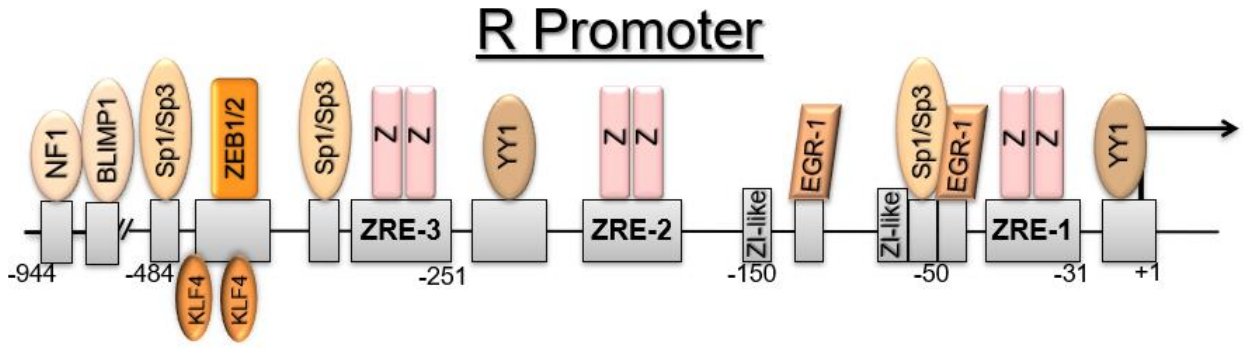

Figure 3: The R promoter map. The grey squares represent the *cis*-acting elements, with the transcription factors that interact with these elements indicated above or below them. (Adapted from Kenney and Mertz, Seminars in Cancer Bio, 2014 (90)).

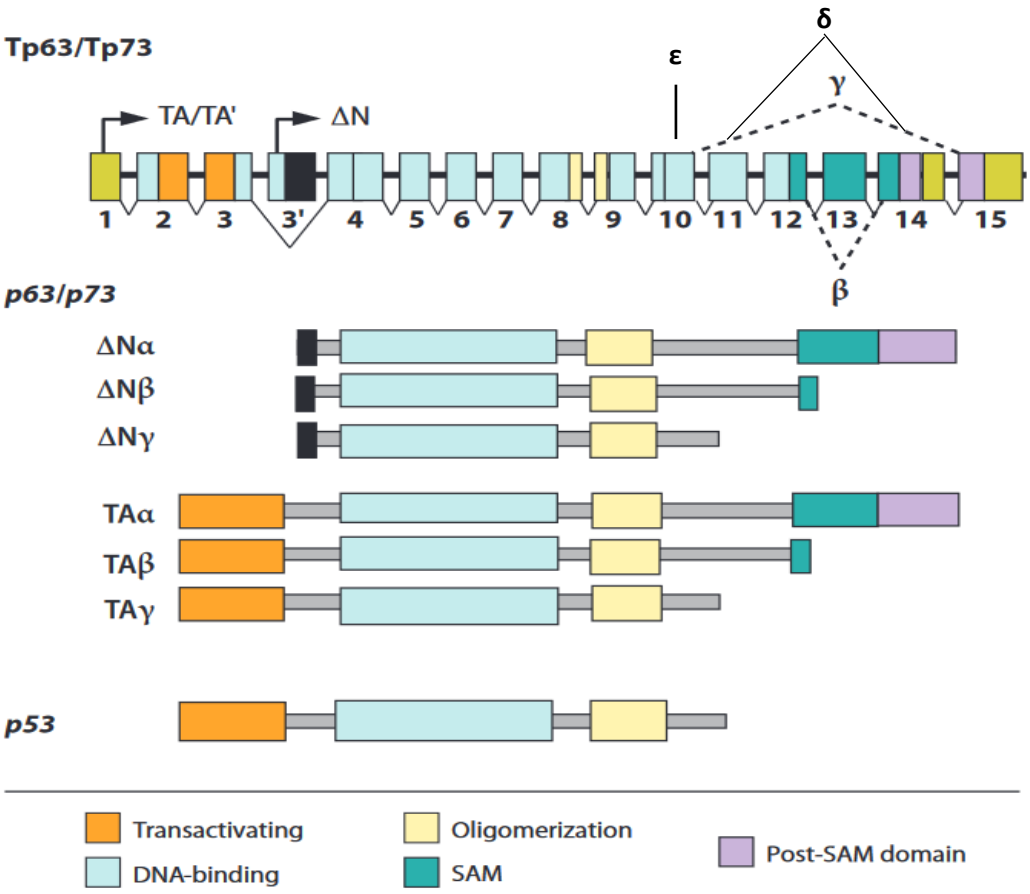

Figure 4: p63/p73 and p53 isoforms protein structure. p63 is a part of the p53 protein family. It has two types of isoforms, TA and ΔN. TAp63 is the full-length protein, while ΔNp63 is synthesized from an alternative transcription start site. There are three original types of C-terminal isoforms: α, β, and γ. The length of these C-terminal isoforms dictates if the isoform contains a SAM and a post-SAM domain. Additionally, two additional isoforms ε and δ were recently described (not shown). Isoform ε is formed from a stop codon in exon 10, and δ is alternatively spliced and lacking exon 13. All isoforms contain an oligomerization domain, and a DNA-binding domain. ΔNp63α is predominantly expressed in epithelial cells, while TAp63α is expressed in some lymphomas and oocytes. TAp63 contains the “transactivating domain” which was thought to enable the transcriptional effects of this protein. (Adapted from McKeon and Crum, Annu. Rev. Pathol. Mech, 2010)

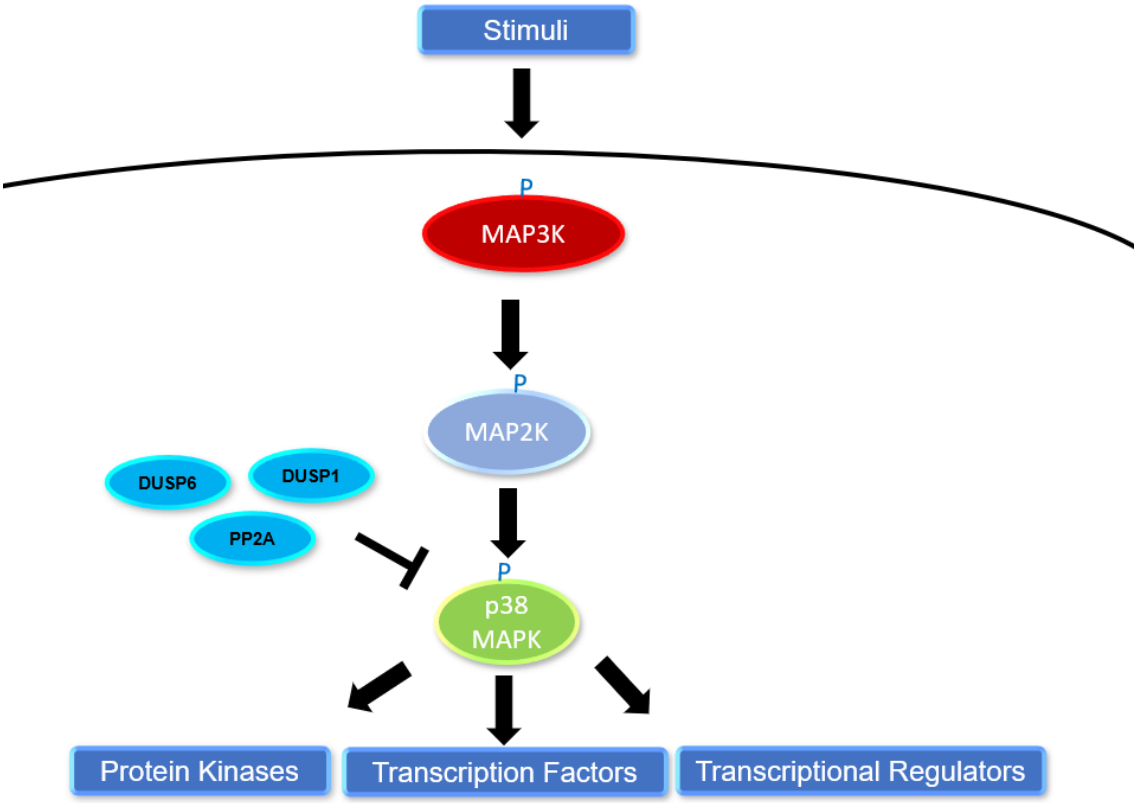

Figure 5: The canonical p38 MAPK activation pathway. The p38 MAPK kinase cascade begins when MAP3Ks are stimulated by stress events such as heat shock and DNA damage. The MAP3Ks subsequently phosphorylate the MAP2Ks, which go on the phosphorylate the MAPKs. Once MAPKs are phosphorylated, they can phosphorylate other protein kinases such as some PKC isoforms, transcription factors such as ATF2, or the SWI/SWF component BAF60.

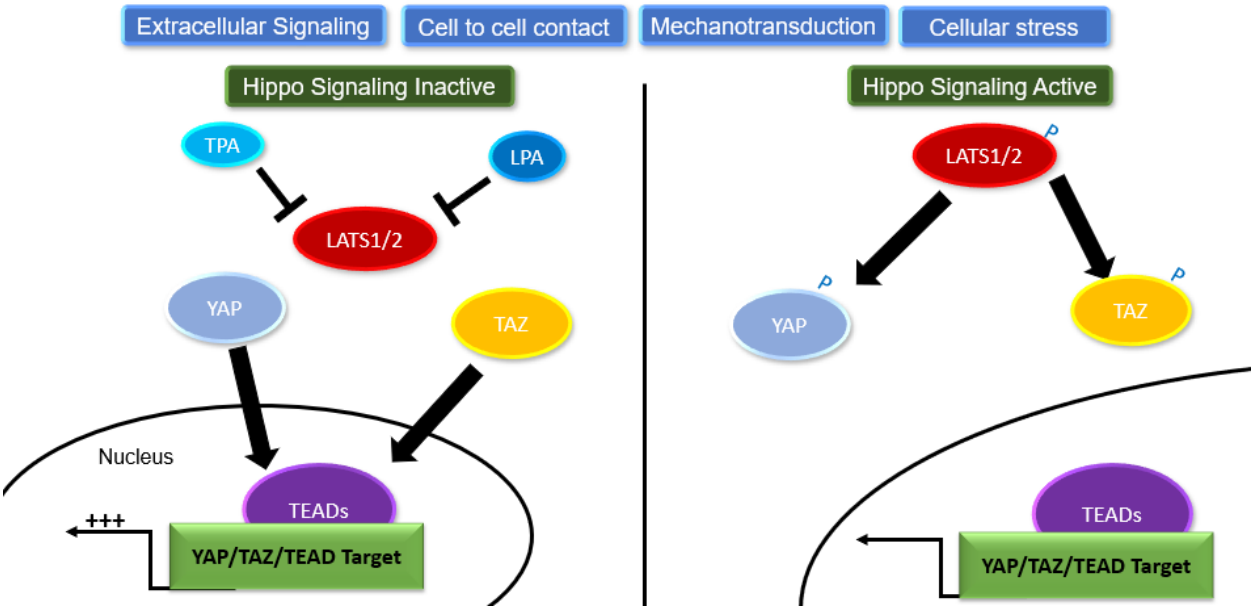

Figure 6: A simplified schematic of Hippo signaling and upstream regulators of this kinase cascade. Hippo signaling refers to the upstream kinases that dictate the activities of transcriptional effectors YAP and TAZ. The function of these kinases is modulated in turn by cellular signaling such as cell-to-cell contact, cell polarity, extracellular signaling, mechanotransduction, and cellular stress. If Hippo signaling is activated, MST1/2 phosphorylates LATS1/2 which, in turn, phosphorylate YAP/TAZ. Phosphorylated YAP/TAZ interacts with 14-3-3 sequestering them in the cytoplasm where they are degraded. Conversely, if Hippo signaling is inhibited, none of this phosphorylation will occurs, leaving YAP/TAZ to be free to shuttle to the nucleus. Once in the nucleus YAP and TAZ bind to the transcriptional co-activator TEADs (and other co-activators not shown here) to induce the expression of YAP/TAZ target genes. (Adapted from Hansen, Moroishi, and Guan, Trends in Cell Biology, 2015 (220))

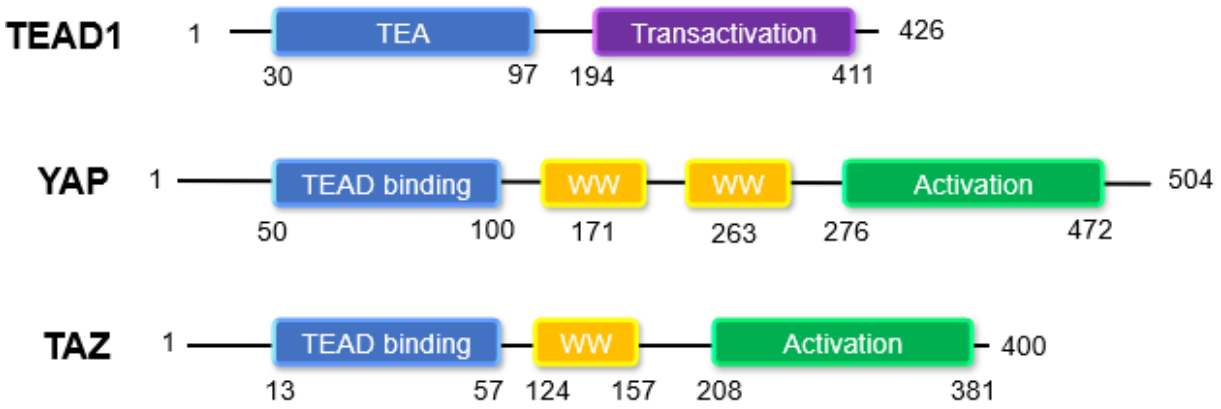

Figure 7: TEAD1, YAP, and TAZ protein structures. TEAD comprises two major domains: the TEA domain from amino acid residues 30-97; and the transactivation domain from amino acids 194–411. YAP has three major regions: the TEAD-binding domain from amino acids 50-100, the WW motifs around residues 171 and 263; and the activation domain at position 276 to 472. TAZ is structurally similar to YAP, with a TEAD-binding domain from amino acid residues 13 to 57, with a WW motif at residues 124 -157, and an activation domain at residues 208 to 381. (Adapted from Pobbati and Hong, Cancer Biology and Therapy, 2013 (252))

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Chapter 2

ΔNp63α promotes Epstein-Barr virus latency in undifferentiated epithelial cells

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Abstract

Epstein-Barr virus (EBV) is a human herpesvirus that causes infectious mononucleosis and contributes to both B-cell and epithelial-cell malignancies. EBV-infected epithelial cell tumors, including nasopharyngeal carcinoma (NPC), are largely composed of latently infected cells, but the mechanism(s) maintaining viral latency are poorly understood. Expression of the EBV BZLF1 (Z) and BRLF1 (R) encoded immediate-early (IE) proteins induces lytic infection, and these IE proteins activate each other's promoters. ΔNp63α (a p53 family member) is required for proliferation and survival of basal epithelial cells and is over-expressed in NPC tumors. Here we show that ΔNp63α promotes EBV latency by inhibiting activation of the BZLF1 IE promoter (Zp). Furthermore, we find that another p63 gene splice variant, TAp63α, which is expressed in some Burkitt and diffuse large B cell lymphomas, also represses EBV lytic reactivation. We demonstrate that ΔNp63α inhibits the Zp promoter indirectly by preventing the ability of other transcription factors, including the viral IE R protein and the cellular KLF4 protein, to activate Zp. Mechanistically, we show that ΔNp63α promotes viral latency in undifferentiated epithelial cells both by enhancing expression of a known Zp repressor protein, c-myc, and by decreasing cellular p38 kinase activity. Furthermore, we find that the ability of cis-platinum chemotherapy to degrade p63 contributes to the lytic-inducing effect of this agent in EBV-infected epithelial cells. Together these findings demonstrate that the loss of ΔNp63α expression, in conjunction with enhanced expression of differentiation-dependent transcription factors such as BLIMP1 and KLF4, induces lytic EBV reactivation during normal epithelial cell differentiation. Conversely, expression of ΔNp63α in undifferentiated nasopharyngeal carcinoma cells and TAp63α in Burkitt lymphoma promotes EBV latency in these malignancies.

Importance

Epstein-Barr virus (EBV) is an important cause of both epithelial cell and B cell human cancers. EBV-infected tumors have predominantly latent viral infection, allowing them to escape the cell killing that occurs during lytic viral infection. EBV is highly lytic in normal differentiated oral epithelial cells. Thus, an important question is how the virus maintains the latent form of viral infection in EBV-associated epithelial cell tumors such as undifferentiated nasopharyngeal carcinoma (NPC). This study demonstrates that the cellular transcription factor ΔNp63α, which is specifically expressed in undifferentiated basal epithelial cells and is over-expressed in NPC tumors, maintains EBV latency by inhibiting the activity of the viral immediate-early (IE) promoter (Zp) that drives expression of the BZLF1 IE protein. A related splice variant, TAp63α, found in some EBV+ lymphomas, has a similar inhibitory effect. Our findings reveal that ΔNp63α and TAp63α contribute to EBV latency in both epithelial and B cell tumors. Furthermore, since differentiation results in loss of ΔNp63α expression, our results help to explain why lytic EBV reactivation is promoted by epithelial cell differentiation.

Introduction

Epstein-Barr virus (EBV) is a gamma-herpesvirus that infects over 90% of the human population by adulthood and causes the clinical syndrome, infectious mononucleosis. EBV primarily infects B cells and epithelial cells and is associated with both B-cell and epithelial-cell malignancies, including Burkitt lymphoma, diffuse large B cell lymphoma, Hodgkin lymphoma, gastric carcinoma, and nasopharyngeal carcinoma (NPC) (1). Like all herpesviruses, EBV can infect host cells in either latent or lytic forms and persists in the host for life. In latently infected cells, the virus expresses only a small subset of viral genes and replicates once per cell cycle using the host cell DNA polymerase. In lytically infected cells, the full viral gene complement is expressed, the virus is replicated using the virally-encoded DNA polymerase and infectious virions are produced. The major site of persistent latent EBV infection in humans is memory B cells, although the lytic form of infection can be induced by B-cell receptor stimulation, plasma cell differentiation or various other stimuli in B cells (2–6).

In contrast, normal oropharyngeal epithelial cells support the lytic form of EBV infection, and it remains uncertain whether latent EBV infection of epithelial cells normally occurs in humans (7–9). Lytically-infected epithelial cells are thought to be the major source of infectious EBV particles in saliva (10) and thus are essential for the spread of the virus from host to host. EBV infection of non-transformed tongue epithelial cells causes the clinical syndrome, oral hairy leukoplakia (OHL), in immunocompromised patients (7,11). Analysis of OHL lesions has revealed that EBV infection is confined to the more differentiated epithelial cell layers, and that the infection is completely lytic without evidence of concomitant latent infection (7,11). Nevertheless, the EBV-associated epithelial tumor, undifferentiated nasopharyngeal carcinoma, contains largely latent EBV infection, and the ability of the virus to maintain latency in the tumor cells is likely required for NPC development. Thus, understanding how EBV stays latent in a cell type where it is usually lytic is important for understanding NPC.

Lytic EBV infection is initiated by expression of the two EBV immediate-early (IE) proteins, BZLF1 (Z) and BRLF1 (R) (12–14). The Z and R IE genes are driven by the Zp and Rp promoters, respectively, and activation (or repression) of these promoters by cellular transcription factors serves as the major control point in determining if EBV infection is latent versus lytic in cells (15–22). Once expressed, the Z and R proteins function as viral transcription factors that activate each other's promoters, as well as their own promoters, in a positive feedback loop (12,23–25). Z and R then synergistically activate early lytic viral gene promoters to induce early gene expression and lytic viral DNA replication.

Z is a part of the bZip protein family and binds to AP-1-like sites called Z-response elements (ZREs), while R has no cellular homolog and binds to R-responsive elements (RREs) (12,26,27). R can also activate some promoters, including Zp, indirectly through non-DNA binding mechanisms that are still not totally understood (25,28). Many ZREs are preferentially bound by Z in the methylated form, helping Z to induce lytic viral reactivation even when the viral genome becomes highly methylated (as occurs normally in EBV-infected B cells and in EBV-positive B cell and epithelial cell tumors)(29). In contrast, R preferentially activates unmethylated lytic EBV promoters (29), and in EBV-infected hTERT-immortalized normal oral keratinocyte cells (NOKs), where the viral genome remains hypomethylated, over-expression of R, but not Z, induces lytic reactivation (12,13,29–32). Regardless of the viral genome methylation state, Z and R synergistically activate many early lytic gene promoters and expression of both proteins is required for the virus to lytically replicate and complete the lytic cycle (29,33).

We and others have previously demonstrated that epithelial cell differentiation induces EBV lytic reactivation in stably infected oral keratinocyte cells (15,16,34–39). Similarly, EBV infection of primary oral epithelial cells grown in stratified "raft" cultures resulted in no detectable latent or lytic infection in undifferentiated layers and a robust lytic infection in the differentiated cell layers (8). We showed the cellular transcription factors KLF4 and BLIMP1, which are

preferentially expressed in differentiated epithelial cells, synergistically activate the Z, R, and LMP1 EBV promoters, at least partly explaining why EBV reactivation is induced by epithelial cell differentiation (15,16,36,40,41). However, given the robust latency of EBV infection in undifferentiated epithelial cell models (16,34–36), we hypothesized that additional factors expressed in undifferentiated (but not differentiated) epithelial cells may also contribute to the differentiation-dependent phenotype by inhibiting lytic viral reactivation in undifferentiated cells.

Here we show that expression of the master-regulator of epithelial cell identity, p63, in undifferentiated epithelial cells is a potent negative regulator of lytic EBV reactivation. The cellular p63 protein is a member of the p53 family that transcriptionally regulates many different epithelial cell-specific genes (42). The major epithelial cell p63 isoform, ΔNp63α, is a critical regulator of proliferation and differentiation in keratinocytes and a marker for the epithelial stem cell compartment (42–47). ΔNp63α mutations result in developmental defects in limb development, while deletion of ΔNp63α in mice is lethal after birth due to a lack of a stratified epidermis (42,46,48). Like p53, p63 contains a DNA binding domain and, through this domain, can bind to thousands of sites on the human genome to regulate transcriptional function (49,50). This DNA binding domain retains homology to p53, and p63 can bind to some of the same promoter sites as p53 (51). However, unlike p53, ΔNp63α is rarely mutated in cancer and instead has been reported to be over-expressed in several epithelial tumor types, including NPC (52,53). We find that ΔNp63α inhibits lytic EBV reactivation in epithelial cells by decreasing activity of the Zp IE promoter and show that this effect is mediated both by reduced cellular p38 kinase activity and increased c-myc expression. In addition, we show that another isoform of p63, TAp63α, which is expressed in some EBV+ B cell lymphomas, likewise inhibits EBV reactivation. These findings not only further elucidate how EBV uses differentiation-dependent transcription factors (ΔNp63α, KLF4 and BLIMP1) to ensure differentiation-dependent lytic reactivation in epithelial cells, but also reveal an important mechanism for promoting viral latency in EBV-infected tumor cells.

Results

∆Np63α and immediate-early protein Z are expressed in distinct sections of organotypic raft cultures of NOKs-Akata cells. NOKs-Akata is an EBV-infected telomerase-immortalized normal oral keratinocyte cell line that retains the ability to differentiate. We previously showed that lytic reactivation of EBV occurs only in the differentiated layers of rafted NOKs-Akata cells (16,34,36). In contrast, ΔNp63α is primarily expressed in the basal epithelial layer of normal stratified epithelium. ΔNp63α expression is lost during epithelial cell differentiation due to IRF6-mediated protein degradation as well as the antagonistic effect of the differentiation-dependent miR-203 (54–56). To determine if expression of the IE lytic EBV protein Z overlaps that of ΔNp63α in rafted NOKs-Akata cells, we performed immunohistochemistry (IHC) using anti-Z or anti-p63α antibodies. p63α was expressed primarily in the basal layer and immediate suprabasal layers of the NOKs-Akata cell raft cultures, and p63α expression ceased in the more differentiated layers, congruent with previous literature (54–56) (**Fig 1A**). In contrast, Z expression was observed exclusively in the raft's more differentiated layers (**Fig 1B**) with no overlap in expression of the p63α and Z proteins in the raft cultures. From these observations, we hypothesized that EBV-infected NOKs cells expressing p63α may be impaired for the ability to enter EBV lytic reactivation.

Knockdown of ∆Np63α expression results in EBV lytic reactivation in EBV+ carcinoma and immortalized keratinocyte cell lines. To determine if ΔNp63α expression inhibits EBV lytic reactivation in the context of undifferentiated epithelial cells, lentivirus vectors expressing shRNAs targeting p63 (or control vectors) were used to infect the CNE-2-Akata cell line (a carcinoma cell line incapable of differentiation) and stably infected cells were selected with puromycin. As shown in **Fig 2A**, ΔNp63α expression was successfully decreased with the p63 targeting shRNAs. More importantly, loss of ΔNp63α expression resulted in increased

expression of EBV IE proteins Z and R as well as the early lytic protein BMRF1 in comparison to control lentivirus infections (**Fig 2A**).

To confirm this finding, siRNAs targeting ΔNp63α were delivered into a second EBV-infected epithelial cell line, NOKs-Akata cells, which retain the ability to differentiate (16). As shown in **Fig 2B**, knockdown of ΔNp63α expression also increased expression of EBV IE proteins, Z and R, in NOKs-Akata cells. These results indicate ΔNp63α expression represses the EBV lytic cascade and that this repression can occur even in carcinoma cell lines that are incapable of differentiation.

ΔNp63α inhibits lytic reactivation during epithelial cell differentiation. EBV lytically reactivates during epithelial cell differentiation, and this effect is at least partially mediated through enhanced expression of the transcription factors KLF4 and BLIMP1 (15,16,36). ΔNp63α is known to impede keratinocyte differentiation through transcriptional repression of cellular genes required for differentiation, including KLF4 (57). To determine if ΔNp63α over-expression reduces EBV lytic reactivation during epithelial cell differentiation, we stably infected NOKs-Akata cells with either a lentivirus over-expressing ΔNp63α or with a control lentivirus vector, and then seeded the cells onto collagen-treated membranes in air-liquid interface culture conditions. After three days of differentiating the cells, immunoblot analysis was performed to assess EBV lytic reactivation and cellular differentiation. While EBV lytically reactivated in the NOKs-Akata cells infected with the control lentivirus, cells infected with the ΔNp63α expressing lentivirus had reduced expression of the lytic EBV proteins, R, Z, and BMRF1 (**Fig 3**). Interestingly, the levels of involucrin, KLF4, and BLIMP1 did not change between the control and ΔNp63α- expressing lentiviruses, indicating that ΔNp63α repression of EBV lytic reactivation may be directly impeding viral processes as opposed to reducing cellular differentiation in this experiment.

ΔNp63α over-expression inhibits R- but not Z- mediated induction of lytic reactivation. To begin to examine the mechanism(s) for the ΔNp63α effect on lytic EBV reactivation, we next compared the effect of co-transfected ΔNp63α protein on the ability of the Z versus R EBV IE proteins to induce lytic EBV reactivation when transfected into EBV-infected epithelial cell lines that can be reactivated by either Z or R transfection. In the gastric carcinoma cell line AGS-Akata, which lacks endogenous ΔNp63α expression, co-transfected ΔNp63α inhibited the ability of transfected R protein to induce expression of endogenously expressed lytic viral proteins, Z and BMRF1 (**Fig 4A)**. In contrast, co-transfected ΔNp63α protein did not inhibit the ability of transfected Z protein to induce expression of endogenous lytic proteins R and BMRF1 (**Fig 4A**) even though more ΔNp63α was present in this condition. A repeat experiment confirmed that ΔNp63α does not inhibit the ability of transfected Z to induce lytic protein expression in AGS-Akata cells (**Sup Fig 3**). This result suggests that ΔNp63α may inhibit lytic EBV reactivation through effects on the R IE protein function and/or the BZLF1 Zp promoter, since R must first induce Z expression in order to turn on early lytic genes such as BMRF1 (33).

To confirm that ΔNp63α inhibits R-mediated lytic reactivation, we examined its effect in NOKs-Akata cells, in which R transfection, but not Z transfection, can initiate the lytic cascade (29). Similar to the results in AGS-Akata cells, ΔNp63α co-transfection inhibited the ability of transfected R protein to induce expression of the Z, and BMRF1 proteins, as well as the late viral capsid protein, p18 (**Fig 4B, 4C**). Co-transfected ΔNp63α likewise decreased the ability of transfected R to induce expression of the Z and BMRF1 lytic proteins in CNE-2-Akata cells (**Fig 4D**).

While R activates many lytic EBV promoter targets through a direct DNA binding mechanism (58), it is not known to bind directly to the IE Zp promoter and instead is thought to activate this promoter through indirect effects on cellular transcription factors (24,25,59,60). In addition, while some early lytic EBV promoters (such as the BMRF1 promoter) contain both Z and R binding sites and require both Z and R for efficient expression (29,61,62), maximal

expression of the SM early lytic promoter has been shown to only require R binding to the promoter, and is not further enhanced by Z expression (63,64). Interestingly, we found that while ΔNp63 inhibits the ability of co-transfected R to activate expression of the Z and BMRF1 proteins in CNE-2-Akata cells, it does not affect R activation of the SM protein (**Fig 4D**) (58,63). These results suggest that ΔNp63α primarily inhibits lytic EBV reactivation by preventing R's ability to induce the Z IE promoter, since the concomitant decreased expression of the BMRF1 and p18 proteins would both be the expected outcome from loss of Z expression.

ΔNp63α repression of EBV lytic reactivation is independent of p53. ΔNp63α is a member of the p53 protein family, and approximately 60% of ΔNp63α's DNA binding domain is homologous to the DNA binding domain of p53 (54). Furthermore, p53 and ΔNp63α bind to some of the same promoters, and ΔNp63α can repress p53 activation at these promoters (51,54,65). Since p53 was previously reported to be a positive regulator of EBV lytic reactivation and binds to the Z promoter via the Sp1 transcription factor and HIF-1α (66–69), we determined if ΔNp63α's repression of EBV lytic reactivation is due to competition with p53. For these experiments, ΔNp63α expression was inhibited by siRNAs in a NOKs-Akata cell line that had CRISPR-Cas9 mediated knockout of p53 (**Fig 5A**). As shown in **Fig 5B**, immunoblot analysis revealed that loss of ΔNp63α expression upregulates Z and R expression even in the absence of p53 expression. These results suggest that ΔNp63α represses EBV lytic reactivation independently of competitive inhibition of p53 in EBV infected cells.

ΔNp63α does not directly bind to the EBV immediate-early promoters. ΔNp63α can function as either a transcriptional repressor or transcriptional activator, and is known to bind to promoters of cellular genes such as KLF4, p21, and p16 to prevent their expression and maintain proliferation of the keratinocyte stem cell compartment (57,65,70). To determine if ΔNp63α inhibits lytic EBV replication by binding directly to the Zp and/or Rp IE viral promoters,

we conducted chromatin-immunoprecipitation quantitative PCR (ChIP-qPCR) assays. AGS-Akata cells were transfected with a FLAG-tagged ΔNp63α vector or vector control and ChIP-qPCR assays were performed two days later using an anti-FLAG antibody as described in the methods. As shown in **Fig 6A,** we found that ΔNp63α binds to the cellular promoter for NECTIN1, a known ΔNp63α binding target (71), but we did not detect ΔNp63α binding to either the Z promoter or the R promoter on the EBV genome (**Fig 6A**). This result suggests that ΔNp63α does not bind to the immediate-early gene promoters and inhibits EBV reactivation through a different mechanism.

∆Np63α does not prevent R binding to the EBV genome. R binds to RREs on the EBV genome during lytic reactivation to induce lytic gene expression (58). To determine if ∆Np63α prevents R binding to RREs on the EBV genome during lytic reactivation, CNE-2-Akata cells were transfected with a FLAG-tagged R vector with or without ∆Np63α. We then performed ChIP-qPCR on known RRE sites using primers against the SM (BMLF1) promoter, BMRF1 promoter, BARF1 promoter, and the Z and R promoters (which do not contain RRE sites) to determine if R binding is affected by co-transfection with ∆Np63α (58). Interestingly, we found that R binds to the EBV genome RRE sites in the SM, BMRF1 and BARF1 promoters equivalently with or without ∆Np63α (**Fig 6B**). These results are consistent with our finding that R activates SM gene expression in the presence or absence of co-transfected ∆Np63α (**Fig 4D**), and again suggest that a major ∆Np63α inhibitory effect may be mediated by decreasing the ability of R to indirectly activate Zp.

The C-terminal domain of TAp63 and ∆Np63 inhibits R-induced lytic reactivation. There are two primary p63 isoforms, ∆N and TA, which are defined by their N-terminal regions. The isoforms are derived from alternative transcription start sites, which either lack or contain the N-terminal transactivation domain, respectively (42). An additional five isoform subtypes (α, β, δ, ε,

and γ) can also arise from alternative splicing (or in the case of ε, a premature stop codon in codon 10) (54,72,73). The ΔNp63α isoform is the primary isoform expressed in epithelial cells. Interestingly, TAp63α is expressed in some B-cell lymphomas, including Burkitt lymphoma and diffuse large B cell lymphomas (DLBCL), and is a potential marker for poor patient prognosis (42,54,74–77).

To determine the region(s) of ΔNp63 responsible for the suppression of EBV lytic reactivation, we transfected human ΔNp63α, mouse ΔNp63α, and mouse ΔNp63β isoforms into NOKs-Akata cells with or without R. We found that, like human ΔNp63α, mouse ΔNp63α inhibits R-mediated initiation of the lytic replication cycle. However, the mouse ΔNp63β isoform, which is missing 121 amino acids of the C-terminal domain of ΔNp63α, failed to do so (**Fig 7A**). Additionally, TAp63α also inhibited R-induced lytic reactivation similarly to ΔNp63α, indicating that TAp63α may also be an inhibitor of lytic reactivation. ΔNp63α and TAp63α are identical at the C-terminal domains, which contain sterile alpha motif (SAM) and the post-sterile alpha motif domains, features that ΔNp63β lacks. SAM motifs in p63α isoforms provide docking sites for proteins containing SAM motifs, indicating that a protein-protein interaction could be mediating ΔNp63α and TAp63α lytic repression (78).

TAp63α acts as a repressor of EBV lytic reactivation in the Akata Burkitt lymphoma cell line. Since TAp63α is expressed in some Burkitt lymphomas and diffuse large B cell lymphomas (75,76) and TAp63α over-expression inhibits lytic reactivation in NOKs-Akata cells, we asked whether TAp63α helps maintain EBV latency in a lymphoma cell line that expresses TAp63α. The level of TAp63α protein expression in various EBV-infected lymphoma cell lines or lymphoblastoid cell lines (including the ILB1 DLBCL line, the Akata BL cell line, an Akata EBV strain virus transformed lymphoblastoid cell line (LCL), and an AG876 EBV strain virus transformed LCL) was examined by immunoblot analysis. We found that the ILB1 DCBL and Akata BL cell lines express TAp63α, which has a higher molecular weight compared to the ΔNp63α protein in the control NOKs-Akata epithelial cell sample (**Fig 8A**).

To determine if TAp63α represses EBV lytic reactivation in the B-cell environment, we knocked down TAp63α expression in the Burkitt Akata cell line using an shRNA against p63 or infected the cells with two separate control shRNAs. EBV lytic protein expression was increased in cells expressing the shRNAs against TAp63α compared to the control conditions (**Fig 8B**). These results suggest that expression of TAp63α, like ΔNp63α, inhibits EBV lytic reactivation in EBV-infected human lymphoma cells and thus contributes to maintenance of EBV latency in this cell type.

ΔNp63α inhibits constitutive Zp activity in AGS gastric cells and prevents KLF4-mediated activation of Zp. Since R activates the Zp through indirect mechanism(s) involving poorly defined cellular transcription factors (25)**,** we next asked if the constitutive activity of Zp in the gastric AGS cell line is also inhibited by ΔNp63α. EBV negative AGS cells were chosen for these studies since Zp activity is very high in these cells (in contrast to most cell lines). Luciferase reporter constructs containing either no promoter, or various portions of the Zp promoter, were transfected into AGS cells with or without a ΔNp63α expression vector, and the amount of luciferase activity was quantified two days later. As shown in **Fig 9A**, ΔNp63α decreased the activity of each of the Zp reporter constructs tested, including a construct containing only 83 base pairs upstream of the BZLF1 transcription initiation site. In contrast, ΔNp63α did not repress activity of the promoterless luciferase vector, and, as previously reported (79), increased the activity of the EBV BARF1 promoter in the same reporter vector (**Fig9B**).

We also examined the effect of ΔNp63α on the ability of co-transfected KLF4 to activate Zp in reporter gene assays. KLF4 is thought to activate Zp through a direct binding mechanism (16). ΔNp63α decreased the ability of co-transfected KLF4 to activate Zp-driven luciferase activity but did not affect the total level of transfected KLF4 protein (**Fig 9C**). Together, these

results strongly suggest that ΔNp63α inhibits Z promoter activation by positively acting regulators, including the EBV R protein and KLF4.

Lytic repressor c-myc is increased by ΔNp63α. Since our results are consistent with a model in which ΔNp63α indirectly inhibits the activity of the Zp promoter by upregulating expression of a cellular protein that inhibits Zp activity, we examined the effect of ΔNp63α over-expression, or ΔNp63α knock-down, on the level of c-myc in NOKs-Akata or CNE2-AKata cells. C-myc was recently shown to be a potent inhibitor of EBV reactivation via repressive effects on Zp activity (19). We observed that knockdown of ΔNp63α expression is associated with a concomitant decrease in c-myc expression **(Fig 10A**), while over-expression of ΔNp63α increases c-myc expression **(Fig 10B),** consistent with previous reports that Np63α helps maintain c-myc expression in basal epithelial cells (19,80).

To determine if this alteration in c-myc expression is sufficient to completely explain the inhibitory effect of ΔNp63α on lytic reactivation, we knocked down c-myc expression using siRNAs in NOKs-Akata cells, and then transfected cells with an R expression vector in the presence or absence of a ΔNp63α expression vector. As shown in **Fig 10C**, although we confirmed that knock-down of c-myc alone does increase R-mediated lytic reactivation, we also found that ΔNp63α still inhibits R-mediated lytic reactivation even when c-myc expression is knocked down. Thus, while alterations in c-myc expression may well contribute to the ΔNp63α inhibitory effect, additional inhibitory mechanisms are also likely involved.

ΔNp63α inhibits p38 MAPK activity, which also contributes to ΔNp63α suppression of lytic EBV reactivation. ΔNp63α was recently reported to inhibit activity of cellular p38 MAP kinase by increasing expression of the cellular DUSP6 phosphatase (81). Since ΔNp63α inhibits the ability of transfected R, but not Z, to induce lytic reactivation in epithelial cells (Fig 4), and we previously showed that the cellular p38 MAP kinase is required for R-mediated (but not Z-mediated) activation of the BZLF1 and BMRF1 lytic promoters in latently infected epithelial cells

(25), we next asked whether ΔNp63α at least partially inhibits R-mediated activation of BZLF1 and BMRF1 expression by decreasing p38 kinase activity. To examine this possibility, we over-expressed ΔNp63α in NOKs-Akata cells (using a lentivirus vector) and examined the effect on p38 kinase activity. We confirmed that phosphorylated (activated) p38 is substantially decreased in p63 over-expressing NOKs-Akata cells compared to control cells (**Fig 11A**).

Cellular p38 kinase is encoded by four different highly homologous cellular genes (MAPK 11-14). As shown in **Fig 11B,** knock-down of MAPK14 (p38α) expression (the most highly transcribed p38 encoding gene in NOKs-Akata cells (35)) inhibited constitutive Z and BMRF1 expression in NOKS-Akata cells, while R expression was only slightly affected. To examine how loss of p38 kinase activity affects the ability of R to induce various different lytic EBV promoters, NOKs-Akata cells were transfected with R in the presence or absence of a p38 kinase inhibitor (SB 202190, which affects both MAPK11 and MAPK14 p38 kinase activity), or an siRNA directed against the MAPK14 p38α kinase. As shown in **Fig 11C**, similar to the effect of ΔNp63α, treatment of cells with a p38 inhibitor greatly decreased R's ability to activate expression of both the BZLF1 IE protein and BMRF1 early lytic proteins. As expected, inhibition of p38 kinase activity decreased expression of its known downstream target, TTP (ZFP36). Specific knock-down of MAPK14 (p38α) expression inhibited R's ability to activate BMRF1 expression while having a lesser effect on BZLF1 expression **(Fig 11D**). Interestingly, the finding that specific knock-down of the p38α (MAPK14) form of p38 kinase affected BZLF1 expression less than the p38 kinase inhibitor suggests that additional p38 kinase protein(s) may contribute to inhibition of BZLF1 expression. Together, these results reveal that p38 kinase activity is crucial for R-mediated lytic viral reactivation in NOKs-Akata cells and suggest that ΔNp63α inhibition of p38 kinase activity is an important mechanism by which it inhibits lytic reactivation.

P38 kinase activation of lytic EBV infection does not require activation of the MK2 pathway. Although we previously showed that one mechanism by which p38 MAP kinase enhances lytic EBV reactivation is via phosphorylation and activation of the cellular ATF2 transcription factor (which directly binds to and activates the BZLF1 (Zp) promoter)(25), growing evidence suggests that p38 kinase commonly activates gene expression not only by enhancing transcription factor activity, but also by increasing stability ARE-containing RNA transcripts (82). Since the major mechanism by which the p38 kinase promotes RNA stability is through phosphorylation and activation of the MK2 kinase (MAPKAPK2) (83), we examined the ability of a MK2 inhibitor to affect R-mediated lytic reactivation in NOKs-Akata cells. As shown in **Fig 11E**, inhibition of MK2 activity using a chemical inhibitor did not suppress the ability of transfected R protein to induce BZLF1 or BMRF1 expression. Loss of TTP (ZFP36) expression (which requires MK2 activity for high level expression (82)) in this experiment confirmed that MK2 activity was decreased (**Fig 11F**) (82).

ΔNp63α over-expression during cis-platinum treatment of CNE-2-Akata and NOKs-Akata cells inhibits EBV lytic reactivation. Chemotherapy agents such as cis-platinum have been shown to potently induce EBV lytic reactivation (67,84,85), and the treatment of cells with cis-platinum is known to degrade ΔNp63α (86). We hypothesized that ΔNp63α over-expression may therefore inhibit cis-platinum-induced lytic reactivation in epithelial cells. To examine this, we over-expressed ΔNp63α in CNE-2-Akata cells and then treated with 10 μM cis-platinum for two days to induce lytic reactivation. Overexpression of ΔNp63α resulted in a striking complete loss of lytic induction in response to cis-platinum (**Fig 12A**); similar results were obtained in NOKS-Akata cells (**Fig 12B**). These results indicate that ΔNp63α is a major repressor of cis-platinum-induced lytic reactivation and conversely, loss of intact ΔNp63α protein expression contributes to the lytic inducing effect of cis-platinum.

Discussion

Infection of oropharyngeal epithelial cells plays an essential role in the EBV life cycle, particularly since these cells are the major source of transmissible virus. However, while EBV infection of normal oropharyngeal epithelial cells is highly lytic, and likely confined to the more differentiated cell layers, EBV infection of nasopharyngeal carcinoma cells is largely latent. Furthermore, the ability of NPC tumor cells to maintain EBV latency is likely required for their growth and survival. A major difference between NPC cells and the non-transformed oropharyngeal epithelial cells normally infected by EBV is that NPC cells are undifferentiated basal cells that express high levels of the basal cell transcription factor, ΔNp63α, which is lost when stratified epithelial cells differentiate. Here we demonstrated that both the basal epithelial cell specific ΔNp63α transcription factor, and the related isoform TAp63α (expressed in some EBV positive lymphomas) act as potent negative regulators of EBV lytic reactivation. These findings help to explain how EBV can achieve latent infection in undifferentiated NPC and B cell lymphomas.

EBV has two different IE proteins, Z and R, which encode viral transcription factors that cooperate to induce lytic reactivation of EBV. Our studies here show that the major inhibitory effect of ΔNp63α on lytic EBV reactivation occurs via negative regulation of the BZLF1 gene promoter (Zp). We found that ΔNp63α prevents the ability of transfected R protein to induce Z protein expression in latently infected epithelial cells, while not inhibiting the ability of transfected Z protein to induce R expression and downstream early lytic EBV proteins (**Fig 4**). These results indicate that Z over-expression (under the control of a heterologous promoter) is sufficient to bypass the inhibitory effect of ΔNp63α. Interestingly, in contrast to the effect on R-mediated early lytic BMRF1 protein expression, we found that ΔNp63α does not inhibit R-mediated activation of the early lytic SM protein (**Fig 4D**). This difference likely reflects the fact that the SM lytic gene can be turned on by R alone (even in the absence of Z expression

(63,87), while activation of BMRF1 gene expression requires both the Z and R proteins (29,61,62). Our finding that ΔNp63α also inhibits late lytic protein expression in R-transfected cells reflects the known requirement for Z to complete the lytic form of viral DNA replication (29,33) and to initiate subsequent late viral gene expression. Thus, loss of sufficient Z expression is the key defect impeding lytic viral reactivation in ΔNp63α expressing cells.

Although ΔNp63α is an essential regulator of epithelial cell differentiation and is well known to inhibit differentiation of epithelial cells by repressing expression of key cellular transcription factors such as KLF4 (57) and ZNF750 (88) that are required for epithelial cell differentiation, we found here that ΔNp63α inhibits Zp activity even in cells that cannot differentiate such as AGS cells and CNE-2 cells (**Figs. 4A, 4D, 9**). Thus, although EBV uses differentiation-dependent cellular transcription factors such as KLF4 and BLIMP1 to ensure that Zp and Rp are both activated when EBV-infected epithelial cells differentiate (15,16), it has usurped the functions of the basal cell specific transcription factor ΔNp63α to inhibit Zp activity even in the absence of KLF4 and BLIMP1. We show here that ΔNp63α inhibits the constitutive activity, as well as KLF4-induced activity, of Zp-driven reporter gene constructs in EBV-negative AGS gastric carcinoma cells (**Fig 9**).

EBV-negative AGS cells do not normally express ΔNp63α and in contrast to most other cell types are remarkable for their high level of constitutive Zp activity (89). We found that ΔNp63α strongly inhibits constitutive Zp activity in AGS cells and this effect was observed even with the smallest promoter Zp promoter construct studied (which contains only 83 base pairs upstream of the transcriptional initiation site). Of note, each of the Zp luciferase constructs used in this study contains the AP1-like "ZII" motif, which binds to the AP-1 and ATF2 (25) transcription factors and is required for constitutive Zp activity. ΔNp63α also inhibits the ability of transfected KLF4 protein to enhance Zp activity in AGS cells, without affecting the level of transfected KLF4 (**Fig 9**). We previously showed that KLF4 binds directly to Zp and contributes

to differentiation-dependent lytic EBV reactivation in epithelial cells (16). Together, these results suggest that ΔNp63α inhibits the ability of multiple different stimuli to induce Zp activity.

To examine whether ΔNp63α inhibits lytic EBV reactivation by binding directly to the Zp or Rp IE promoters, we performed ChIP assays using a Flag-tagged ΔNp63α expression vector (**Fig 6A**). Although we confirmed that ΔNp63α bound to a positive control cellular promoter in these assays, we did not observe binding of ΔNp63α to the Zp or Rp EBV IE promoters. To determine whether ΔNp63α blocks R binding to known RRE sites in the early lytic BMRF1 and SM promoters, we also performed ChIP assays using a Flag-tagged R expression vector to compare R binding to these promoters in the presence and absence of co-transfected ΔNp63α (**Fig 6B**). ΔNp63α co-transfection did not affect R binding to either the BMRF1 or SM promoters. Together, these results suggest that the inhibitory effect(s) of ΔNp63α on Zp activity do not require either direct ΔNp63α binding to the Zp or Rp IE promoters, or inhibition of R binding to other EBV promoters.

To further examine potential mechanism(s) for the inhibitory effect of ΔNp63α on Zp activity, we compared the ability of different p63 isoforms to block R-mediated lytic EBV reactivation in NOKs-Akata cells (**Fig 7**). These studies revealed that the C-terminal domain of ΔNp63α is required for the inhibitory effect. Since the TAp63α isoform (which is expressed in some EBV+ lymphomas) shares this inhibitory C-terminal domain, we also asked whether this isoform can block lytic viral reactivation. Importantly, we found that the Burkitt Akata lymphoma line expresses TAp63α, and that knockdown of TAp63α in these cells increases constitutive lytic EBV protein expression (**Fig 8**). Thus, other p63 family members may also play a role in maintaining EBV latency in some EBV-infected lymphomas.

Our results here suggest that one mechanism by which ΔNp63α promotes viral latency is by increasing expression of c-myc (**Fig 10**). ΔNp63α is known to enhance c-myc expression in basal epithelial cells (90), and c-myc was recently shown to potently inhibit Z expression and lytic reactivation in EBV-infected cells (19). We confirmed that ΔNp63α activates c-myc

expression in NOKs-Akata cells and showed that siRNA-mediated knock-down of c-myc increases the ability of transfected R protein to induce Z and BMRF1 expression (**Fig 10**). Nevertheless, since we found that ΔNp63α still inhibits R-mediated lytic reactivation in NOKs-Akata cells even when c-myc expression is greatly decreased using siRNA (**Fig 10**), the ΔNp63α effect on c-myc expression is unlikely to be the only mechanism by which ΔNp63α promotes viral latency.

Instead, our experiments here suggest that another major mechanism by which ΔNp63α promotes viral latency in epithelial cells is by decreasing p38 kinase activity. We found that ΔNp63α expression inhibits p38 kinase activity in NOKs-Akata cells, and that inhibition of p38 kinase activity reduces both constitutive, and R-mediated activation of the Z and BMRF1 proteins in NOKs-Akata cells (**Fig 11**). Although the mechanism(s) by which R increases Zp activity in the context of the intact viral genome are not completely understood, direct DNA binding of R to the Zp promoter does not appear to be involved, and instead R activation of Zp is thought to be mediated through indirect effects of R on cellular transcription factors (28,59,60,67). We previously showed that inhibition of p38 kinase activity blocks the ability of over-expressed R protein, but not over-expressed Z protein, to induce lytic EBV reactivation in latently infected epithelial cells, and correlated this effect with the ability of p38-phosphorylated ATF2 transcription factor to bind to and activate the BZLF1 promoter (25). More recently, the ability of the late lytic BGLF2 tegument protein to promote lytic reactivation was also shown to be at least partially mediated through p38 kinase activation (91,92).

In addition to its requirement for R-mediated and BGLF2-mediated lytic EBV reactivation in epithelial cells, a growing literature indicates that p38 kinase activity is required for the ability of multiple different types of stimuli to induce lytic EBV reactivation in both epithelial cells and B cells. Indeed, p38 kinase inhibitors have been shown to block viral reactivation in response to such diverse stimuli as B-cell receptor activation, chemotherapy agents, phorbol esters, reactive oxygen species, and TGF-β (93–98). Given the large number of direct p38 kinase substrates,

and the multiple downstream pathways that are indirectly affected by this kinase, we speculate that p38 kinase contributes to lytic EBV reactivation through multiple different mechanisms. Our results here suggest that one of the primary effects of this kinase on EBV reactivation is to enhance BZLF1 promoter activation in response to both viral and cellular transcription factors. Interestingly, a number of different cellular transcription factors that have previously been shown to bind to and activate the BZLF1 promoter (including ATF1, ATF2, c-jun, c-fos, XBP1, HIF-1α, and p53) are known to be directly phosphorylated and activated by p38 kinase (99). Furthermore, p38 kinase more globally enhances transcription by activating downstream kinases such as MSK1 and MSK4 that directly phosphorylate histone sites such as H3 S28**;** this phosphorylation then reduces inhibitory modifications on H3 K27 that recruit the polycomb complex (100–102).

In addition, p38 kinase activity is increasingly recognized to exert many of its cellular effects by increasing stability of a subset of cellular RNAs (83). This effect is mediated largely through activation of the MK2/MKK3 kinases, which phosphorylate and inactivate a cellular protein, ZFP36 (TTP), that induces degradation of RNAs containing AU-rich 3' LTRs (82). However, treatment of NOKS-Akata with an MK2 inhibitor did not impact R-induced lytic reactivation, indicating that p38 may facilitate lytic reactivation through pathways independent of effects on RNA stability (**Fig 11E**). Interestingly, p38 kinase was also recently reported to inhibit nonsense mediated decay (NMD) of RNA in cells with DNA damage (103). Since NMD was recently shown to promote degradation of the transcript encoding the BZLF1 and BRLF1 genes in latently infected cells (104), decreasing NMD of this transcript in the context of the intact viral genome could be another post-transcriptional mechanism by which p38 kinase promotes lytic EBV reactivation.

Finally, we showed here that the recently described ability of the chemotherapy agent cis-platinum to induce ΔNp63α degradation in epithelial cells (86) can potentially be used to potentiate "lytic induction" therapy for EBV-positive epithelial tumors. Lytic induction therapy of

latently infected EBV-positive tumor seeks to identify small molecules that can reactivate the lytic form of viral infection in tumors, thereby using the virus itself to help kill the tumor cell. Interestingly, we previously showed that p38 kinase activity is required for chemotherapy to induce lytic EBV reactivation in certain epithelial cell lines (84). Here we confirmed that cis-platinum treatment of EBV-infected epithelial cells does, indeed, result in loss of intact ΔNp63α expression and showed that restoration of ΔNp63α expression in cis-platinum treated cells reduces the amount of lytic EBV reactivation (**Fig 12A and 12B**). Thus, the use of chemotherapy drugs such as cis-platinum that can not only induce DNA damage and p53/ATM activation (known activators of lytic EBV reactivation (67,69)), but can simultaneously degrade ΔNp63α, is predicted to be the most effective approach for achieving efficient lytic induction therapy in EBV-positive NPC tumors.

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Materials and Methods

Cell culture

The normal oral keratinocytes (NOKs) cell line (a generous gift from Karl Munger of Tufts University via Paul Lambert of the University of Wisconsin) is a telomerase-immortalized normal oral keratinocyte cell line, grown in keratinocyte serum-free media supplemented with 12.5 mg bovine pituitary extract, and 0.1 µg epidermal growth factor per 500ml of media (KSFM, Lifetech). NOKs were derived as previously described (105). p53KO NOKs were derived by CRISPR-Cas9 mutagenesis of the p53 gene as previously described (69). EBV-infected NOKs-Akata cells were created as previously described (29) and were maintained with 50 µg/ml G418 antibiotic selection in addition to the media/growth supplements used to grow NOKs cells. The CNE-2 cell line was derived initially from NPC tumors but has been subsequently shown to have HeLa cell genome contamination and HPV infection (106,107). CNE-2-Akata cells (a generous gift from K.W. Lo of the Chinese University of Hong Kong via Diane Hayward) are stably EBV-infected with the Akata strain of EBV (containing a G418 resistance gene cassette and GFP gene inserted into the EBV BXLF1 gene) and were grown in DMEM with 10% fetal bovine serum, 1% pen-strep, and 400 µg/ml G418 antibiotic selection. AGS cells are an EBV-negative gastric carcinoma cell line that was obtained from the ATCC and were grown in F12 media with 10% fetal bovine serum and 1% pen-strep. AGS-Akata cells are AGS cell stably infected with the Akata strain of EBV (containing a G418 resistance gene cassette and GFP gene inserted into the EBV BXLF1 gene derived as previously described (108) and were a kind gift from Lindsay Hutt-Fletcher. AGS-Akata cells were grown in F-12 media with 10% fetal bovine serum, 1% pen-strep, and 400 µg/ml G418 antibiotic selection. Both the uninfected and EBV-infected AGS were cured (in the Hutt-Fletcher lab) of the contaminating SV5 virus present in most AGS lines. The Burkitt lymphoma cell line Akata (Burkitt-Akata) (a gift from Lindsay Hutt-Fletcher) was derived as previously described by super-infecting an EBV-negative Akata Burkitt

lymphoma cell clone with the Akata strain of EBV (containing a G418 resistance gene cassette and GFP gene inserted into the EBV BXLF1 gene)(108) and was maintained with RPMI media with 10% fetal bovine serum with 1% pen-strep and 500 µg/ml G418 antibiotic selection. The IBL1 diffuse large B cell lymphoma line is a gift from Ethel Cesarman and was derived as previously described (64). The AG876 and Akata lymphoblastoid cell lines (LCLs) were derived from peripheral blood B cells transformed with either the AG876 or Akata strains of EBV, respectively, as previously described (65) and were maintained with RPMI supplemented with 10% fetal bovine serum and 1% pen-strep.

Collagen Membrane Differentiation

Approximately $5x10^5$ total NOKs-Akata cells were seeded onto a collagen-treated transwell membrane (Corning #3460) with KSFM media on both the basal and apical surfaces of the membrane. After 24 hours, when cells were 100% confluent, apical media was removed and the basal media exchanged for Epilife media (Thermofisher # MEPICF500) supplemented with 10% FBS, 1.4mM $CaCl_2$, and 5 ug/ml ascorbic acid. Cell extracts on the collagen were harvested with sumo lysis buffer for immunoblot analysis membranes after three days of differentiation.

Immunoblots

Immunoblots were performed as previously described (111). Briefly, cell lysates were harvested with sumo lysis buffer with protease inhibitors (cOmplete, Roche). Quantitation of protein concentration was conducted with a sumo protein assay (Biorad). The lysates were separated using a 10% polyacrylamide gel and then transferred onto a nitrocellulose membrane. The membranes were subsequently blocked with 5% milk consisting of .1% Tween 20 and 1X PBS for one hour. Membranes were then incubated with primary antibody overnight. The following day the antibodies were removed and the membrane was washed with wash buffer (1X PBS, .1% Tween 20) three times for 5 minutes. The membrane was then incubated with secondary antibody suspended in 5% milk for one hour, before washing with wash buffer three times for 10 minutes before treatment with ECL (Thermofisher) and imaging.

Immunohistochemistry

Immunohistochemistry (IHC) was conducted as previously described (112). To summarize, paraffin-embedded slides were initially heated on 70°C heat blocks and deparaffinized in xylene two times for 10 minutes. The slides were then hydrated in a series of alcohols (100%, 90%, 70%) for a period of 5 minutes each. The slides were then boiled in 1X sodium citrate buffer for 20 minutes and allowed to cool for an hour before blocking with horse serum. Primary antibodies were added to the slides for an hour, and then the primary was washed off in 1X PBS before adding secondary for thirty minutes. DAB was added for a period of approximately 15 seconds, and then horseradish peroxidase for approximately 10 seconds. Hematoxylin was used as a counterstain and the slides were dehydrated in alcohols before mounting. Z antibody was used at 1:200, and p63α antibody was used at 1:200.

Chemicals

Cisplatin (Sigma, catalog#1134357 was made up at 10 mM and used at 10 µM. SB202190 p38 kinase inhibitor was purchased from Santa Cruz (sc-202334) was used at 10 µM. Phorbol 12-myristate 13-acetate (TPA) was purchased from Sigma (catalog#P8139) used at 20 ng/ml. MK2 inhibitor PF-364402 hyrdate was purchased from Millipore-Sigma (catalog#PZ0188) and used at 10 µM. Control conditions were treated equal amounts of the solvent.

Antibodies

The following antibodies were used for immunoblot analyses in this study: anti-R rabbit polyclonal antibody directed against the R peptide (peptide sequence EDPDEETSSQAVKALREMAD 1: 2500), anti-p63α (Cell Signaling Technologies catalog # 13109 1:1000), anti-KLF4 (Cell Signaling Technologies, catalog #4038 1: 1000), anti-BZLF1 (Santa Cruz, catalog # sc-53904, 1:500), anti-BMRF1 (Millipore, catalog # MAB8186, 1: 2500), anti-p18 (Thermo-Fisher Scientific, # PA1-73003, 1:2000), anti-BLIMP1 (Cell Signaling Technologies catalog # 9115S 1:1000), anti-involucrin (Sigma, catalog #19018, 1:3000), anti-c-

myc (Abcam catalog# ab32072, 1:10000), p38 MAPK14 antibody (Cell Signaling Technologies catalog#9218 1:1000) and phospho-p38 MAPK (Cell Signaling Technologies catalog #9211 1:1000), anti- EBV SM polyclonal rabbit antibody (1:750), a generous gift from Sankar Swaminathan of the University of Utah, anti-Tristetraprolin (Cell Signaling Technologies catalog# #71632 1:1000). The secondary antibodies used were Horseradish peroxide (HRP)-labeled goat anti-mouse antibody (Thermo Scientific# 31430, 1:5000), HRP- labeled donkey anti-goat antibody (Santa Cruz#sc-2056, 1:5000), and HRP- labeled goat anti-rabbit antibody (Fisher Scientific 1:10000).

Plasmids

All plasmid DNA was prepared using the Qiagen Maxi-prep kit according to the manufacturer's instructions. The plasmid pSG5 was purchased from Stratagene. pSG5-R and pSG5-Z (kind gifts from Diane Hayward of John Hopkins University) contain the BZLF1 (Z) and BRLF1 (R) immediate-early genes driven by the SV40 promoter as previously described (14,113). ΔNp63α-FLAG (Addgene # 26979, a gift from David Sidransky of John Hopkins University) encodes the ΔNp63α gene with a FLAG tag. ΔNp63α pReceiver-Lv105 (Genecopoeia product # Z57540-Lv105) is a ΔNp63α lentiviral expression vector, and plenti is the lentiviral empty control (Addgene #39481, a gift from Ie-Ming Shih of John Hopkins University). ΔNp63α-CMV was purchased from Genecopoeia (catalog #EX-Z5740-M02). pCMV3FC and BRLF1-pCMV3FC (containing a FLAG-tagged BRLF1 protein) are both kind and generous gifts from Lori Frappier of the University of Toronto (114). Mouse ΔNp63α-myc, TAp63α-myc, and ΔNp63β-myc plasmids are generous gifts of Xiaohua Su at MD Anderson and Elsa Flores at the Moffitt Cancer Center.

siRNAs

siRNAs against ΔNp63α (sc-36161A, sc-36161B), c-myc (sc-29226, sc-44248) and control siRNAs A and C (sc-37007, sc-44231). MAPK14 siRNA (sc-29433) was purchased from Santa

Cruz. siRNAs were transfected in 12 well plates using RNAiMAX (Invitrogen) according to the manufacturer's protocol. After two days, the cells were harvested with sumo lysis buffer for immunoblot analysis.

Transient Transfections

DNA was transfected into NOKs-Akata, CNE-2-Akata, AGS-, and AGS-Akata using the Lipofectamine 2000 (Thermo Fisher #11668019) system according to the manufacturer's protocol. Generally, 500ng of total DNA with 1.5 µL Lipofectamine 2000 was used per condition to transfect epithelial cells that were approximately 70% confluent in a 12 well plate.

shRNAs and lentivirus packaging

shRNAs against ΔNp63α purchased from Horizon (catalog#RHS4533-EG8626). Lentivirus packaging was done as previously described (16). Packaging components pCMV-VSV-G (Plasmid #8454, a gift from Bob Weinberg of the Massachusetts Institute of Technology) and pSPAX2 (Plasmid #12260, a gift from Didier Trono from the École Polytechnique Fédérale de Lausanne) were purchased from Addgene. To package the lentiviruses, 4 µg of the vector, 0.6 µg of VSV-G, and 1.4 µg of psPAX2 were transfected into 293T cells plated in a 10cm dish. The cell's media was changed 24 hours post transfection and viral supernatant was harvested on days 2 and 3 to infect either NOKs-Akata or CNE-2-Akata cells and was selected with puromycin to derive a stable cell line.

Organotypic rafting

Cells were stratified by organotypic rafting as described previously (16). To briefly summarize, a dermal equivalent was created using transwell inserts (24mm diameter, 0.4µM pore Costar) that were coated with 1ml collagen mix (3 mg/ml Wako) also containing F-media, 10% FBS and 1% pen-strep. This layer was coated with an additional 2.5 ml collagen mix containing F-12 media, 10% FBS, 1% pen-strep, and 4.5 X 10^5 early- passage human fibroblasts (EF-1-F). This dermal equivalent was suspended in F-12 medium supplemented with 10% FBS and 1% pen-strep. Four days later, 2.1 x 10^5 NOKs-Akata cells were plated on the dermal equivalent suspended in

keratinocyte plating media (F-medium [1.88 mM $Ca2^{+}$]) supplemented with 0.5% FBS, adenine (24 µg/ml), cholera toxin (8.4 ng/ml), hydrocortisone (2.4 µg/ml), and insulin (5 µg/ml). The cells were allowed to grow for four days to reach confluence, and at this point the media was exchanged for cornification media (keratinocyte plating medium containing 5% FBS and 10 µM $C_{8:0}$), and the cells were lifted to the air liquid interface so that only the basal cells were supplied with media. After 11 days of differentiation in cornification media that was replaced every other day, the cells were harvested and embedded in 2% agar-1% formalin, and fixed in 10% neutral buffered formalin overnight. The raft tissues were subsequently embedded in paraffin and sectioned in 4 µM cross sections.

Chromatin immunoprecipitation (ChIP) and quantitative PCR

ChIP assays were performed as described previously (115). For p63 ChIP assays, AGS-Akata cells (using three 10cm dishes per condition) were transfected with a pCDNA empty vector control or a p63-FLAG expression vector. For the R FLAG ChIP assays, CNE-2-Akata cells were transfected with BRLF1-pCMV3FC (which contains a FLAG tag), BRLF1-pCMV3FC with ΔNp63α-CMV, or pCDNA empty vector control. 24 hours post-transfection, the cells were fixed with 1% paraformaldehyde for 10 minutes and quenched with 125 mM glycine for 5 minutes. The cells were then pelleted by centrifugation at 1500 rpm for 10 minutes, washed with PBS. The cell pellets were then lysed in cell lysis buffer (10mM Tris pH 8.0, 10mM NaCl, 0.2% NP40) with protease inhibitors (cOmplete, Roche) to remove cytoplasmic protein. Nuclear pellets were lysed in nuclei lysis buffer (50mM Tris-HC pH 8.0, 10mM EDTA pH 8.0, 1% SDS) with protease inhibitors and the mixture was left on ice for 10 minutes. The nuclear lysates were then diluted in IP dilution buffer (20mM Tris-HCl pH 8.0, 2mM EDTA, 150mM NaCl, 1% Triton X100, and 0.01% SDS) and sonicated 4 cycles (30 seconds ON at 10 watts/ 90 seconds OFF) (Fisher Scientific, Sonic Dismembrator Model 100). Approximately 25 µg DNA were diluted with IP dilution buffer, blocked with magnetic A/G beads (Thermo-Fisher, 88802) for one hour, and

immunoprecipitated with M2 FLAG (Sigma-Aldrich, M8823-1ml) beads overnight. The samples were then washed with low salt, high salt, and lithium chloride wash buffer for 15 minutes each, followed by two 15-minute washes with $T_{10}E$ buffer. Protein/chromatin complexes were eluted with elution buffer (0.1M $NaHCO_3$, 1% SDS). Crosslinked protein DNA complexes were reverse cross-linked with 0.3M NaCl for 4 hours at 65°C with RNAse A, then proteinase K were added to all samples. Sample DNA was purified with phenol-chloroform, precipitated with EtOH, and resuspended in $T_{10}E$ for real-time PCR. Purified DNA was quantified using following primers (Z promoter: 5' CCGGCAAGGTGCAATGTTTAG/ 3' CATCACAGAGGAGGCTGGTG, R promoter: 5' TGCCGGCTGACATGGATTACT/ 3' GATGCTGATGCAGAGTCGCC, BMRF1 promoter: 5' CACTGCGGTGGAGGTAGAG/ 3' GGTGGTGTGCCATACAAGG, NECTIN1, 5' TGAGCCTGTAGGACCAGAATCA/ 3' TTTCCCACTCAAGCTGTGTCTCT) and iTaq universal SYBR green supermix (Bio-Rad) using a CFX96 touch real-time PCR detection system (Bio-Rad). Purified input DNAs were used in real-time PCR for standardization. The experiment shown is representative of two independent experiments and error bar indicating standard error of the mean within experiments.

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Figures

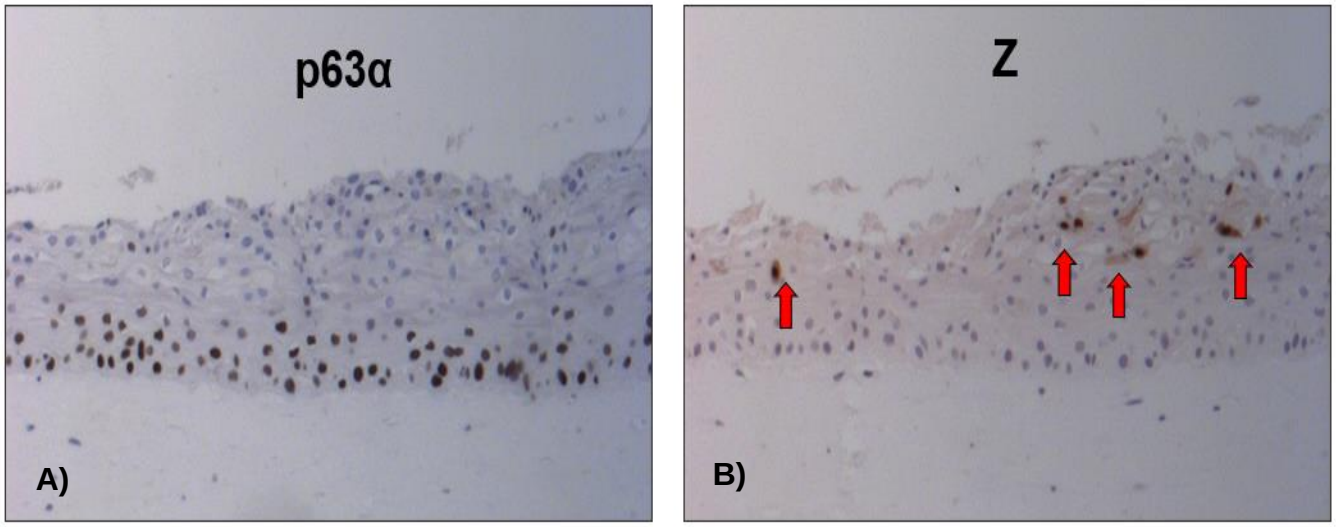


Figure 1: p63α and Z expression occurs in different sections of organotypic raft cultures. Immunohistochemistry (IHC) was performed on adjacent sections of organotypic rafts of differentiated NOKs-Akata cells using antibodies against **A)** p63α and **B)** Z. Arrows show examples of Z expressing cells.

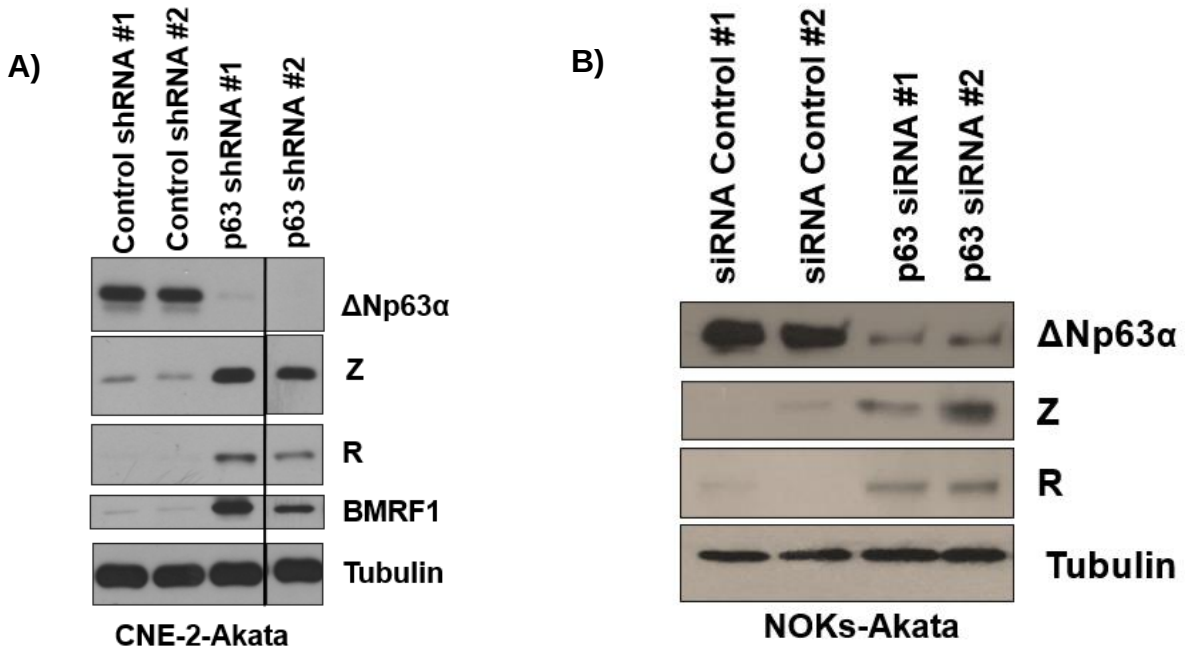


Figure 2: ∆Np63α depletion results in EBV lytic reactivation in EBV+ carcinoma (CNE-2) and telomerase-immortalized oral keratinocyte (NOKs) cell lines. A) CNE-2-Akata cells were infected with lentiviruses expressing shRNAs against p63 or a control sequence, selected with puromycin, and harvested for immunoblot analyses to measure expression levels of the EBV Z, R, and BMRF1 lytic proteins, ΔNp63α, and tubulin proteins as indicated. **B)** NOKs-Akata cells were transfected with siRNAs against p63 or a control siRNA. Two days after transfection, the cells were harvested for immunoblot analyses and expression of the Z, R, ΔNp63α and tubulin proteins was examined as indicated. The black line indicates where irrelevant lane(s) were removed. The original western blots used to construct this figure are shown in supplemental figure 1.

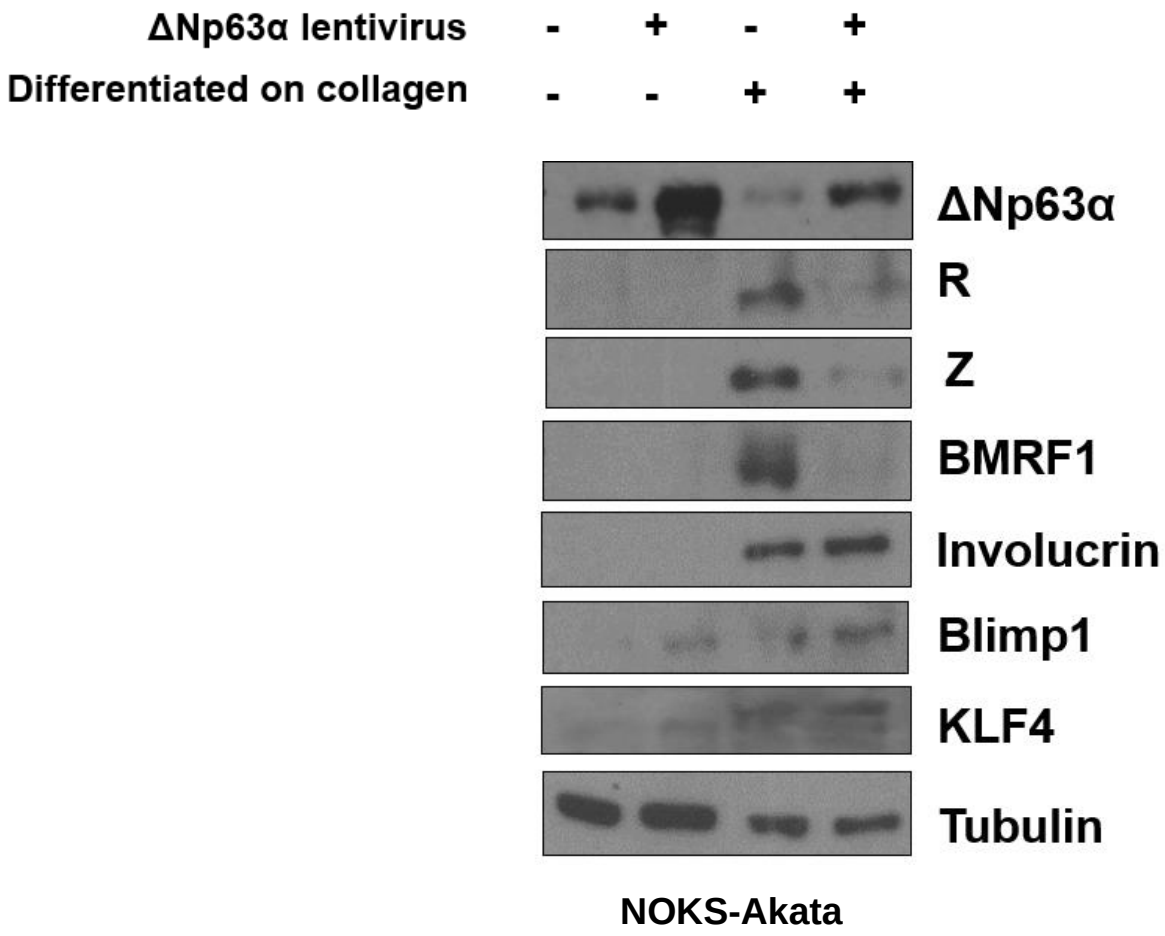


Figure 3: ∆Np63α over-expression in NOKs-Akata cells during differentiation decreases EBV lytic reactivation. NOKs-Akata cells expressing either ΔNp63α from a lentivirus or infected with a control lentivirus were differentiated on collagen-treated membranes in air-liquid interface culture for three days in the presence of media containing vitamin C, calcium, and 10% serum. The cells were then harvested for an immunoblot and expression levels of the Z, R, BMRF1, ΔNp63α, involucrin, BLIMP1, KLF4, and tubulin proteins was determined as indicated.

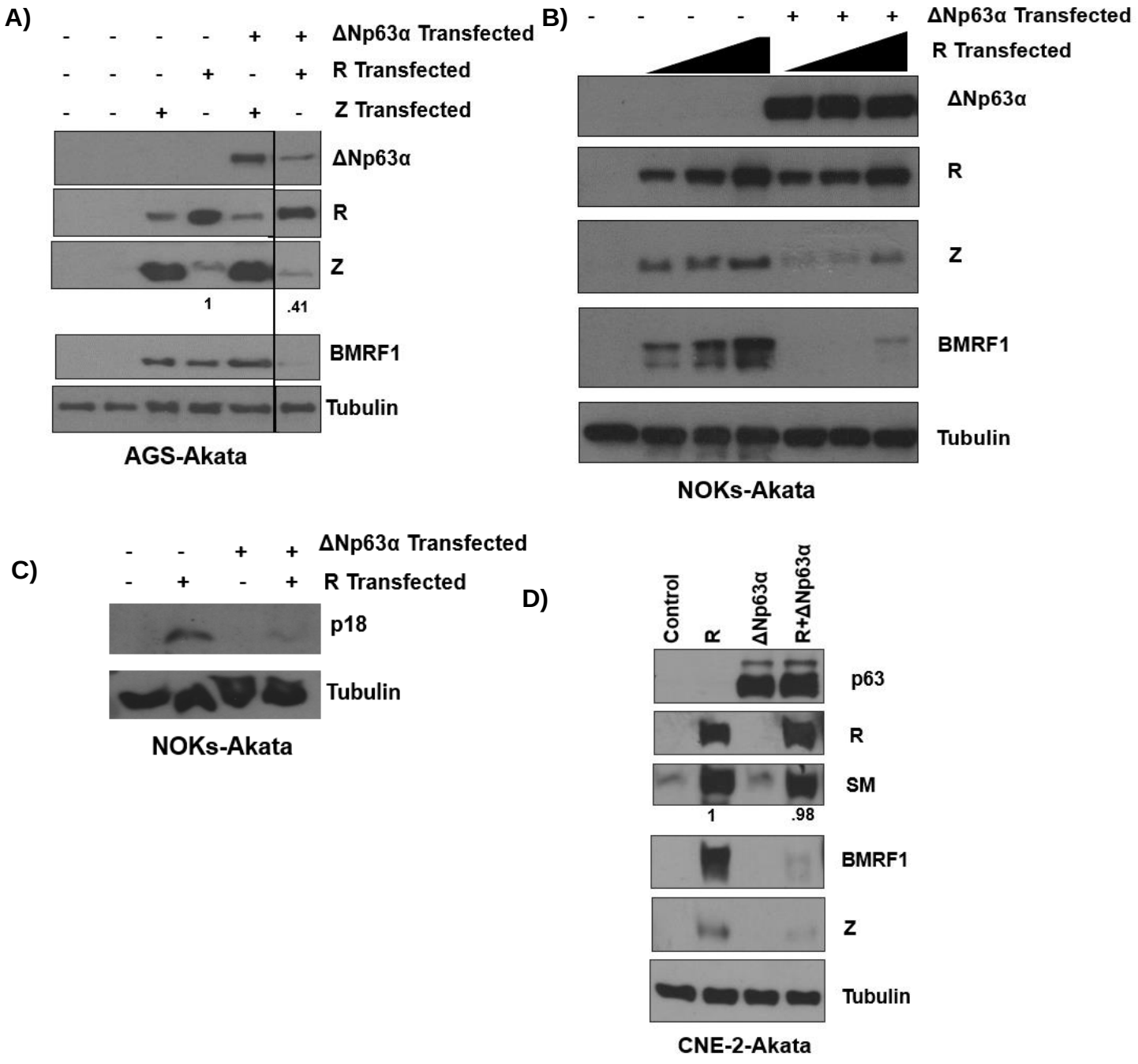


Figure 4: ΔNp63α over-expression inhibits R-mediated, but not Z-mediated, lytic reactivation. A) AGS-Akata cells were transfected with Z or R expression vectors, with or without a co-transfected ΔNp63α expression vector. Immunoblots were performed to assess expression levels of the EBV lytic proteins Z, R, and BMRF1 as indicated, as well as the loading

control, tubulin. Z expression was quantitated by ImageJ software, normalized to tubulin and the Z alone condition was set as 1. **B)** NOKs-Akata cells were transfected with 5 ng, 10 ng, or 50 ng of an R expression vector with or without a co-transfected ΔNp63α expression vector. Immunoblot was performed to examine expression levels of the EBV lytic proteins Z, R, and BMRF1, as well as ΔNp63α and tubulin as indicated. **C)** NOKs-Akata cells were transfected with 10 ng of an R expression vector with or without a co-transfected ΔNp63α expression vector or the ΔNp63α expression vector alone (same extracts as in figure B) and an immunoblot was performed to examine expression of the late viral protein, viral capsid protein p18. Tubulin was used as a loading control. **D)** CNE-2-Akata cells were transfected with an R expression vector in the presence or absence of a co-transfected ΔNp63α vector. An immunoblot was performed to examine expression levels of the ΔNp63α, R, Z, BMRF1, SM, and tubulin proteins as indicated. SM expression was quantified using ImageJ, normalized to tubulin, with the R transfected by itself set as 1. The black line in Figure 4A shows where irrelevant lane(s) were removed; the original western blots used to construct figure 4A are shown in supplemental figure 2.

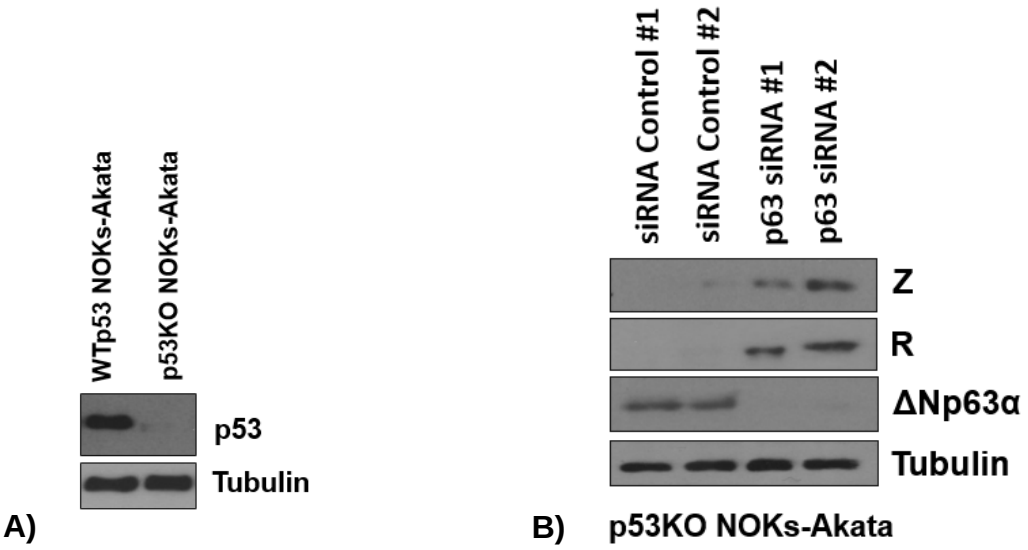


Figure 5: ΔNp63 inhibits lytic reactivation in NOKs-Akata cells even when the p53 gene is deleted. A) The CRISPR/CAS9 technique was used to knock out p53 expression in NOKs-Akata cells, as described in the methods section, and expression of the p53 protein and tubulin loading control was examined by immunoblot to confirm knock-out of p53 expression. **B)** NOKs-Akata cells in which the p53 gene was knocked-out were transfected with siRNAs against ΔNp63α or a control sequence. Two days later expression of the Z, R, ΔNp63α and tubulin proteins was examined by immunoblot.

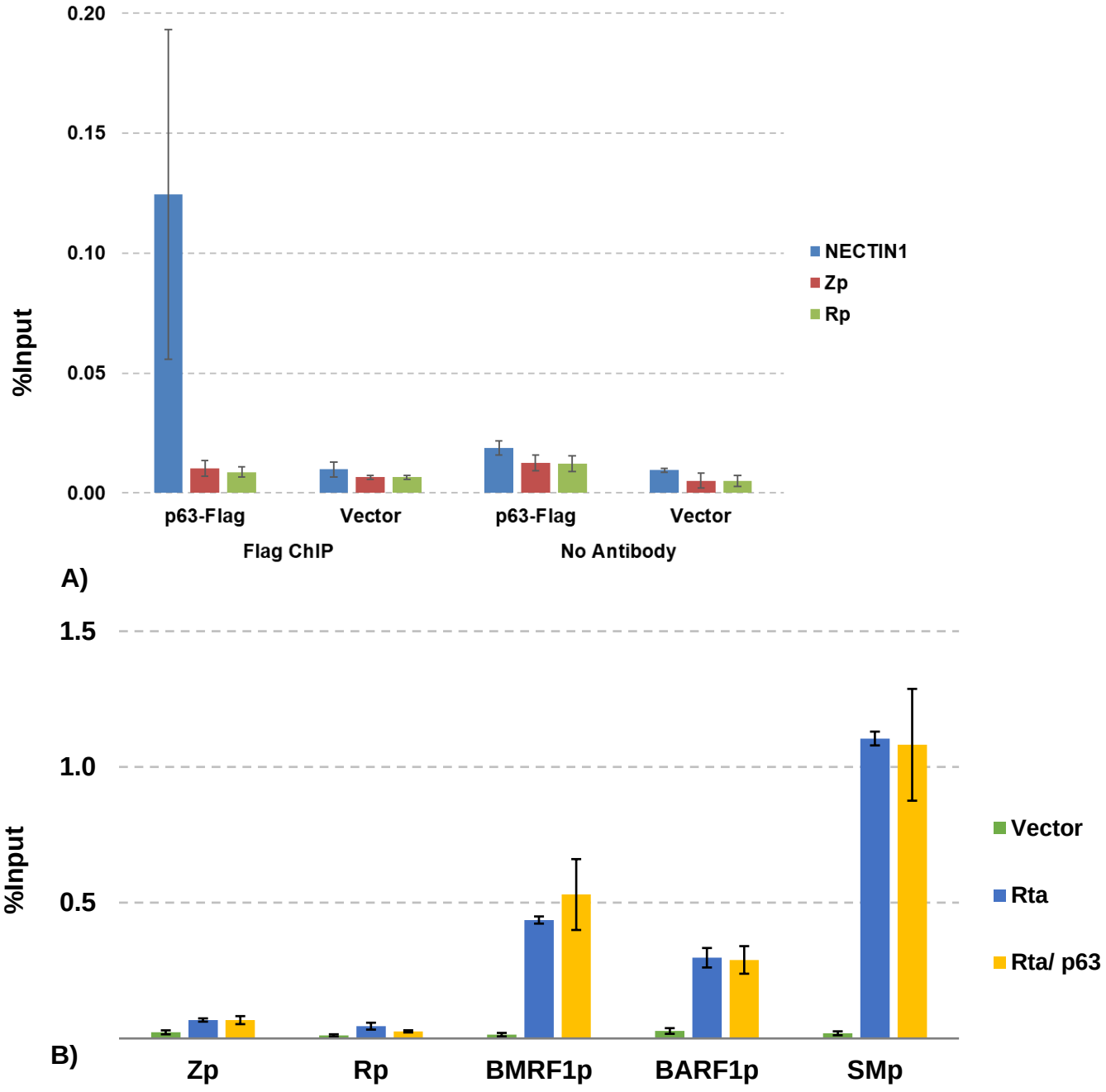


Figure 6: ΔNp63α does not bind directly to the EBV immediate-early gene promoters or prevent R binding to the EBV genome. A) AGS-Akata cells were transfected with a FLAG-tagged ΔNp63α vector or a control vector sequence, and two days after transfection, and a ChIP assay was performed as described in the methods using an anti-FLAG antibody. qPCR was conducted to determine ΔNp63α occupancy of EBV lytic gene IE promoters Zp and Rp, in

addition to NECTIN1, a known ΔNp63α binding site. **B)** CNE-2-Akata cells were transfected with vector control or with a FLAG-tagged R expression vector in the presence or absence of ΔNp63α, and a ChIP assay was performed as described in the methods using an anti-FLAG antibody. qPCR was performed in the ChIP samples to examine R association with the EBV Z promoter, R promoter, BMRF1 promoter, BARF1 promoter, and BMLF1 (SM) promoter.

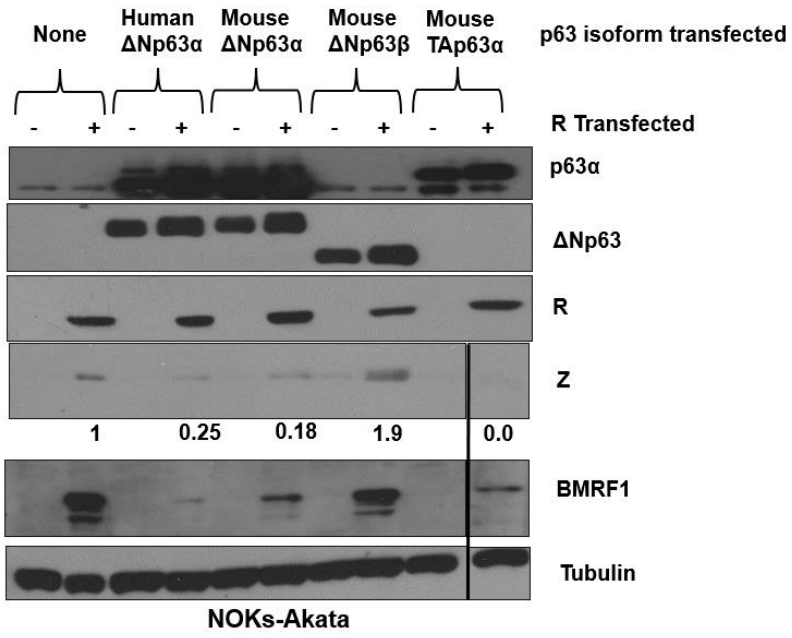


Figure 7: The C-terminus of p63 protein is required for the ability to inhibit R-mediated lytic reactivation. NOKs-Akata cells were transfected with or without an R expression vector in the presence or absence of various different co-transfected p63 isoform vectors, including the human ΔNp63α protein, the mouse ΔNp63α protein, the mouse ΔNp63β isoform (homologous to ΔNp63α but lacking 121 amino acids at the C-terminal domain), or the mouse TAp63α isoform (which is identical to ΔNp63α except for the addition of an N terminal transactivating domain). Two days after transfection, immunoblots were performed to assess expression levels of the Z, R, BMRF1, p63 protein isoforms, and tubulin as indicated. The quantification of Z protein expression was determined through ImageJ, normalized to tubulin, with the R alone condition set as 1. The black lines show where irrelevant lanes were removed; the original western blots used to construct this figure are shown in supplemental figure 4.

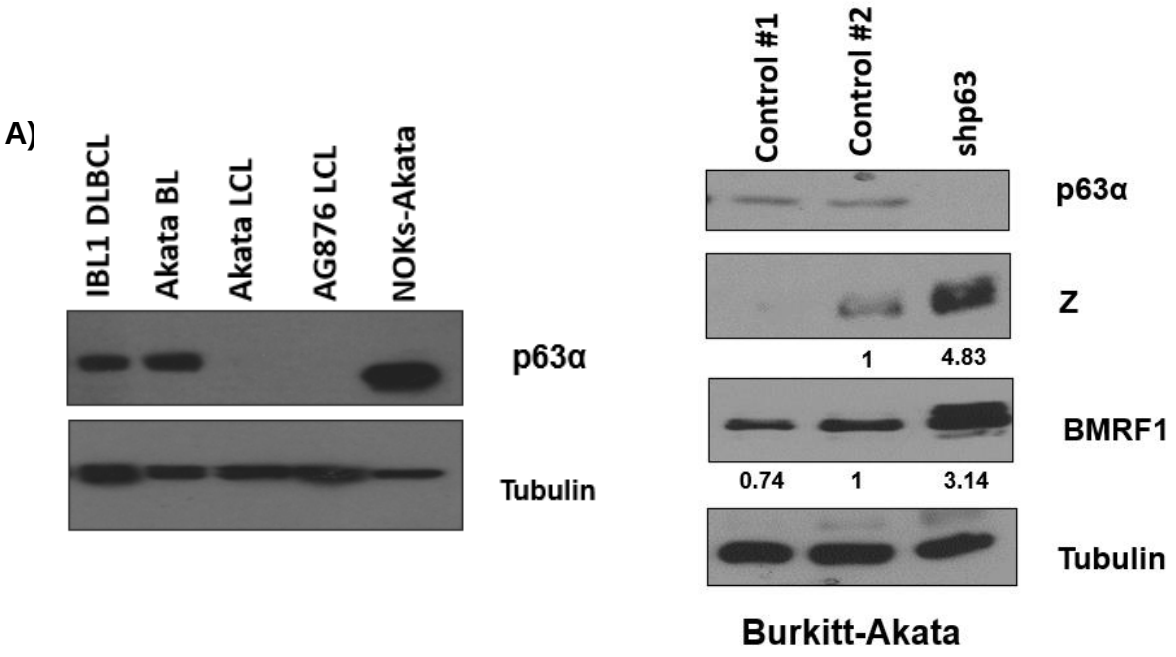


Figure 8: **The TAp63α isoform of p63 inhibits EBV lytic reactivation in Akata Burkitt lymphoma cells. A)** The level of TAp63α isoform expression in various EBV-infected B-cell lines were examined by immunoblot analysis; NOKs-Akata epithelial cells were used as a control for expression of the shorter ΔNp63α isoform. **B)** Akata Burkitt lymphoma cells were infected with two different lentiviral vectors expressing control shRNAs or a lentivirus vector expressing shRNA against p63, selected for puromycin resistance, and then assessed by immunoblot analysis for expression levels of the Z, R, BMRF1, TAp63α and tubulin proteins. The quantification of Z and BMRF1 protein levels was determined through ImageJ, normalized to tubulin, with the control #2 condition set as 1.

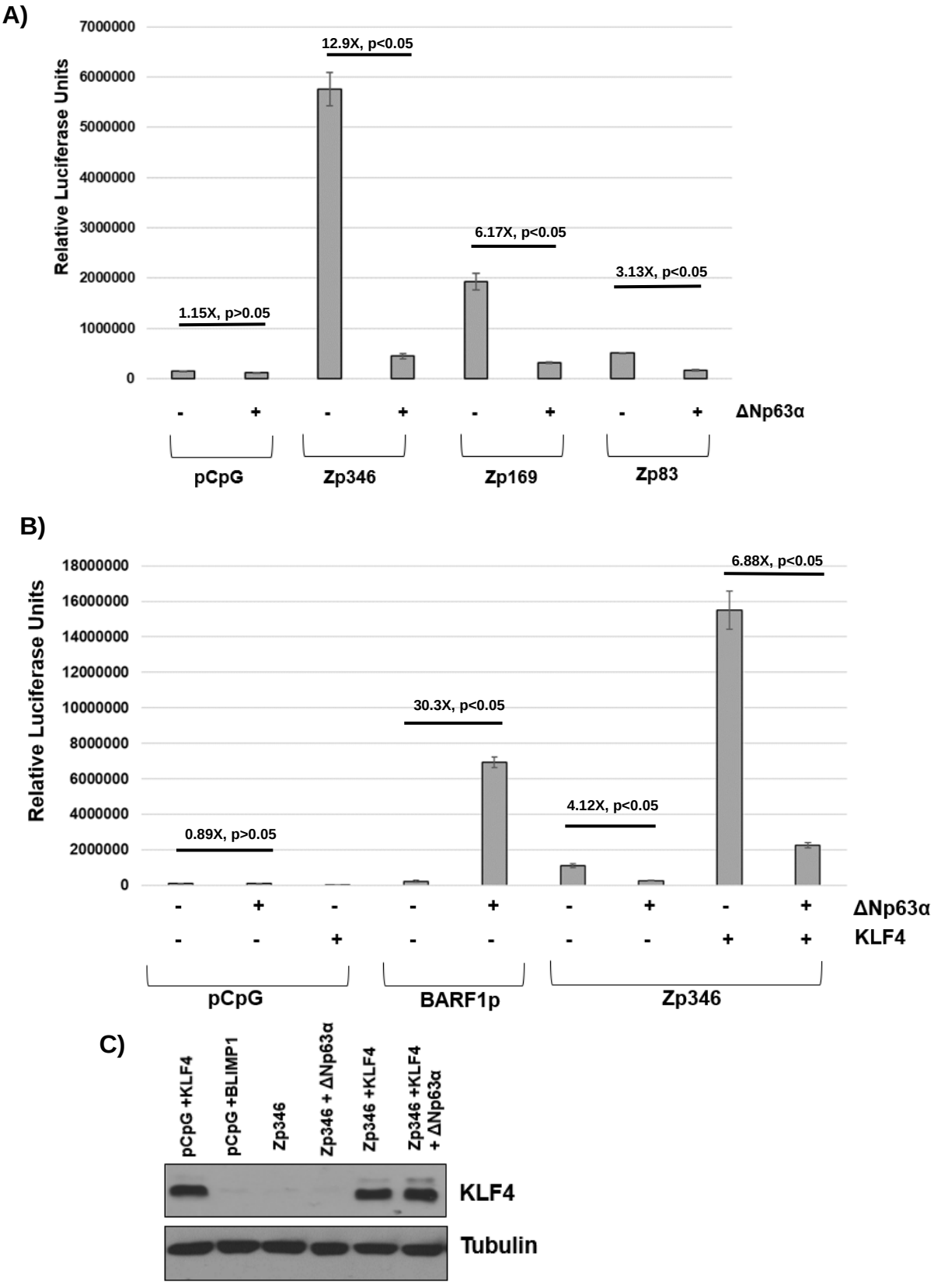

A)
Relative Luciferase Units
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
12.9X, p<0.05
6.17X, p<0.05
3.13X, p<0.05
1.15X, p>0.05
- + - + - + - +
ΔNp63α
pCpG
Zp346
Zp169
Zp83
B)
Relative Luciferase Units
18000000
16000000
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
6.88X, p<0.05
30.3X, p<0.05
4.12X, p<0.05
0.89X, p>0.05
- + - - + - + - +
ΔNp63α
- - + - - - - + +
KLF4
pCpG
BARF1p
Zp346
C)
pCpG +KLF4
pCpG +BLIMP1
Zp346
Zp346 + ΔNp63α
Zp346 +KLF4
Zp346 +KLF4 + ΔNp63α
KLF4
Tubulin

Figure 9: ΔNp63α inhibits Z promoter activity in reporter gene assays. A) Luciferase vectors containing various amounts of the Zp promoter sequence, or the pCpG (promoterless) negative control vector, were transfected into EBV-negative AGS gastric carcinoma cells with or without an ΔNp63α expression vector. Luciferase activity was measured two days later. The average fold change in luciferase activity in ΔNp63α transfected cells versus control vector transfected cells for each promoter construct is shown, with error bars indicating standard error. **B)** AGS cells were transfected with the pCpG negative control luciferase vector, or luciferase vectors driven by the EBV early lytic BARF1 promoter, or the Zp (Zp-346) promoter, in the presence or absence of a KLF4 expression vector, with or without co-transfected ΔNp63α, as indicated. The average fold change in luciferase activity for each condition (relative to each promoter construct transfected with control vector) is shown, as well as the standard error. **C)** Immunoblot analysis of the Zp-luciferase assay samples shown in Fig. 9B above was performed to assess KLF4 and tubulin levels.

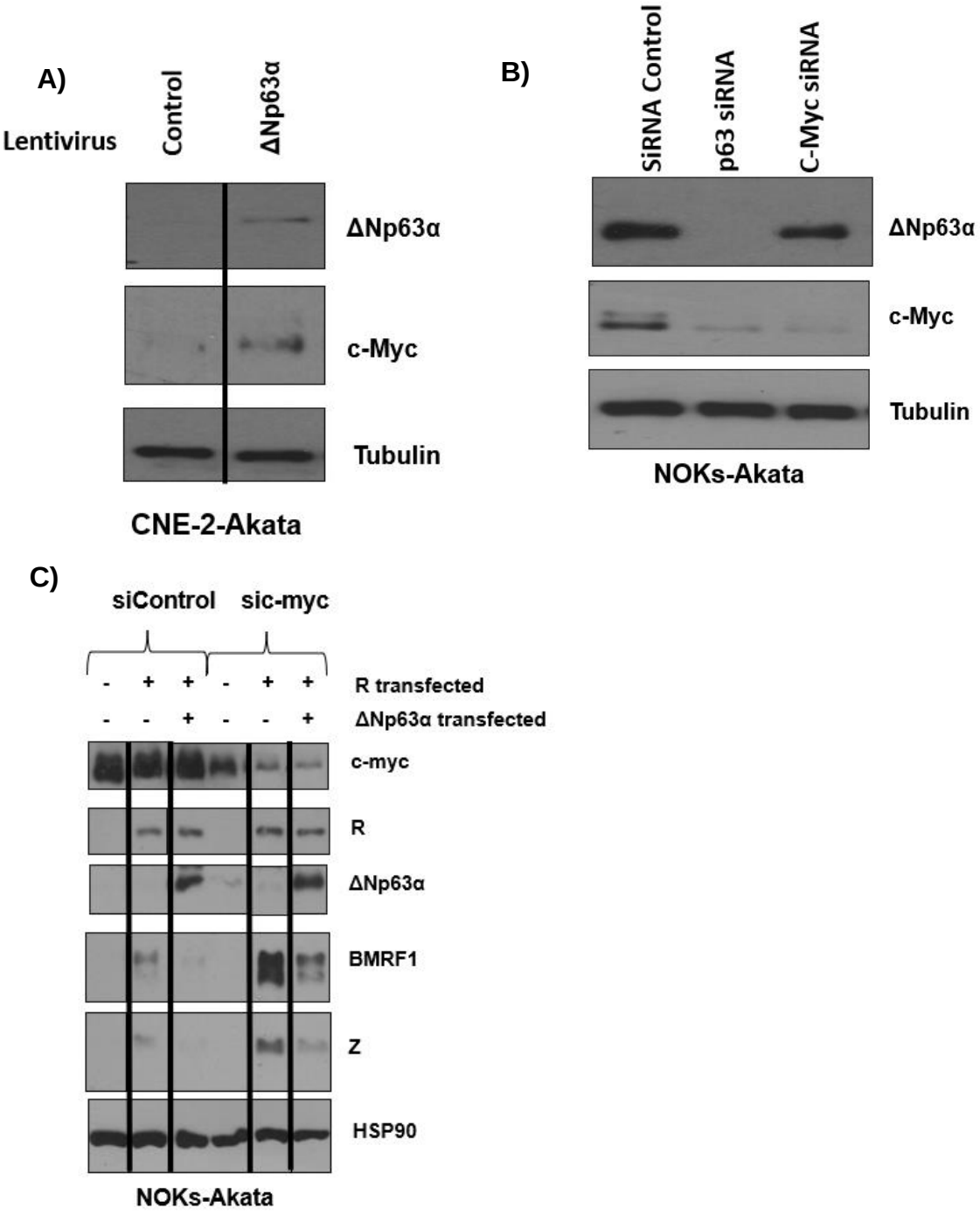

A)
Lentivirus
Control
ΔNp63α
ΔNp63α
c-Myc
Tubulin
CNE-2-Akata
B)
SiRNA Control
p63 siRNA
C-Myc siRNA
ΔNp63α
c-Myc
Tubulin
NOKs-Akata
C)
siControl
sic-myc
R transfected
ΔNp63α transfected
c-myc
R
ΔNp63α
BMRF1
Z
HSP90
NOKs-Akata

Figure 10: ΔNp63α expression increases the expression of the lytic repressor c-myc, but c-myc is not required for ΔNp63α mediated repression of lytic reactivation. A) CNE-2-Akata cells were infected with a ΔNp63α expressing lentivirus or empty control. After five days of puromycin selection, the cells were harvested and an immunoblot performed to assess expression of the ΔNp63α, c-myc and tubulin proteins. **B)** NOKs-Akata cells were transfected with either ΔNp63α or c-myc targeting siRNAs or a control nonspecific siRNA. Two days after siRNA transfection, the cells were harvested for an immunoblot to determine the expression levels of ΔNp63α, c-myc, and tubulin (as a loading control). **C)** NOKs-Akata cells were transfected with siRNAs against c-myc or a control siRNA for two days, and then transfected with or without an R expression vector in the presence or absence of ΔNp63α. Immunoblot was performed one day later to examine expression of c-myc, ΔNp63α, R, BMRF1, Z, and tubulin proteins. Black lines indicate where irrelevant lanes in the blot were removed; the original western blots used to construct this figure are shown in supplemental figure 5.

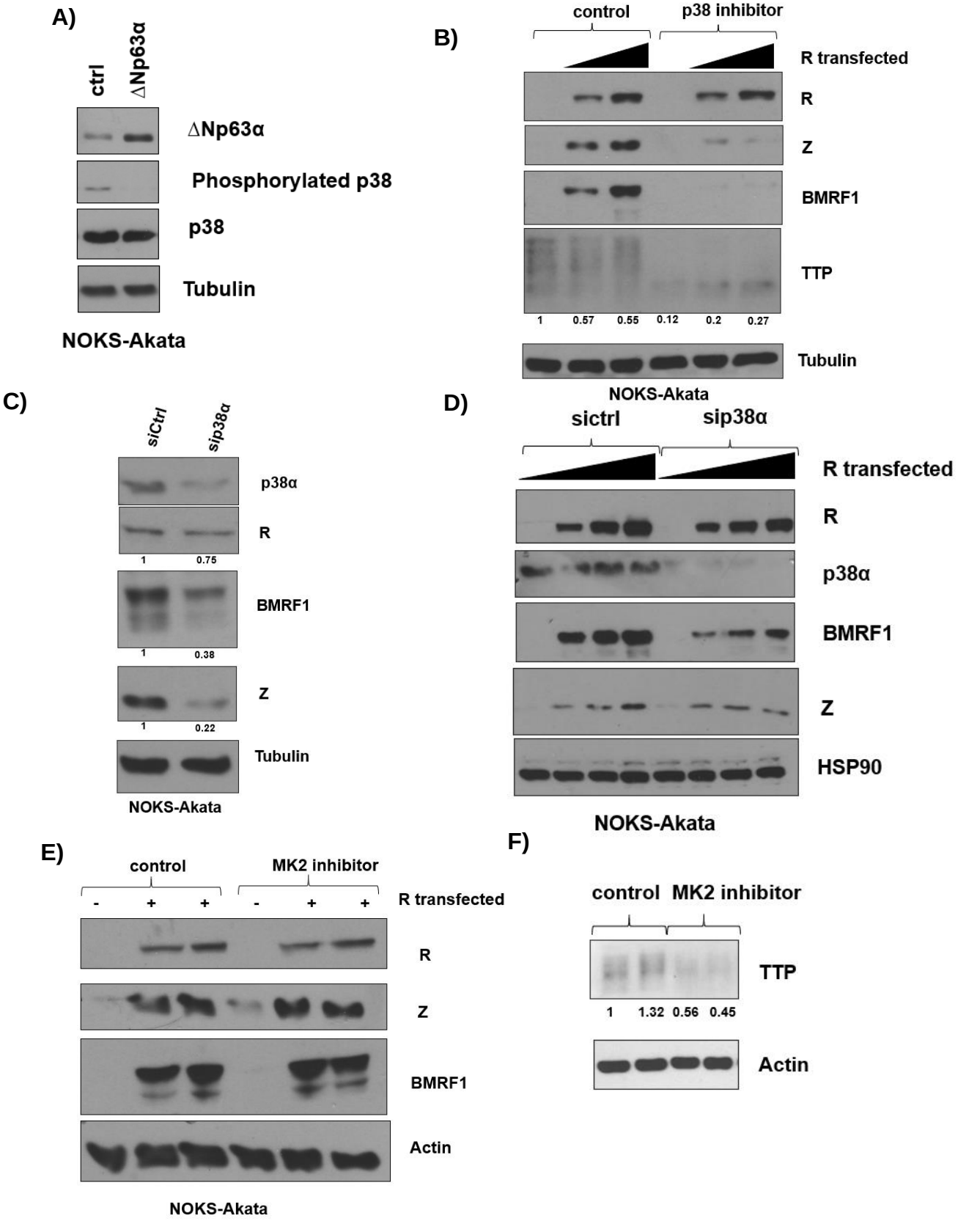

A)
ctrl
ΔNp63α
ΔNp63α
Phosphorylated p38
p38
Tubulin
NOKS-Akata
B)
control
p38 inhibitor
R transfected
R
Z
BMRF1
TTP
1 0.57 0.55 0.12 0.2 0.27
Tubulin
NOKS-Akata
C)
siCtrl
sip38α
p38α
R
1 0.75
BMRF1
1 0.38
Z
1 0.22
Tubulin
NOKS-Akata
D)
sictrl
sip38α
R transfected
R
p38α
BMRF1
Z
HSP90
NOKS-Akata
E)
control
MK2 inhibitor
- + + - + +
R transfected
R
Z
BMRF1
Actin
NOKS-Akata
F)
control MK2 inhibitor
TTP
1 1.32 0.56 0.45
Actin

Figure 11: ∆Np63α overexpression decreases p38 kinase activity, which is required for R mediated lytic reactivation. A) Immunoblot analysis was performed on protein lysates harvested from NOKS-Akata cells infected with a lentivirus vector expressing ∆Np63α, or a control lentivirus vector, and expression of ∆Np63α, phosphorylated (activated) p38, total p38 alpha (MAPK14), and tubulin (a loading control) was assessed. **B)** NOKS-Akata cells were transfected with or without an R expression vector in the presence or absence of 10 μM p38 MAPK inhibitor SB202190. Immunoblot analysis was performed two days later to examine expression levels of Z, R, BMRF1, TTP and tubulin as indicated. Expression of TTP was quantitated with ImageJ, normalized to tubulin with the first lane set as 1. **C)** NOKs-Akata cells were transfected with either a control siRNA or an siRNA targeting p38 alpha (MAPK14), and two days later immunoblot analysis was performed to examine expression of p38 alpha (MAPK14), R, BMRF1, Z, and tubulin as indicated. Quantitation of R, BMRF1 and Z expression was determined through ImageJ, normalized to tubulin, and the siRNA control sample was set as 1. **D)** NOKs-Akata cells were transfected with either a control siRNA or an siRNA targeting p38 alpha (MAPK14) and then transfected with or without an R expression vector one day later. Immunoblots were performed one day after R transfection to examine expression of p38 alpha (MAPK14), Z, R, BMRF1, and tubulin. **E)** NOKS-Akata cells were transfected with or without an R expression vector, in the presence or absence of 10 μM MK2 (MAPKAPK2) inhibitor (PF-364402 hyrdate). Two days later immunoblot analysis was performed to examine expression of the Z, R, BMRF1, and actin proteins as indicated. **F)** NOKS-Akata cells treated with 10 μM MK2 (MAPKAPK2) inhibitor (same extracts used in figure E) were also examined for expression of TTP (ZFP36) and tubulin as indicated. Quantitation of TTP expression was determined through ImageJ, normalized to tubulin, and the first control sample was set as 1.

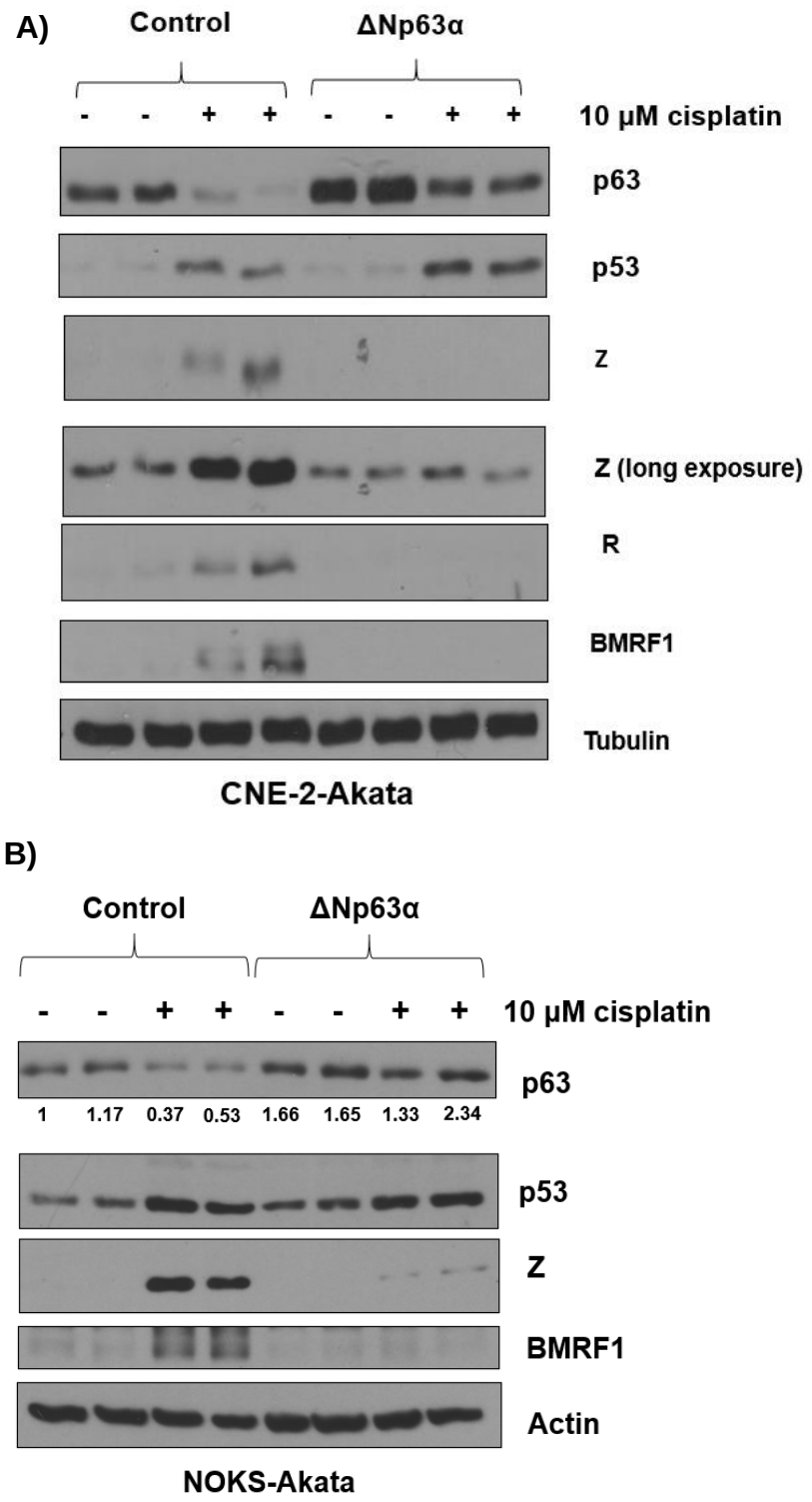
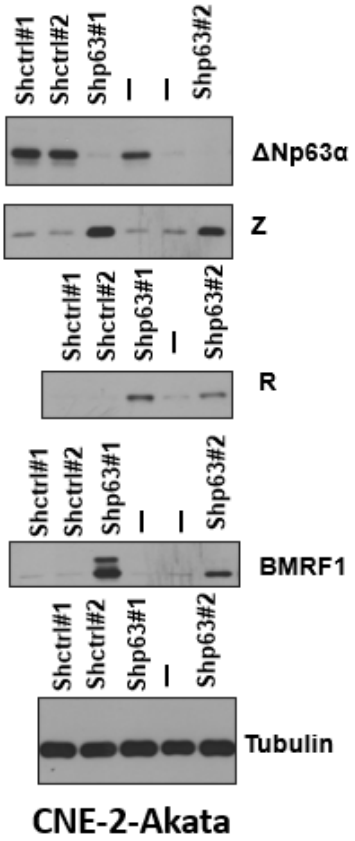


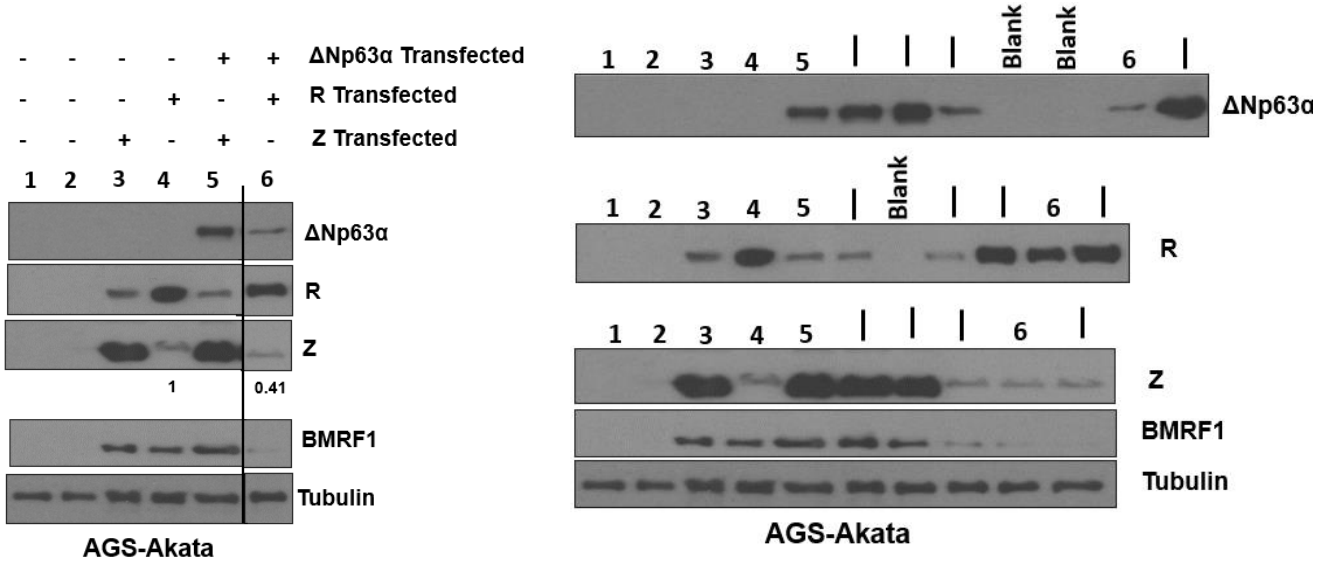
Figure 12: Cisplatin mediated lytic reactivation is curtailed during ΔNp63α overexpression. A) CNE-2-Akata cells infected with a lentivirus expressing ΔNp63α, or a

control lentivirus, were treated with or without 10 μM cis-platinum and then examined by immunoblot analysis two days later for expression of p63, p53, Z, R, BMRF1, and the loading control tubulin. **B)** NOKs-Akata cells infected with a lentivirus expressing ΔNp63α, or a control lentivirus, were treated with or without 10 μM cis-platinum and then examined by immunoblot analysis two days later for expression of p63, p53, Z, BMRF1, and the loading control actin. The expression of p63 was quantified using ImageJ, normalized to tubulin, with the first control treated sample set as 1.

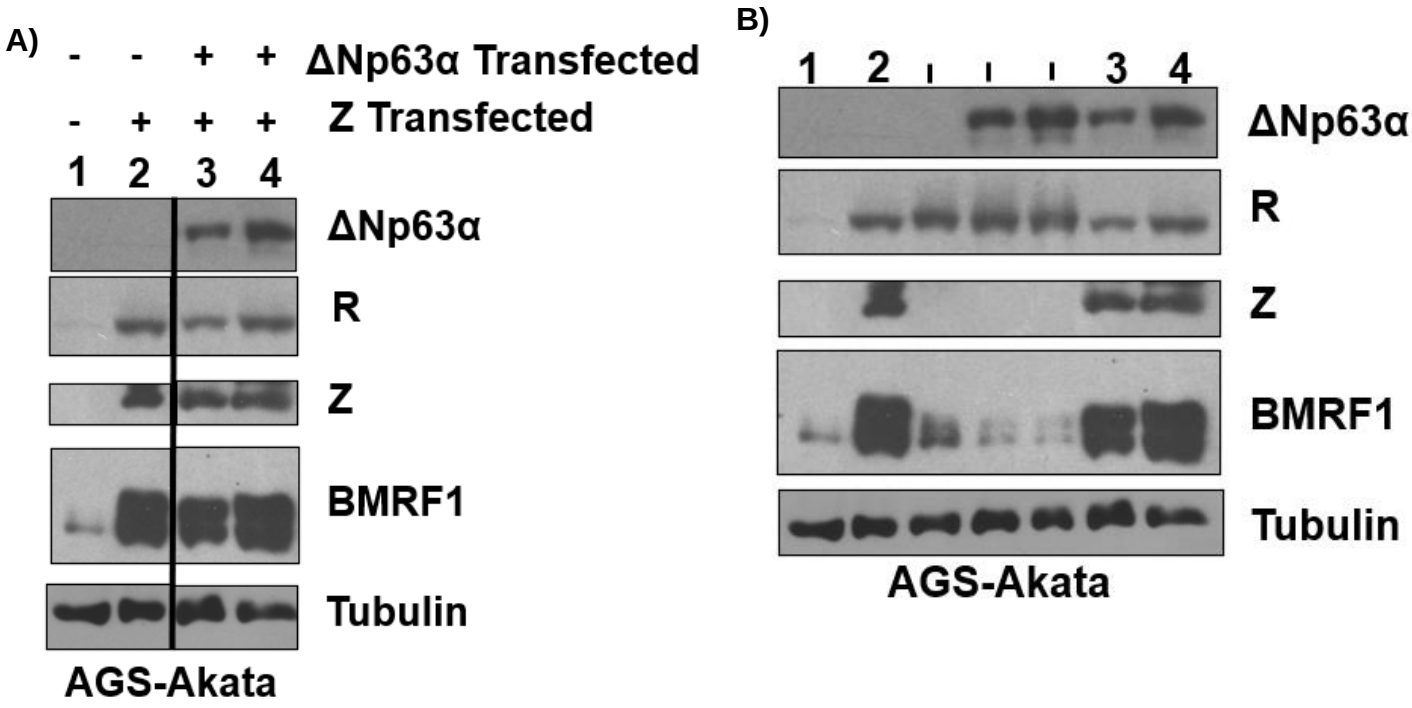
Supplemental Figure Legends



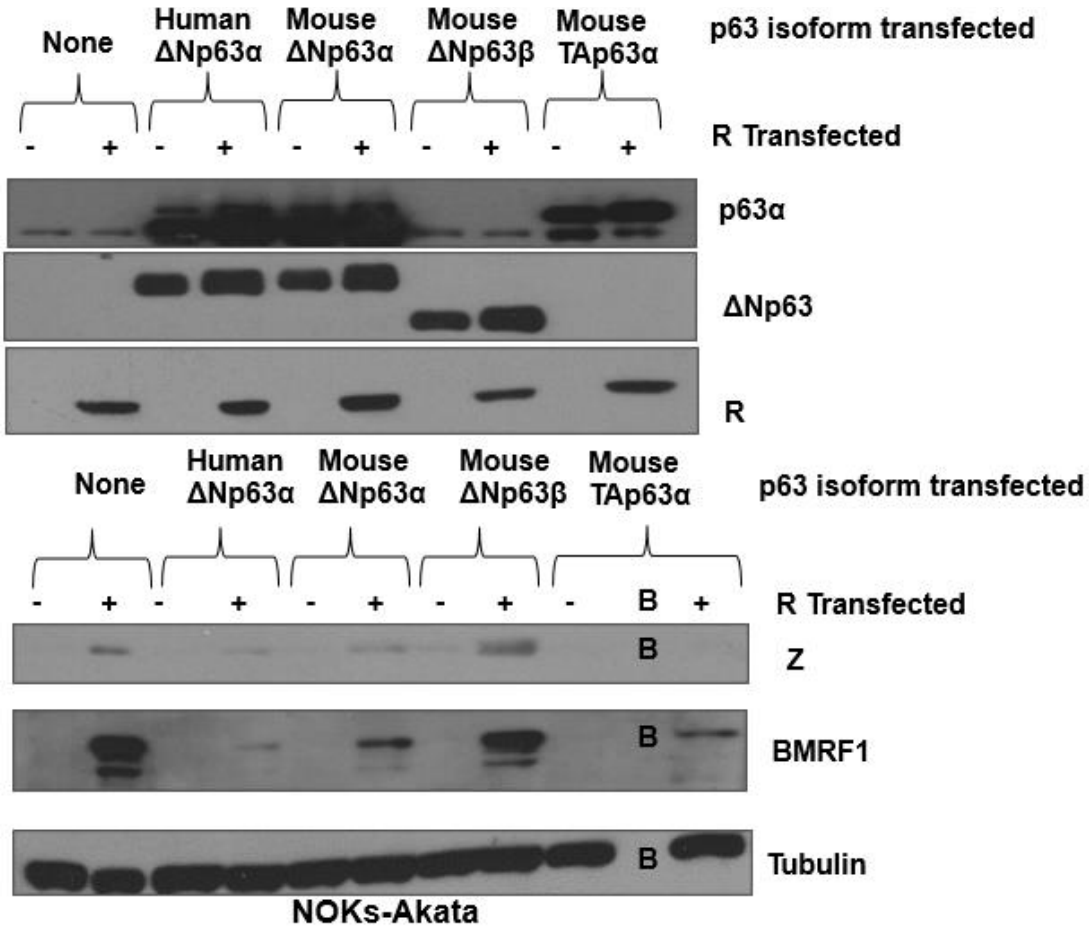
Sup. Figure 1. The original western blots used to construct Figure 2A are shown. The lanes used in the blot shown in figure 2A are labelled. Lanes not used in the final figure are indicated by a “l” symbol.



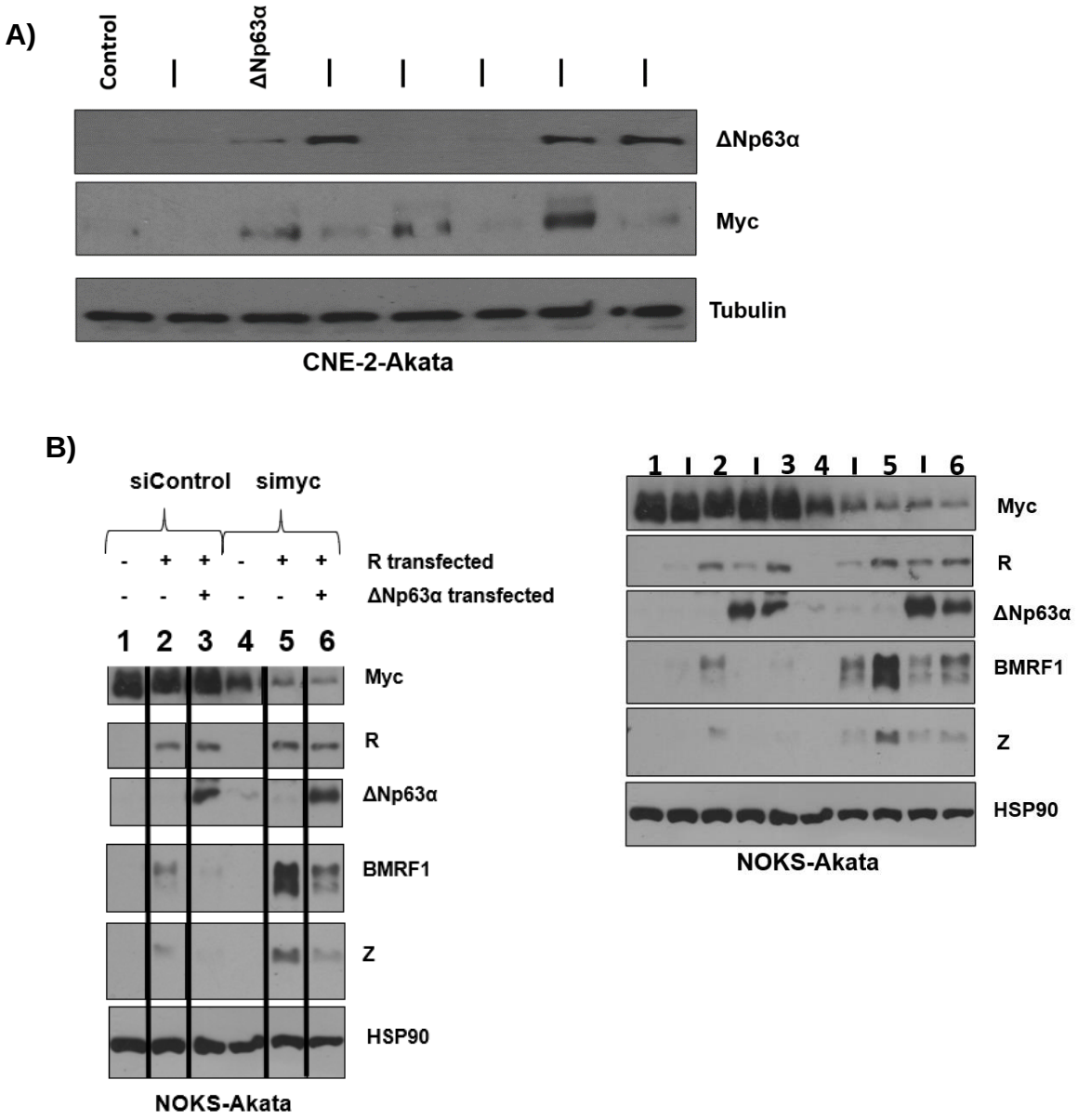
Sup Figure 2. The original western blots used to construct Figure 4A are shown in the right panel. The lanes used in the blot shown in figure 4A are labelled 1-6 as indicated. Lanes not used in the final figure are indicated by a "I" symbol. Different western blots were used to derive the ΔNp63α and R expression levels whereas the same three western blots were used to derive the Z, BMRF1, and tubulin expression levels. "Blank" refers to lanes where no protein was loaded. Note that the same protein lysates were used in each of the western blots shown.



Supplemental Figure 3. A) AGS-Akata cells were transfected with a vector control or Z expression vector in the presence of absence of ΔNp63α expression vector, as indicated. Western blots were performed to examine the expression level of transfected Z and ΔNp63α proteins, R, BMRF1, and tubulin. **B)** The original western blots used to generate the supplemental figure 3A are shown; lanes are numbered to indicate their position in the figure A. Lanes not used are indicated by an "I".



Sup Figure 4 The original western blots used to construct Figure 7 are shown. The lanes used in the blot shown in figure 7 are labelled. Different western blots were used to derive the ΔNp63α, p63 alpha and R expression levels, and another western blot was used to derive the Z, BMRF1, and tubulin expression levels. “Blank” (B) refers to lanes where no protein was loaded. Note that the same protein lysates were used in each of the western blots shown.



Supplemental Figure 5. A) The original western blots used to construct Figure 10A are shown. The lanes used in the blot shown in figure 10A are labelled. Lanes not used in the final figure are indicated by a "I" symbol. **B)** The original western blots used to construct Figure 10C are shown on the right panel, along with Figure 10C on the left panel with lanes number 1 through 6. The lanes used in the original blots shown in figure 10C are numbered 1 through 6. Lanes not used in the final figure are indicated by a "I" symbol.

Chapter 3

Hippo signaling effectors YAP and TAZ induce Epstein-Barr virus (EBV) lytic reactivation through TEADs in epithelial cells

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Abstract

The Epstein-Barr virus (EBV) human herpesvirus is associated with B-cell and epithelial-cell malignancies, and both the latent and lytic forms of viral infection contribute to the development of EBV-associated tumors. Here we show that the Hippo signaling effectors, YAP and TAZ, promote lytic EBV reactivation in epithelial cells. The transcriptional co-activators YAP/TAZ (which are inhibited by Hippo signaling) interact with DNA-binding proteins, particularly TEADs, to induce transcription. We demonstrate that depletion of either YAP or TAZ inhibits the ability of phorbol ester (TPA) treatment, cellular differentiation or the EBV BRLF1 immediate-early (IE) protein to induce lytic EBV reactivation in oral keratinocytes, and show that over-expression of constitutively active forms of YAP and TAZ reactivate lytic EBV infection in conjunction with TEAD family members. Mechanistically, we find that YAP and TAZ interact with, and activate, the EBV BZLF1 immediate-early promoter**.** Furthermore, we demonstrate that YAP, TAZ, and TEAD family members are expressed at much higher levels in epithelial cell lines in comparison to B-cell lines, and find that EBV infection of oral keratinocytes increases the level of activated (dephosphorylated) YAP and TAZ. Finally, we have discovered that lysophosphatidic acid (LPA), a known YAP/TAZ activator that plays an important role in inflammation, induces EBV lytic reactivation in epithelial cells through a YAP/TAZ dependent mechanism. Together these results establish that YAP/TAZ are powerful inducers of the lytic form of EBV infection and suggest that the ability of EBV to enter latency in B cells at least partially reflects the extremely low levels of YAP/TAZ and TEADs in this cell type.

Author summary

EBV causes infectious mononucleosis and persists in latently infected B cells for life. The virus periodically reactivates to a lytic form that produces infectious virus particles, allowing the virus to infect new cells and be transmitted to new hosts. Oral epithelial cells are an important site of lytic viral infection, promoting secretion of infectious virus into saliva. Rarely, EBV infection results in B-cell and epithelial-cell human tumors, and both the latent and lytic forms of infection contribute to these malignancies. However, the viral and cellular factors that determine whether the virus remains latent or lytic are still incompletely understood. Here we have discovered that the Hippo signaling pathway is an important regulator of lytic EBV reactivation, particularly in epithelial cells. We show that transcriptional co-activators YAP and TAZ (which are turned off by Hippo signaling) are strong inducers of lytic EBV reactivation, collaborating with DNA-binding TEAD proteins to activate EBV immediate-early genes. Furthermore, we demonstrate that epithelial cells express much higher levels of YAP/TAZ/TEADs in comparison to B cells, helping to explain why B cells support latent infection. Finally, we find that lysophosphatidic acid, a YAP/TAZ activator, induces lytic reactivation through a YAP/TAZ-dependent mechanism.

Introduction

Epstein-Barr virus (EBV) is a gamma herpesvirus that causes the clinical syndrome, infectious mononucleosis, and infects over 90% of the human population. EBV primarily infects B cells and oropharyngeal epithelial cells. While the vast majority of EBV-infected individuals experience no additional symptoms after recovery from the initial viral infection, a minority of individuals go on to develop EBV-associated B-cell and epithelial-cell cancers such as Burkitt lymphoma (BL), Hodgkin lymphoma, nasopharyngeal carcinoma (NPC), and gastric carcinoma (1–4).

Like all herpesviruses, EBV can infect host cells in either latent or lytic forms. During latency, EBV expresses relatively few genes, and the latency proteins produced enable the viral genome to persist and the infected cell to survive. However, once lytic replication is initiated EBV's full gene complement is expressed, and infectious virions are produced (5,6). EBV persists in the memory B-cell population in a tightly latent form for the life of the host, but can periodically reactivate to the lytic form of viral infection when B cells are stimulated by antigen and/or differentiate into plasma cells. In contrast, EBV-infected epithelial cells in the oropharynx generally undergo lytic replication and shed virus into the saliva (7–9).

Worldwide, the number of EBV-infected epithelial cell tumors greatly exceeds the number of EBV-infected B cell tumors (3). The underlying molecular mechanisms that control EBV lytic reactivation, particularly in epithelial cells, remain incompletely characterized. Although excessive lytic EBV infection may play an important early role in promoting EBV-induced tumors by increasing the total number of EBV-infected cells, fully formed tumors are largely maintained by latent EBV infection (10–14). Indeed, suppression of excessive lytic EBV infection (which generally kills the host cell) is likely required for the development of these tumors. Therefore, a better understanding in regard to mechanisms by which cellular and viral factors regulate the latent-to-lytic EBV switch at different time points during tumor development

may provide insights for preventing or controlling these malignancies. For example, inducing EBV lytic replication in latently infected tumor cells has been proposed as a method for specifically blocking the growth of EBV-infected malignancies (15–17).

EBV switches from the latent to the lytic form of infection when cellular transcription factors activate the EBV immediate-early (IE) promoters, BZLF1 (Zp) and BRLF1 (Rp) (6,18–20). The BZLF1 (Z) and BRLF1 (R) IE proteins are viral transcription factors that collaboratively induce expression of the EBV early lytic genes that encode proteins required for lytic viral replication (6,21–23). Following viral DNA replication, expression of the EBV late lytic genes, which encode structural viral proteins, occurs, allowing release of infectious virion particles. Z preferentially binds and activates promoters with methylated DNA, whereas R preferentially activates promoters with unmethylated (or 5-hydroxymethylated) DNA (24–26). Z expression efficiently initiates lytic EBV reactivation in B cell lines (in which the viral genome becomes highly methylated), while R expression is required for initiation of lytic reactivation in the EBV-infected telomerase-immortalized oral keratinocyte (NOKs) cell line, in which the viral genome remains hypomethylated (6,24,27,28). Regardless of cell type, once expressed the Z and R proteins activate each other's promoters, and both proteins are required for expression of most lytic viral genes and viral genome replication.

A number of different cellular stimuli (including cellular differentiation, hypoxia, B cell receptor engagement, DNA damage and caspase activation) can initiate lytic EBV infection (18–20,29–34), while other factors (including CAF1, HIRA, myc, ZEB 1 and ZEB2) can inhibit it (35–38). Two master regulators of stratified epithelial cell differentiation, KLF4 and BLIMP1, synergistically induce lytic EBV reactivation in differentiated cells by collaboratively activating the Zp and Rp IE promoters (18,19). Nevertheless, lytic EBV infection also occurs in a small subset of undifferentiated NPC tumor cells and may even contribute to the development of this cancer type (10). Two recent studies examining EBV infection of pseudostratified primary

nasopharyngeal respiratory epithelial cells grown in air-liquid interface culture (which may be a good model for early NPC lesions) found predominantly latent viral infection in the undifferentiated basal epithelial cell layer, and predominantly lytic infection in suprabasal layers (39,40). However, there was no obvious association between high level KLF4 and BLIMP1 expression and lytic EBV gene expression in this model (40), and some lytic gene expression was found in the undifferentiated basal cells (39). Thus, the cellular transcription factors important for inducing lytic EBV infection in undifferentiated and differentiated epithelial cells have not yet been fully defined.

Yes-associated protein (YAP) and its paralog TAZ (also known as WWTR1) are the Hippo signaling pathway's transcriptional effector genes. YAP and TAZ are considered oncogenes and are overexpressed or amplified in numerous epithelial cancers (41–45). Nevertheless, the effects of YAP and TAZ are highly tissue-specific and context-dependent, as in some cases YAP and TAZ have been reported to enhance cellular differentiation (46–48). The tumor suppressor Hippo signaling pathway negatively regulates YAP/TAZ function via activation of the LATS1/2 kinases, which phosphorylate YAP/TAZ at multiple sites (41,49–52), allowing YAP/TAZ to interact with 14-3-3 proteins (sequestering YAP/TAZ in the cytoplasm) and be degraded by the proteasome (41,49,53). YAP/TAZ dephosphorylation, which can be induced by many different types of stimuli, results in localization of these proteins to the nucleus, where YAP/TAZ can activate gene expression. YAP and TAZ do not contain a DNA-binding domain and require a DNA-bound cofactor (most commonly one of the four TEAD family members) to activate gene expression (46,54–56). Transcription factors such as p73, ErbB4, KLF4, SMAD 2/3 and MRTF can also facilitate YAP/TAZ transcriptional activities (46,53–55,57–61).

Here we show that both YAP and TAZ are potent activators of the EBV lytic cascade. We find that loss of constitutive YAP or TAZ expression reduces differentiation-induced or phorbol ester-induced lytic EBV reactivation in epithelial cell lines and inhibits BRLF1-mediated

reactivation in EBV-infected NOKs cells. Conversely, we show that over-expression of either constitutively active YAP or TAZ induces lytic reactivation in EBV-infected epithelial cell lines and demonstrate that YAP and TAZ cooperate with TEAD family members to activate the Z IE promoter. Furthermore, we find that YAP, TAZ and TEAD proteins are expressed at high levels in epithelial cell lines, but not B-cell lines, and show that EBV infection enhances YAP/TAZ activity in NOKs cells. Finally, we have discovered that lysophosphatidic acid (LPA), a known YAP/TAZ activator that plays an important role in inflammation, induces EBV lytic reactivation in epithelial cells through a YAP/TAZ dependent mechanism. These results reveal that YAP and TAZ are novel inducers of lytic EBV reactivation in epithelial cells.

Results

YAP expression is essential for constitutive lytic protein expression in an EBV-infected AGS gastric carcinoma cell line. Since we previously showed that AGS gastric carcinoma cells stably infected with the Akata EBV strain (AGS-Akata cells) have an unusually high level of lytic infection (62), and gastric carcinomas (including AGS) often express constitutively active YAP (63,64), we asked whether YAP expression is required for constitutive lytic EBV protein expression in these cells. AGS-Akata cells were treated with control siRNA or siRNA targeting YAP and the level of various lytic EBV proteins was examined two days later using immunoblot analysis. As shown in Fig. 1, knockdown of YAP expression resulted in decreased expression of the Z and R immediate-early lytic EBV proteins as well as the BMRF1 early lytic protein. A similar result was obtained in another experiment (supplemental Fig. 1). These results suggest that activated YAP is required for efficient lytic EBV protein expression in AGS-Akata cells.

YAP and TAZ are both required for the ability of the phorbol ester, TPA, to induce EBV lytic reactivation in NOKs-Akata cells. The phorbol ester TPA is a well-characterized inducer of EBV lytic reactivation, and has been previously shown to induce YAP dephosphorylation and activation in a cell type-dependent manner by dephosphorylating LATS and inhibiting their function (65–67). To determine if YAP and/or TAZ is required for TPA to induce lytic reactivation of EBV-infected NOKs cells, NOKs cells stably infected with Akata strain EBV (NOKs-Akata cells) were treated for 24 hours with control siRNAs or siRNAs targeting YAP and/or TAZ, and then treated with or without TPA for another 24 hours before harvesting cells for immunoblot analysis. As shown in Figs. 2A and 2B**,** knockdown of either YAP or TAZ decreased the ability of TPA to induce expression of the EBV Z, R, and BMRF1 proteins. We confirmed that YAP and TAZ were depleted in their respective conditions as expected, although in some cases knockdown of TAZ also reduced YAP expression to a lesser extent (suggesting that TAZ may

enhance YAP expression in these cells). Interestingly, depletion of either YAP or TAZ alone was as effective at inhibiting TPA-induced lytic reactivation in NOKs cells as YAP/TAZ double knockdown (Fig. 2B). Similar results were obtained in another experiment (Supplemental figure 2). These results demonstrate that the ability of TPA to induce lytic EBV reactivation in NOKs cells requires both YAP and TAZ, suggesting that YAP and TAZ each contribute independently to TPA-induced lytic reactivation.

YAP and TAZ are both required for differentiation-induced EBV lytic reactivation in NOKs cells. We next asked if both YAP and TAZ are required for the ability of EBV to lytically reactivate during epithelial cell differentiation. NOKs-Akata cells were treated for 24 hours with either control siRNA or siRNAs directed against YAP or TAZ, plated on collagen treated membranes for another 24 hours, and then transfected again with the various siRNAs and lifted to the air-liquid interface to induce differentiation before harvesting 48 hours later for immunoblot analysis. As shown in Fig. 3, NOKs-Akata cells treated with control siRNA differentiated (as determined by expression of the differentiation proteins involucrin and BLIMP1) and lytically reactivated (as determined by expression of the lytic EBV proteins, Z, R and BMRF1). NOKs-Akata cells treated with YAP siRNA or TAZ siRNA had decreased expression of the lytic EBV proteins Z, R, and BMRF1 (Fig. 3A, 3B), although expression of the differentiation markers involucrin and BLIMP1 was not altered. These results suggest that YAP and TAZ also facilitate EBV lytic reactivation during epithelial cell differentiation although they are not required for differentiation of this cell type per se.

BRLF1-mediated disruption of latency in NOKs-Akata cells also requires YAP and TAZ expression. We previously showed that over-expression of the R IE protein, but not the Z IE protein, can initiate EBV lytic reactivation in NOKs-Akata cells (24,25). During R-mediated viral reactivation, R initially activates expression of the Z IE gene, and then the R and Z proteins together collaboratively induce expression of the early lytic EBV genes. To assess if YAP and/or

TAZ facilitate R induction of lytic reactivation, we treated NOKs-Akata cells with either YAP, TAZ or control siRNAs, and transfected cells 24 hours later with an R expression vector or empty vector control, and then performed immunoblot analysis at 24 hours after R transfection. We found that the depletion of either YAP or TAZ did not affect the level of transfected R protein, but greatly reduced the ability of R to induce the expression of the Z or BMRF1 lytic proteins (Fig. 4A). Similar results were obtained in other experiments (supplemental Fig. 3). Since induction of BMRF1 early lytic gene expression requires both the Z and R proteins, these results suggest that YAP and TAZ are required for efficient R-mediated activation of the Z IE promoter. Consistent with this, we found that YAP and TAZ knockdown did not significantly inhibit the ability of transfected Z and R proteins together to induce BMRF1 expression (Fig. 4 B, C and supplemental Fig. 3).

Constitutively activated YAP and TAZ are sufficient to induce EBV lytic reactivation in epithelial cell lines. We next asked if over-expression of constitutively active forms of YAP and/or TAZ is sufficient to induce lytic EBV reactivation in epithelial cells. AGS-Akata cells were transfected with expression vectors for constitutively active forms of YAP (YAP(5SA)) or TAZ (TAZ (S89A)) that are each missing specific serine residues that inhibit YAP and TAZ nuclear translocation when phosphorylated by LATS1/2 (51,52,54,55). As shown in Fig. 5A and 5B, both YAP(5SA) and TAZ(S89A) strongly induced expression of the EBV immediate-early and early lytic proteins Z, R, and BMRF1, and the late lytic viral protein VCA-p18, compared to the vector control. Similar results were found in NOKs-Akata cells (Fig. 5C). Both YAP(5SA) and TAZ(S89A) also induced expression of the EBV lytic proteins Z, R, and BMRF1 in the EBV-infected SNU-719 gastric carcinoma cell line (which was EBV positive in the original patient tumor and has remained EBV-positive in culture (68)(Fig. 5D and supplemental Fig. 4A). These findings indicate that YAP and TAZ (when they are not phosphorylated by LATS and thus are

transcriptionally active) are indeed sufficient to induce EBV lytic reactivation in both gastric and oral epithelial cell types.

YAP cooperates with TEAD family members to induce lytic EBV reactivation in epithelial cells. YAP and TAZ commonly cooperate with TEAD family members to activate target genes, as both YAP and TAZ lack a DNA binding domain and cannot access the DNA without a DNA-binding partner (54,55,59,69). To determine if YAP cooperates with TEADs to induce EBV lytic reactivation, we transfected NOKs-Akata cells with either the constitutively active YAP(5SA) vector or a mutant form of this vector (YAP(5SA-S94A)) that specifically prevents YAP from interacting with TEAD family members (54). We found that the YAP(5SA-S94A) protein is deficient in inducing lytic EBV reactivation in comparison to the YAP(5SA) vector (Fig. 6A and supplemental Fig. 5A).

To determine if over-expression of TEADs synergizes with YAP to induce lytic EBV reactivation, we transfected HONE-Akata cells with the constitutively active YAP vector in the presence or absence of co-transfected expression vectors for TEAD1 or TEAD2. We found that while YAP induces EBV lytic gene expression by itself, the lytic inducing effect is much stronger when YAP is co-transfected with either TEAD1 or TEAD2 expression vectors (Fig. 6B and supplemental Fig. 5B). These results suggest that YAP and TEADs collaborate to activate lytic EBV protein expression.

YAP and TAZ induce Z IE promoter activity. To determine if YAP and/or TAZ can activate the Z (Zp) or R (Rp) IE promoters in reporter gene assays, we transfected HeLa cells with Zp (Zp-346) or Rp (Rp-1068) promoter constructs (driving luciferase gene expression) in the presence of absence of co-transfected constitutively active YAP or TAZ expression vectors and performed luciferase assays. Co-transfection with the TAZ(S89A) vector induced the activity of the Zp-346 promoter, and to a lesser extent the Rp-1068 promoter, while not affecting the negative control Zp-83 promoter vector. Co-transfection with the YAP(5SA) vector also activated the Zp-346

promoter construct (although the effect was weaker than that of the TAZ vector) but did not activate the Rp or Zp-83 promoters (Fig. 7A). In contrast, YAP and TAZ did not significantly activate the early lytic BMRF1 EBV promoter in EBV-negative cells (Fig. 7B). As previously reported by our group, the combination of co-transfected KLF4 and BLIMP1 also potently induced activation of both the Z and R promoters (18,19)(Fig. 7A). These results indicate that TAZ, and to a lesser extent YAP, activates the Z promoter in EBV-negative cells.

YAP and TAZ activate the Z IE promoter via TEAD binding motifs. To further define the Zp promoter sequences required for YAP and TAZ activation *in vitro*, we compared the ability of co-transfected YAP and TAZ to activate a series of 5 ‘deletions of the Zp-346 promoter construct as depicted in Fig. 7C. Examination of this region of Zp suggested three potential TEAD motifs between -218 and -266. As shown in Fig. 7D, removal of two of the three TEAD motifs in the Zp-226 construct decreased YAP and TAZ activation by approximately 50%, and removal of all three TEAD motifs in the Zp-218 construct eliminated the ability of YAP and TAZ to activate Zp. In contrast, removal of the TEAD motifs did not inhibit the ability of co-transfected BLIMP1 to activate Zp (Fig. 7E). These results suggest that both YAP and TAZ activate the Zp promoter though TEAD binding motifs. Since these TEAD binding motifs are located near to the Rp promoter (within about 2000 basepairs downstream of the transcription start site) in the intact EBV genome, they may also serve to mediate YAP/TAZ activation of Rp transcription in the context of the intact viral genome.

YAP and TAZ are complexed to the EBV immediate-early gene region *in vivo*. To determine if YAP and/or TAZ can associate with the EBV genome IE region in EBV-infected cells *in vivo*, we performed ChIP assays in HONE-Akata cells co-transfected with TEAD1 and FLAG-tagged YAP(5SA) or FLAG-tagged TAZ(S89A) vectors, or control vectors, and then performed qPCR assays to assess YAP or TAZ association with various regions of the EBV genome. Both YAP and TAZ were found to be preferentially associated with the EBV Zp

sequence compared to other regions of the EBV genome examined (including the R and BMRF1 lytic promoter sequences and the latent Cp promoter sequence) (Fig. 8A, 8B). Transfected myc-tagged TEAD1 protein also preferentially associated with the EBV Zp sequence (compared to three other negative control regions of the EBV genome that lack local putative TEAD motifs) in ChIP assays (Fig. 8C). These results suggest that YAP/TAZ are complexed to TEAD motifs in the YAP-responsive region of the EBV BZLF1 IE promoter *in vivo* and that direct TEAD binding to Zp is at least partially responsible for its ability to reactivate lytic EBV infection. Nevertheless, since the binding of the YAP/TAZ/TEAD proteins to Zp is somewhat weak, we cannot totally exclude the possibility that YAP/TAZ/TEAD also indirectly activate Zp by binding/activating a promoter driving an as yet unknown cellular transcription factor that activates Zp.

YAP, TAZ, and TEADs are expressed at much higher levels in EBV-positive and EBV-negative epithelial cell lines in comparison to EBV-positive B cell lines. We next performed immunoblot analyses to assess the levels of total YAP and TAZ expression in various EBV-infected and uninfected epithelial cell lines. As shown in Figs. 9A, 9B, and 9C, although the ratio of YAP versus TAZ expression varied among the different cell lines, YAP and/or TAZ were found to be expressed in every epithelial cell line examined, including EBV-positive and EBV-negative AGS gastric carcinoma cells, EBV-infected SNU-719 gastric carcinoma cells, EBV-positive and EBV-negative NOKs cells, and EBV-positive CNE and HONE cells (cell lines originally thought to be NPCs but which are primarily composed of HeLa cells)(70). EBV-infected 293 cells (a cell line currently thought to be derived from fetal mesenchymal stem cells) (48) also expressed some YAP and TAZ. EBV infection did not alter the total level of YAP or TAZ in either the AGS or NOKs cell lines.

We next examined the levels of total YAP and TAZ in four different EBV-infected B-cell lines, including two type 1 EBV-transformed lymphoblastoid cell lines (“Mutu” and “Akata”), two

type 2 EBV-transformed lymphoblastoid cell lines (“AG876” and “BL5”) and one EBV-infected Burkitt lymphoma line (Akata BL) (Figs. 9A and 9B). Interestingly, we found that none of the EBV-infected B cell lines express detectable YAP protein, and only a subset of the LCL lines express detectable TAZ. These results suggest that protein expression levels of YAP (and to some degree TAZ) are very cell type dependent and are very low in EBV-infected B cells.

We also compared the expression levels of TEADs in various different epithelial cell lines versus different B cell lines using a pan-TEADs antibody that detects all four TEAD family members. As shown in Figs. 9A, B, and C, we found that TEAD protein(s) are expressed in all epithelial cell lines surveyed (including EBV-infected NOKs, uninfected AGS cells, EBV-infected HONE and CNE cells, and EBV-infected SNU-719 cells). In contrast, we did not detect TEAD protein expression in any of the B cell lines, including three different EBV-negative Burkitt lymphoma lines (Daudi, Mutu and Akata), three different EBV-positive Burkitt lines (Akata, Mutu I and P3HR1) and four different EBV-transformed lymphoblastoid cell lines (Mutu, Akata, AG876 and BL5). These results indicate that specific signals that induce YAP/TAZ activity and lytic EBV reactivation in EBV-infected epithelial cells are unlikely to do so in EBV-infected B cells due to the generally extremely low levels of YAP, TEAD (and often TAZ) expression in this cell type.

The combination of over-expressed TEADs, YAP and TAZ is sufficient to induce EBV lytic reactivation in an EBV-infected B cell line. We next investigated if YAP or TAZ over-expression, with or without co-transfected TEAD proteins, is sufficient to induce EBV lytic reactivation in a B-cell environment that does not normally express these transcriptional effectors. EBV+ Akata Burkitt lymphoma cells were transfected with YAP(5SA) and/or TAZ(S89A) expression vectors, with or without a TEAD1 expression vector and the levels of EBV lytic proteins, BZLF1 and BMRF1, was examined by immunoblot analysis 24 hours later. In the absence of co-transfected TEAD1, neither YAP(5SA) nor TAZ(S89A) expression vectors could induce lytic EBV protein expression (Fig. 10 and supplemental Fig. 6). However, when

either the YAP or TAZ vectors were co-transfected with the TEAD1 expression vector, lytic EBV protein expression was induced (Fig. 10 and supplemental Fig. 6). These findings further confirm that YAP and TAZ cooperate with TEADs to induce lytic EBV reactivation and suggest that EBV-infected B cells are not intrinsically resistant to the lytic-inducing effects of these transcription factors, assuming they are expressed.

EBV infection increases YAP/TAZ activity in NOKs cells. To determine if the presence of EBV in epithelial cells affects the state of YAP or TAZ activation, we also compared the amount of activated (dephosphorylated) versus inactivated (phosphorylated) YAP and TAZ in EBV-infected versus uninfected NOKs cells. For these assays, cells were plated at sub-confluent density and grown in the absence of growth factors. Immunoblots were performed using antibodies that recognize total YAP or TAZ, versus antibodies that recognize inactivated YAP (phosphorylated at S127) or inactivated TAZ (phosphorylated at S89). We also examined the levels of phosphorylated (active) versus total LATS1 protein. As shown in Fig. 11, the levels of YAP and TAZ phosphorylation were decreased in EBV-infected versus uninfected NOKs, suggesting that EBV infection increases YAP and TAZ activity in NOKs cells. Similar results were obtained in another experiment (supplemental Fig. 7). Interestingly, however, as shown in Fig. 11, we did not find that EBV infection of NOKs cells affected the level of LATS1 phosphorylation (a modification which increases LATS1 function and thus leads to decreased YAP/TAZ phosphorylation), suggesting that EBV acts to inhibit YAP/TAZ phosphorylation through some other mechanism.

Lysophosphatidic acid, a YAP/TAZ activator, induces lytic EBV reactivation through a YAP/TAZ-dependent mechanism. Lysophosphatidic acid (LPA), which is released by a variety of different cell types during inflammatory responses, binds to several different G-protein coupled receptors and induces YAP/TAZ activation by inhibiting LATS1/2 kinases (71,72). To determine if LPA can induce lytic viral reactivation in EBV-infected NOKs in a YAP/TAZ-

dependent manner, cells were treated for one day with control siRNA or siRNAs targeting YAP or TAZ and then treated with or without 10 μM LPA for another 24 hours. As shown in Fig. 12A and supplemental Fig. 8, immunoblot analysis of the various conditions revealed that LPA treatment does indeed reactivate lytic EBV protein expression in NOKs-Akata cells, and that this effect is reduced when either YAP or TAZ expression is inhibited by siRNAs. We also confirmed that treatment of NOKs-Akata cells with LPA reduces the phosphorylation of YAP at serine 127, consistent with the previously described ability of LPA to activate YAP by inhibiting its phosphorylation (Fig. 12B). In contrast, LPA does not induce lytic EBV reactivation in Burkitt Akata B cells (Fig. 12C, 12D), which as shown in Fig. 9 do not express TEAD, YAP or TAZ proteins. These results are the first to show that LPA induces EBV lytic reactivation in epithelial cells, and that this effect is mediated through activated YAP and TAZ. Thus, LPA is likely to be a biologically relevant stimulus by which YAP/TAZ activation can result in lytic EBV reactivation in humans.

Discussion

EBV infection of B cells is generally latent, while infection of differentiated epithelial cells results in lytic infection. Stratified squamous oropharyngeal epithelial cells are a major site of lytic EBV infection in humans (7,9,73,74) and “rafted” oral keratinocytes provide a good *in vitro* system for modeling lytic “oral hairy leukoplakia” lesions that occur in differentiated tongue cells of immunosuppressed patients. We previously showed that differentiation-dependent expression of the cellular KLF4 and BLIMP1 transcription factors induces lytic EBV infection in stratified squamous oral epithelial cells by activating the Z and R IE EBV promoters (18,19). EBV infection of pseudostratified respiratory nasopharyngeal epithelial cells (grown in air-liquid interface culture) also preferentially supports lytic EBV infection in differentiated suprabasal cells (39,40), although in this model system lytic EBV infection is not highly associated with either KLF4 or BLIMP1 expression (40) and some lytic gene expression occurs even in the undifferentiated basal cells (39). Here we demonstrate that the Hippo effectors YAP and TAZ cooperate with TEADs to induce Z IE promoter activity and lytic EBV reactivation in epithelial cells, and show that the lack of YAP/TAZ/TEAD expression in B cells likely contributes to EBV latency in this cell type. Furthermore, we find that LPA, a phospholipid that activates YAP/TAZ function, promotes lytic EBV reactivation in epithelial cells via a YAP- and TAZ- dependent mechanism. Given the very high level of LPA in saliva, LPA may be a biologically important factor that serves to enhance lytic EBV infection in oropharyngeal epithelial cells independent of their differentiation state (75).

The differences in YAP, TAZ, and TEAD expression in epithelial cells versus B cells points to a previously unappreciated mechanism promoting lytic EBV infection in epithelial cells and latent viral infection in B cells (1,5,30). In support of our results here, results presented in the Human Protein Atlas database indicate that RNA transcripts of YAP, TAZ, and the four TEAD family members (TEAD1, TEAD2, TEAD3, and TEAD4) are not detected in the EBV-

infected Burkitt lymphoma cell line, Daudi (76). Furthermore, the RNA transcripts of YAP, TEAD1, TEAD3, and TEAD4 are expressed at only extremely low levels in normal B cells in peripheral blood, although there is some low-level expression of TAZ and TEAD2 in normal B cells (76). Further studies will be required to determine if EBV-encoded latency proteins further repress the already low levels of TAZ/TEAD2 protein expression in EBV-infected B cells, or whether certain stimuli can increase YAP/TAZ/TEAD expression in B cells and contribute to viral reactivation in this cell type.

The effects of activated YAP and TAZ are complex and very context- and cell type - dependent. While YAP and TAZ are known to induce cellular proliferation and support tumor formation in some contexts, there is emerging literature indicating that YAP and TAZ can also be essential for driving cellular differentiation in other contexts (46–48). For example, the adenovirus E1A protein was recently shown to inhibit differentiation of 293 HEK cells into fibroblasts by sequestering YAP and TAZ in an inactive form in the cytoplasm (48). In the case of oral epithelial cells, YAP and TAZ are reported to be expressed at highest levels in the undifferentiated basal cell layer and are thought to inhibit cellular differentiation (77–81). Our finding here that YAP and TAZ are required for the ability of two different epithelial cell differentiation-inducing stimuli (collagen treated membrane/air-liquid interface culture and TPA treatment) to induce maximal lytic protein expression in EBV-infected epithelial cells (Figures 2 and 3) is thus somewhat paradoxical. However, since we did not observe any alterations in the ability of our NOKs cells to differentiate when YAP or TAZ was depleted with siRNAs (Figure 3), the ability of YAP and TAZ to induce lytic EBV protein expression appears to be distinct from their effects on epithelial cell differentiation. A similar paradox is the ability of ROCK inhibitor to prevent epithelial cell differentiation (81,82), even though ROCKs are required to induce YAP/TAZ activation in response to a number of different stimuli such as LPA**,** TGF-β, mechanotransduction and stress fiber formation(71,84–87). Our results are consistent with a

model in which low levels of active YAP/TAZ promote the initiation of lytic EBV reactivation in undifferentiated basal cell epithelium while not completely preventing epithelial cell differentiation.

YAP and TAZ do not bind to and activate target gene promoters independently and thus require DNA-binding co-factors for their transcriptional activity. Previous studies have reported that many co-activating partners, such as Runx2, ErbB4, p73, KLF4, and SMADs, can co-activate YAP and TAZ transcriptional targets, but the best-characterized partners are the four members of the TEAD family (53,54,58,59,88). We determined that TEAD family members are essential for the ability of YAP and TAZ to induce lytic EBV reactivation, since the mutated YAP protein, YAP(5SA-S94A), which specifically cannot interact with TEADs, does not induce lytic reactivation, and co-transfection with a TEAD protein is required for the ability of both YAP and TAZ to induce lytic EBV reactivation in the Akata Burkitt lymphoma line (where YAP/TAZ and TEADs are not endogenously expressed) (Fig.10). Whether other potential mediators of YAP and TAZ transcriptional effects, in particular SMAD2/3 or KLF4, are also involved in their ability to induce lytic EBV reactivation remains to be determined in future studies.

EBV reactivation is initiated by cellular transcription factor-mediated activation of the two EBV immediate-early genes, BZLF1 and BRLF1. We find that the ability of TPA treatment, as well as growth of cells on collagen filters in air-liquid interface culture, to induce lytic EBV reactivation in epithelial cells requires expression of YAP and TAZ. We show that TAZ and YAP can activate the Zp (and to a lesser extent the Rp) in reporter gene assays and mapped a YAP- and TAZ-responsive motif in the Zp construct to several likely TEAD binding motifs located between -218 and -251 relative to the BZLF1 transcriptional initiation site. Since these motifs are within 2000 bp of the BRLF1 transcript start site in the context of the intact viral genome, we speculate that binding of TEADs with TAZ/YAP to this IE gene region in the context of the intact viral genome is sufficient to activate both the Zp and Rp. This is particularly likely to be the case

given our previous finding that Z protein expression alone is not sufficient to induce lytic EBV reactivation in EBV-infected NOKs cells (24), and since we found that knock-down of YAP or TAZ in EBV-infected epithelial cells treated with either TPA or LPA similarly reduced Z and R expression.

Interestingly, we also found that the depletion of YAP and TAZ inhibits the ability of over-expressed transfected R IE protein to induce EBV lytic reactivation in NOKs-Akata cells (Fig. 4A) and noted that this effect was accompanied by a large decrease in R-induced Z expression. Since both Z and R expression are required for induction of many early lytic genes, including the BMRF1 gene, in the context of the intact viral genome, our results suggest that YAP/TAZ may be primarily important for R activation of Zp. Consistent with this model, we found that the ability of the transfected Z and R proteins together to activate BMRF1 expression was not significantly inhibited by TAZ or YAP siRNAs (Figure 4B, 4C). R has been previously proposed to activate Zp indirectly through effects on cellular transcription factors including ATF-2 (89,90). Our results here suggest that R activation of Zp may also be mediated by R modulation of YAP/TAZ activity, or at least require that YAP/TAZ are otherwise activated. Of note, we previously reported that R cannot induce lytic reactivation in EBV-infected B cell lines that do not already have some level of constitutive Z expression (23), which is intriguing given our findings here showing that B cells generally lack expression of TEAD family members and YAP/TAZ. Although we observed decreased YAP/TAZ phosphorylation in EBV-infected NOKs cells relative to the uninfected NOKs cells (suggesting enhanced YAP/YAZ function) (Figure 11), the EBV protein(s) mediating this activation remain to be determined in future studies.

Although YAP did not activate the Zp and Rp as efficiently as TAZ in reporter gene assays, we found that it is similar to TAZ in regard to its ability to reactivate EBV in latently infected cells. Interestingly, our siRNA experiments showed that both YAP and TAZ are individually required for the ability of TPA, R, LPA, and epithelial cell differentiation to induce

lytic EBV reactivation in NOKs cells. Thus, the low level of TAZ (but not YAP) expression in EBV-infected SNU-719 gastric cancer cells (Fig. 9C) may contribute to viral latency in this cell line. YAP and TAZ have around 40% amino acid conservation and have many homologous domains, and activate many of the same genes responsible for organ size, proliferation, and differentiation (46,48,50,57,91–93). However, the cellular functions of YAP/TAZ are context- and tissue type-dependent (94). For example, YAP expression is vital for the differentiation of gut epithelium, whereas TAZ is crucial for the differentiation of airway epithelium after injury (46,47). YAP deletion in mice is embryonic lethal, while mice with a deletion of TAZ are viable (95,96). Furthermore, the ability of 293 HEK cells to differentiate into fibroblasts following knock-down of adenovirus E1A protein was found to require both YAP and TAZ, and the YAP and TAZ regulated cellular genes were found to be only partially over-lapping in this differentiation process (48). It will be interesting in future studies to determine why YAP and TAZ are both required for efficient lytic EBV reactivation in epithelial cells.

YAP, TAZ, and the TEADS are considered oncogenes and are overexpressed or amplified in numerous epithelial cancers (41–45). TEADs are overexpressed 300-fold in Kaposi's Sarcoma, and recent work has shown that lytic KSHV induces YAP expression (97,98). The effect of EBV infection on YAP and TAZ function has not been well characterized in EBV infection, although one group has reported that LMP1 over-expression (outside the context of the viral genome) increases TAZ expression by interacting with the TAZ inhibitor gelsolin (99). Additionally, TAZ has been reported to be expressed in the nuclear compartment of NPC tumor cells, suggesting that TAZ activation may play an oncogenic role during EBV-associated tumorigenesis (99). In this study, we found that EBV-infected NOKs have decreased phosphorylation of YAP serine residue 127 and TAZ serine residue 89 compared to the uninfected NOKs. Since phosphorylation of YAP and TAZ at these sites prevents nuclear YAP/YAZ localization and inhibits cell growth (51), the ability of EBV to inhibit this

phosphorylation in epithelial cells may play a role in the ability of EBV to inhibit epithelial cell differentiation (19,100,101) and/or promote epithelial cell tumors in humans. Although we did not observe a consistent effect of EBV infection in NOKs cells on the phosphorylation of the classic YAP and TAZ inhibitor, LATS1, numerous other mechanisms regulate YAP and TAZ phosphorylation, including various phosphatases such as PR55α that can directly remove these phosphorylation modifications (51,102). Additionally, it is becoming increasingly clear that YAP/TAZ activity can also be activated through mechanisms not involving phosphorylation. For example, nuclear IRF3 and MRTF were recently shown to enhance YAP/TAZ transcriptional activity in a phosphorylation independent manner (60,103).

YAP and TAZ transcriptional activity is regulated by many upstream stimuli such as actin cytoskeleton remodeling, GPCR signaling, receptor tyrosine kinase signaling, cell-to-cell contact, WNT signaling, mechano-transduction, PKC activation, as well as AP-1 activity (67,71,84,85,88,104–106). In this study, we investigated the ability LPA (which activates YAP/TAZ activity by inhibiting LATS1/2 phosphorylation) to induce lytic reactivation in EBV-infected cells. We chose to investigate the effect of LPA in particular since LPA is a biologically relevant stimulus present at very high levels in saliva (75,107). We found that LPA does indeed induce lytic viral reactivation in EBV-infected NOKs cells at physiologically relevant levels, and that this effect requires both YAP and TAZ (Figure 12). Given that EBV is found at very high levels in periodontal lesions (108,109), and that LPA levels are particularly high in these lesions (75), we speculate that LPA may promote the replication of EBV in patients with gingival disease. It will clearly be important in future studies to investigate which of the other multitude of different YAP/TAZ activating pathways, in addition to LPA, can also contribute to lytic EBV reactivation, and in what contexts.

Materials and Methods

Cell Lines

The hTERT-immortalized oral epithelial NOKs cell line (a kind gift from Karl Munger, Tufts University) was derived as previously described (110) and was grown in KSFM supplemented with 0.1 µg epidermal growth factor (EGF) and 12.5 mg bovine pituitary extract (BPE) per 500 ml media. NOKs were infected with the Akata strain of EBV (containing a GFP marker and G418 resistance gene inserted into the viral BXLF1 gene) as previously described (24,111,112). The AGS cell line is an EBV-negative gastric carcinoma cell line that was obtained from the ATCC and was grown in F-12 supplemented with 10% FBS and 1% pen-strep. The AGS-Akata cell line was infected with the Akata strain of EBV as previously described (111). SNU-719 is an authentic EBV-infected gastric carcinoma cell line that was derived as described previously (68), and was maintained in RPMI media with 10% FBS and 1% pen-strep. HeLa is an HPV-positive cervical carcinoma cell line obtained from the ATCC and was maintained in DMEM media supplemented with 10% FBS and 1% pen-strep. EBV-positive and EBV-negative HONE and CNE cells (a gift from Lawrence Young, University of Birmingham) were originally described as EBV-negative nasopharyngeal carcinoma cell lines, but were subsequently shown to be contaminated with HPV-infected HeLa cells (70,113). HONE and CNE cells were maintained in DMEM media supplemented with 10% FBS and 1% Pen-strep. HONE-Akata and CNE-Akata cells (each infected with the Akata strain of EBV) were grown in DMEM media supplemented with 10% FBS, 1% pen-strep and 400 µg/ml G418. The EBV-positive 293 cell line infected with EBV p2089 bacmid was described previously (114) and was grown in DMEM media supplemented with 10% FBS, 1% pen-strep, and maintained with 100 µg/ml hygromycin. The Akata, Mutu, BL5, and AG876 lymphoblastoid cell lines were derived by transforming peripheral B cells with each EBV strain as previously described (115), and were maintained in RPMI with

10% FBS and 1% Pen-strep. Akata-, Mutu-, and Daudi- are EBV-negative Burkitt lymphoma cell lines that were derived as previously described (116,117) (a kind gift from Kenzo Takada of Hokkaido University, Japan via Bill Sugden of the University of Wisconsin). Mutu-I is a Burkitt lymphoma cell line that was originally derived by the Alan Rickinson group at the University of Birmingham, UK, and was a kind gift from Jeff Sample of Penn State University (118). P3HR-1 is a Burkitt lymphoma cell line (119), and was a kind gift from Bill Sugden of the University of Wisconsin. All Burkitt lymphoma cell lines were maintained in RPMI media with 10% FBS and 1% Pen-strep.

Collagen Membrane Differentiation

Approximately $5x10^5$ NOKs-Akata cells were seeded onto a collagen-treated transwell membrane (Corning #3460) with KSFM media on both the basal and apical surfaces of the membrane. When cells seeded on the membrane were 100% confluent, all apical media was removed, and basal media was exchanged for Epilife media (Thermo Fisher #MEPICF500) supplemented with 10% FBS, 1.4mM $CaCl_2$, and 5 µg/ml ascorbic acid. After three days, the membrane cells were harvested with sumo lysis buffer for immunoblot analysis.

siRNAs

siRNAs against YAP (catalogue #sc-38637 and SASI_Hs01_00124477) and TAZ (catalogue #sc-38568B, #sc-38568C) were purchased from Santa Cruz and Millipore-Sigma, respectively. Millipore-Sigma's Universal control (catalog# SIC001-1NMOL) and Santa Cruz siRNA controls A and C (catalog# sc-37007, sc-44231) were used as controls. siRNAs were used at 20 pM with 6 µL of RNAimax transfection reagent, and was delivered according to the RNAiMAX protocol (Invitrogen #13778150). After four hours post-transfection the media was changed to maintain cell viability, and the cells were harvested after two days post-transfection.

DNA Transfection

DNA was transfected into NOKs-Akata, NOKs, HONE-Akata, and AGS-Akata cells using the Lipofectamine 2000 (Thermo Fisher #11668019) system according to the manufacturer's protocol. Generally, 500 ng of DNA total and 1.5 µl of Lipofectamine 2000 was used per condition to transfect epithelial cells that were approximately 70% confluent in a 12-well plate. Akata-Burkitt lymphoma cells were nucleofected using an Amaxa Nucleofector 2b device (Lonza) and program A-016 (with buffer V) with 1 µg of DNA in a 12-well plate. 48 hours after transfection, cells were washed with PBS and harvested with sumo lysis buffer for immunoblot analysis.

Chemical reagents

Lysophosphatidic acid (LPA) was purchased from Thermo-Fisher, suspended in H_2O at 10 mM and used at doses of 10 µM. Phorbol 12-myristate 13-acetate (TPA) was purchased from Sigma, and was suspended in DMSO for use at 20 ng/ml. Control conditions were treated equal amounts of the solvent.

Plasmids

All plasmid DNA was prepared using the Qiagen Maxi-prep kit according to the manufacturer's instructions. The plasmid pSG5 was purchased from Stratagene. The pSG5-R vector contains the EBV BRLF1 gene driven by the SV40 promoter (in pSG5) as previously described and was a gift from Diane Hayward at Johns Hopkins University (22). The pCMV-FLAG YAP(5SA) plasmid (Addgene #27371), pCMV-FLAG YAP(5SA-S94A) plasmid (Addgene #33103), HA-TAZ(S89A) plasmid (Addgene #32840), pRK7-Myc-TEAD1 plasmid (Addgene #33109), and pRK7-Myc-TEAD2 plasmid were all gifts of Kun Liang-Guan at the University of California at San Diego. The 3XFLAG pCMV5-TOPO TAZ (S89A) plasmid (Addgene #24815) was a gift from Jeff Wrana at the University of Toronto. The HA-KLF4 plasmid (Addgene #34593 a gift from Michael Ruppert of West Virginia University) and BLIMP1 expression plasmid (a gift from Ken

Wright of the University of South Florida) were described previously (18,19). The pCpG Zp-83 luciferase, pCpG Zp-346 luciferase, pCpG Rp-1068, and pCpG BMRF1p luciferase expression vectors were all described previously (19).

Construction of 5' Z promoter deletions

5' deletions were inserted into the pCpG Zp -346 luciferase construct using the following primers (with the name of the primer indicating the location in the Z promoter relative to the Zp transcriptional start site): Zp-266 primer ATGAAATCTTGGATACATTTCTAAATGA, Zp-226 primer GCATGCCATGCATATTTCAAC, Zp-218 primer TGCATATTTCAACTGGGCTGTCT. The primer TCGTCCAAATGCTGCAGG was the luciferase vector primer.

Immunoblots

Immunoblots were conducted as previously described (120). In brief, cellular extracts were harvested in sumo lysis buffer, and then proteins were run through a 10% polyacrylamide gel at 150 volts. Transfers to nitrocellulose were either done at 70 minutes at 100 volts or overnight at 22 volts. Once transfers were complete, a ponceau S stain was performed to assess transfer quality. 5% milk in wash buffer (1X PBS and .1% Tween-20) was used to block for an hour. Once blocking was completed, primary antibodies in either 5% milk or bovine serum albumin were added for either an hour or overnight depending on antibody requirements. After incubation, primary antibodies were removed and the membrane was washed 3 x 5 minutes before adding a secondary antibody for a period of one hour. The membrane was then washed again for 3 x 10 minutes before adding ECL (Thermo-Fisher) and imaging.

Immunoblot analysis antibodies

Anti-YAP/TAZ (D24E4) dual antibodies were used at 1:1000 (Cell Signaling Technologies, catalog# 8418S), anti-YAP (D8H1X) antibody used at 1:1000 (Cell Signaling Technologies catalog# 14074T), anti-phosphorylated YAP S127 at 1:1000 (Cell Signaling Technologies,

catalog# 4911), anti-TAZ (Novus, catalog# NB110-58359), anti-phosphorylated TAZ S89 (E1X9C) at 1:1000 (Cell Signaling Technologies, catalog# 59971), anti-BZLF1 antibody was used at 1:500 (Santa Cruz, catalog# sc-53904), anti-BRLF1 antibody at 1:2000 (isolated from rabbits injected with peptide sequence EDPDEETSSQAVKALREMAD), anti-BMRF1 antibody at 1:2000 (Millipore-Sigma, catalog# MAB8186), anti-LATS1 antibody at 1:1000 (Cell Signaling Technologies, catalog# 9153), anti-phosphorylated LATS S909 at 1:1000 (Cell Signaling Technologies catalog# 9157), PAN-TEAD antibodies at 1:1000 (Cell Signaling Technologies catalog# 13295S), anti-tubulin antibody at 1:4000 (Sigma, catalog# T5168), anti-β actin antibody at 1:5000 (Sigma catalog# 5441), and anti-HSP90 (F8) at 1:1000 (Santa Cruz catalog# sc-13119). The secondary antibodies used were Horseradish peroxide (HRP)- labeled goat anti-mouse antibody at 1:5000 (Thermo Scientific catalog# 31430), HRP- labeled donkey anti-goat antibody (Santa Cruz catalog# sc-2056, 1:5000), and HRP- labeled anti-rabbit antibody (Fisher Scientific catalog# 31460 1:10000).

Luciferase Reporter Assays

Luciferase reporter assays were conducted as previously described(19). 48 hours after transfection cells were washed once with PBS, suspended in 200 µl of 1X reporter lysis buffer (Promega) and then flash-frozen once. After pelleting by centrifugation and removal of the supernatant to a new tube, luciferase assays were performed according to the manufacturer's instruction using a BD Monolight 3010 luminometer (BD Biosciences). All experiments were done in triplicate and repeated at least twice in separate experiments.

ChIP assays

ChIP assays were performed as described previously (19). For TEAD and YAP ChIP assays, HONE-Akata cells (using three 10cm dishes per condition) were transfected with a pcDNA empty vector control or pCMV-FLAG -YAP(5SA) plus myc-TEAD1 expression vectors. For the

TAZ ChIP assays, HONE-Akata cells were transfected with the 3XFLAG-TOPO TAZ(S89A) and myc-TEAD1 expression vectors or pcDNA empty vector control. One day post-transfection, the cells were fixed with 1% paraformaldehyde for 10 minutes. After this period, the fixing reaction was quenched with 125 mM glycine for 5 minutes. The cells were then pelleted by centrifugation at 2000 rpm for 10 minutes, washed with PBS, and then spun again at 2000 rpm for 10 minutes. The cells were then harvested with cell lysis buffer (10mM Tris pH 8.0, 10mM NaCl, 0.2% NP40) with protease inhibitors (cOmplete, Roche) and left on ice for 10 minutes. The supernatant was then removed, nuclei lysis buffer (50mM Tris-HC pH 8.0, 10mM EDTA pH 8.0, 1% SDS) with protease inhibitors was added and the mixture was left on ice for 10 minutes before storing at -80C overnight. The samples were then diluted in IP dilution buffer (20mM Tris-HCl pH 8.0, 2mM EDTA, 150mM NaCl, 1% Triton X100, and 0.01% SDS) and sonicated 4x30 seconds at 10 watts, with 90 seconds in between sonication (Fisher Scientific, Sonic Dismembrator Model 100). The samples were then blocked with magnetic A/G beads (Thermo-Fisher, 88802) for one hour. After blocking, immunoprecipitation was done with M2 FLAG (Sigma-Aldrich, M8823-1ml) beads overnight as directed by their protocol. For the myc-TEAD1 ChIP, immunoprecipitation was performed with myc-tag antibody (Cell Signaling Technologies catalog #2276 1:100) that was incubated overnight with the samples, and then mixed with the with magnetic A/G beads for two hours. The samples were then washed with low salt, high salt, and lithium chloride washes for 15 minutes each, followed by two 15-minute washes with $T_{10}E$ buffer. Samples then had elution buffer added to elute the DNA from the beads twice. Reverse cross-linking with 0.3M NaCl for 4 hours at 65C. RNase A and proteinase K were added to all samples. Sample DNA was purified with phenol-chloroform, precipitated with EtOH overnight, and resuspended in $T_{10}E$ for real-time PCR.

Real Time PCR (qPCR)

Real-time quantitative PCR (qPCR) was conducted as previously described (19). To briefly summarize, qPCR was conducted with an ABI Prism 7000 Sequence Detector with SYBR Green. All PCR reactions were done in a 96- well plate in either duplicate or triplicate by adding 2.5 µL sample, and 12.5 µL of SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA). The final concentration of primers was 0.3 µmol/L in a final volume of 25 µL. The PCR amplification protocol began at 50°C for 2 minutes followed by 10 minutes at 95°C and 40 PCR cycles consisting of 15 seconds at 95°C followed by 60°C for 1 minute. To ensure no genomic DNA contamination, each reaction contained an internal control, and separate water samples were run in tandem.

qPCR Primers

Primers used for qPCR were as follows: Z promoter ChIP forward: ATGGCATGCAGCAGACATTCATC, Z promoter ChIP reverse: AACACTAGAGTCCATGACAGAGGA, R promoter ChIP forward TGCCGGCTGACATGGATTACT, R promoter ChIP reverse GATGCTGATGCAGAGTCGCC. BMRF1 promoter ChIP forward: CACTGCGGTGGAGGTAGAG, BMRF1 promoter ChIP reverse: GGTGGTGTGCCATACAAGG, C promoter forward: GCCGTGGGAAAAAATTTATGG, C promoter reverse: CGCCAACAAGGTTCAATTTTCT, negative control (NC) 1 - Forward (134221-134240: CACAGCTGCGTCTAGCCTTC)/ Reverse (134327-134346: AGTACAGCCGGTCGTAGTCA), NC2 - Forward (62159-62178: TTCGCCGCGTTAAAAGCGTA)/ Reverse (62221-62239: GCTGGTGGCCGACACTTAT), NC3 - Forward (13387-13406: CAAGGGCGCCAGCTTTTCTC)/ Reverse (13440-13460: TGGGAGGCTGGACTTTACAGA); the negative control primers are named to reflect their locations in the Akata EBV genome KC207813.1.

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Figures

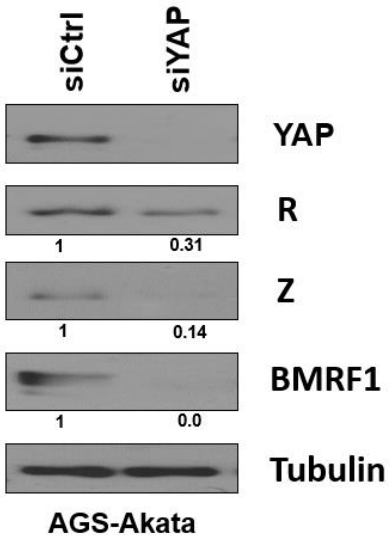


Fig 1: Depletion of YAP decreases constitutive lytic protein expression in AGS-Akata cells. Pooled siRNAs targeting YAP or a control sequence were transfected into AGS-Akata cells. Cells were harvested after two days and immunoblotted for Z, R, BMRF1, YAP and tubulin (loading control). Results were quantitated using ImageJ software; results were normalized to the tubulin result for each condition, with the siRNA control result set as 1.

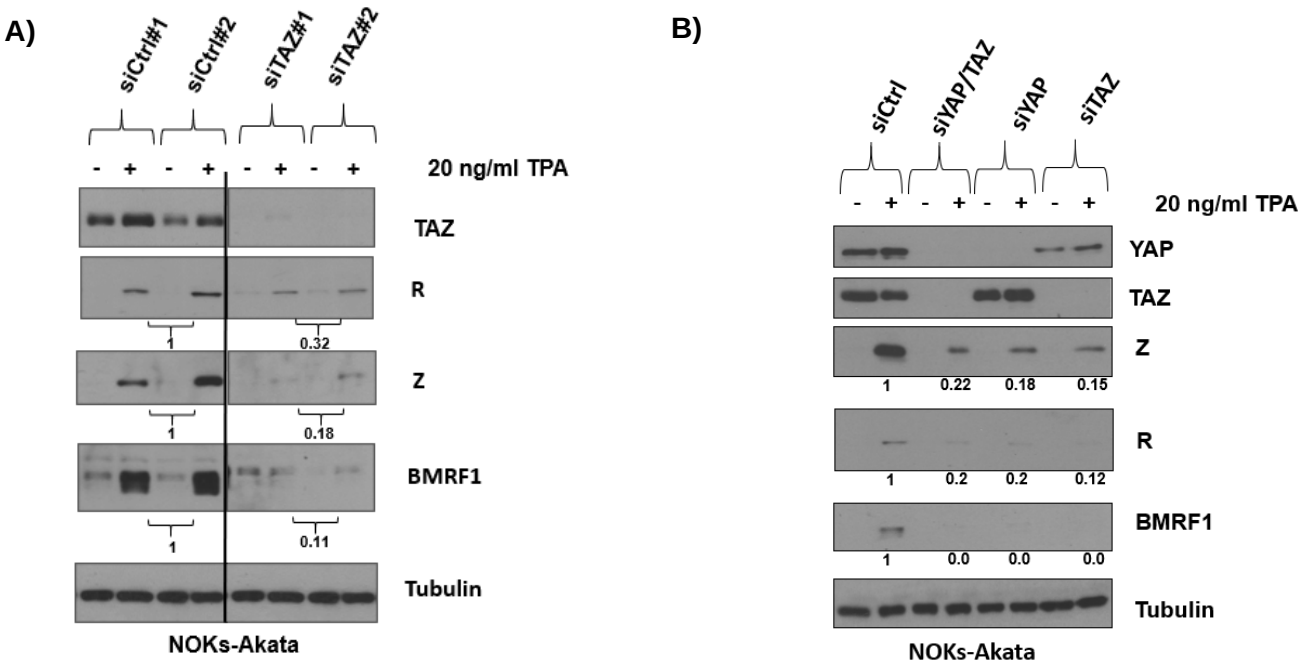


Fig 2: YAP and TAZ expression are both essential for efficient TPA-induced EBV lytic reactivation in NOKs cells. NOKs-Akata cells were treated with either **A)** 20 pM of two different control siRNAs, or 20 pM of two different siRNAs against TAZ or **B)** 20 pM of control siRNA, or 10 pM of YAP and 10 pM TAZ pooled siRNAs (combined), or 20 pM pooled siRNAs against either YAP or TAZ. 24 hours post-siRNA treatment, the cells were dosed with 20 ng/ml TPA. After a subsequent 24 hours the cells were harvested for immunoblots, where the expression of YAP, TAZ, Z, R and BMRF1 was determined. Tubulin was used as a loading control. In figure A, results were quantitated using ImageJ software, with the results for each set of siRNAs averaged and then normalized to the tubulin result for each condition. The average control siRNA result was set as 1. In figure B, quantitated results were normalized to the tubulin result for each condition, and the siRNA control result was set as 1. Black line indicates where irrelevant lanes in the western blot were removed.

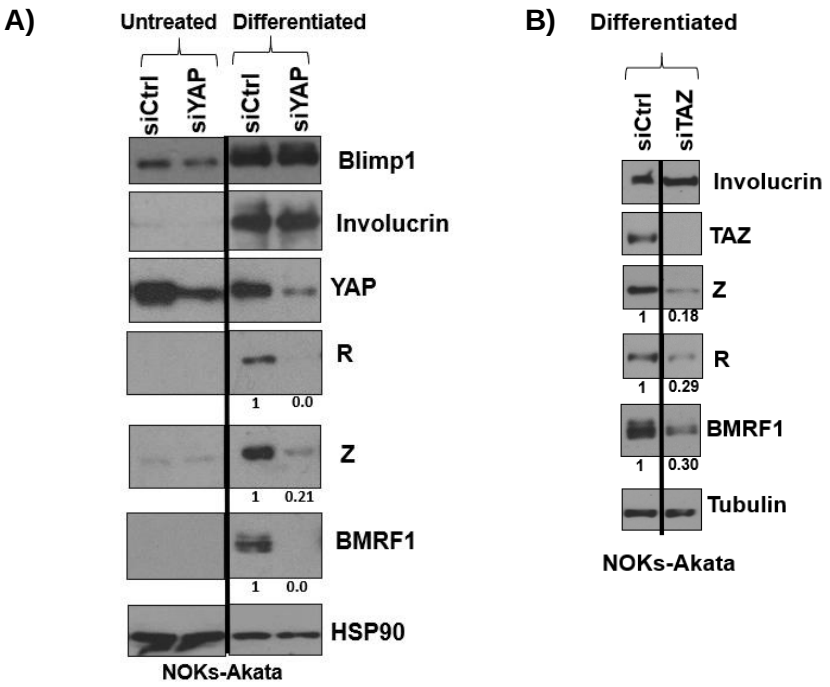


Fig 3: Depletion of YAP or TAZ inhibits EBV lytic reactivation during epithelial cell differentiation. NOKs-Akata cells were transfected with pooled siRNAs targeting YAP or a control siRNA **A)** or **B)** transfected with pooled siRNAs targeting TAZ or a control siRNA. One day after transfection the cells were plated onto type I and type III collagen treated membranes, where they were transfected again with the same siRNAs. The cells were then lifted to the air-liquid interface where they remained for two days before harvesting for an immunoblot to determine the expression levels of BLIMP1, involucrin, YAP, TAZ, Z, R, BMRF1, HSP90 or tubulin as a loading control as indicated. Results were quantitated using ImageJ software and normalized to the loading control result for each condition. The siRNA control result was set as 1. Black lines indicate where irrelevant lanes in the western blot were removed.

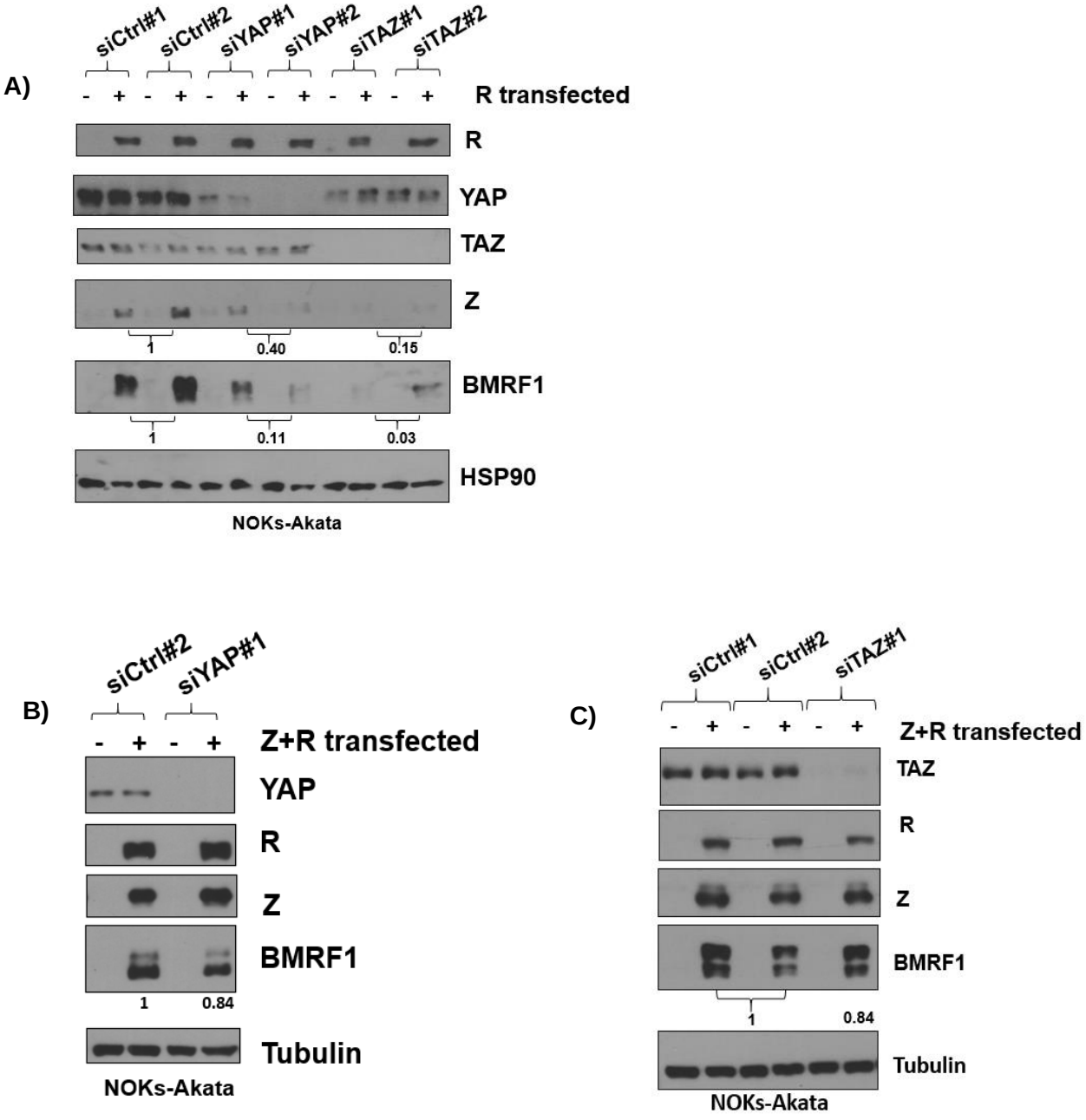


Fig 4: Efficient R-induced lytic reactivation requires expression of YAP and TAZ. A) NOKS-Akata cells were transfected with control siRNA or siRNA against YAP or TAZ, and then 24 hours later cells were transfected with an R expression vector or control vector. After another 24 hours an immunoblot was performed to assess the levels of R, YAP, TAZ, Z, BMRF1, and

HSP90. **B**) NOKS-Akata cells were transfected with control siRNA or siRNA against YAP, and 24 hours later were transfected with both Z and R expression vectors. A western blot was performed to determine expression levels of YAP, Z, R, BMRF1 and tubulin. **C**) NOKS-Akata cells were transfected with control siRNA or siRNAs against TAZ, and were then transfected 24 hours later with Z and R expression vectors. The cells were harvested after 24 hours for western blot analysis to detect expression of TAZ, R, Z, BMRF1, and tubulin. Results were quantitated using ImageJ software and normalized to the loading control result for each condition. The siRNA control result was set as 1.

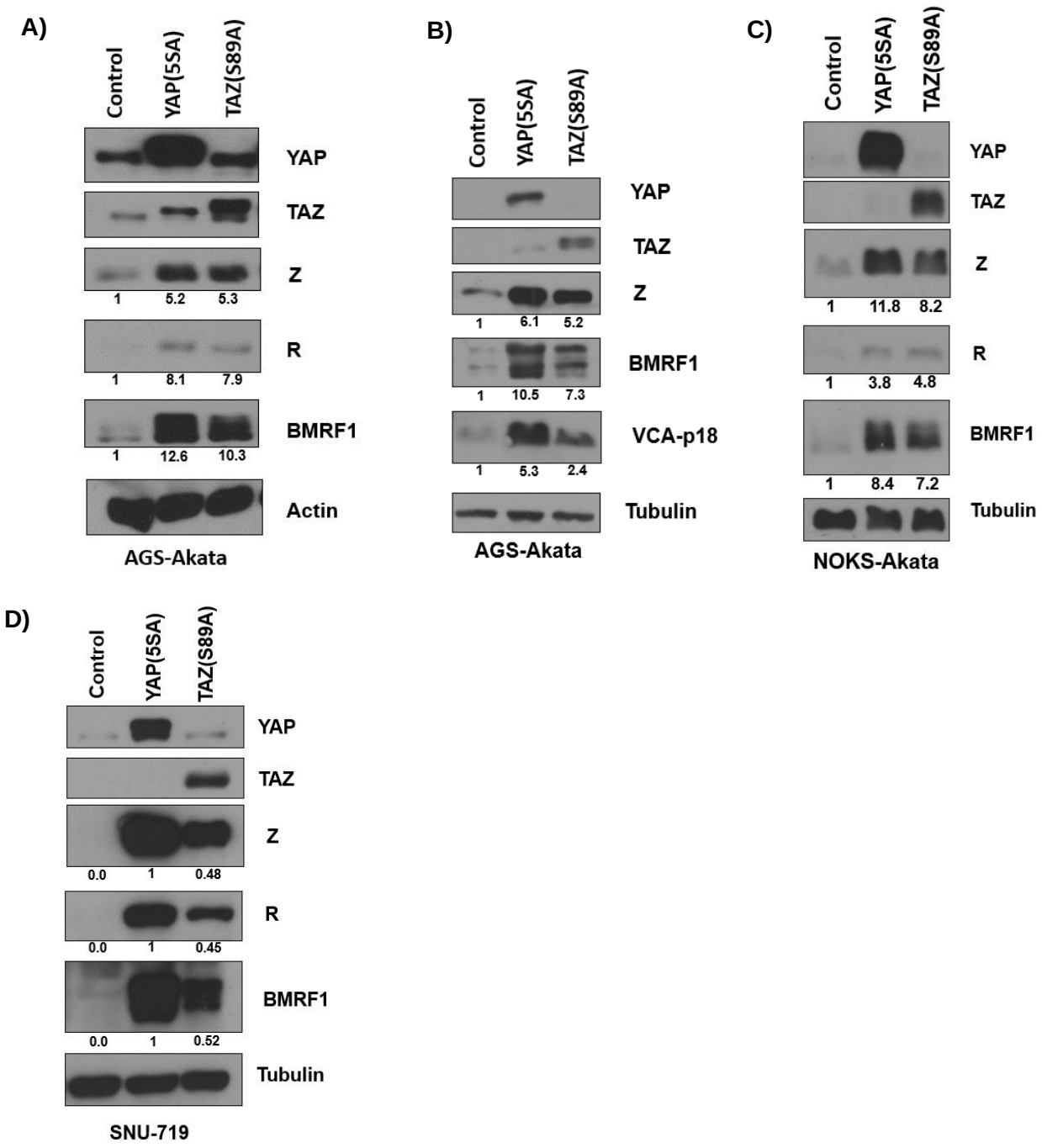


Fig 5: Constitutively activated YAP and TAZ induce lytic EBV reactivation in epithelial cell lines. A) AGS-Akata cells were transfected with constitutively active YAP (YAP(5SA)), or TAZ (TAZ(S89A)) expression vectors, or a vector control. Two days after transfection, the cells were harvested for a western blot and the expression levels of YAP, TAZ, Z, R, BMRF1, and actin (loading control) was examined. **B)** AGS-Akata cells were transfected with constitutively active YAP(5SA), TAZ(S89A), or a vector control. Three days after transfection the cells were

harvested for an immunoblot where the expression of YAP, TAZ, Z, BMRF1, VCA-p18 and tubulin was determined. **C)** NOKs-Akata cells were transfected with YAP(5SA) or TAZ(S89A) expression vectors, or a vector control, and immunoblot was performed to detect expression of transfected proteins YAP and TAZ, and Z, R, BMRF1, and tubulin (loading control). **D)** SNU-719 gastric carcinoma cells were transfected with either YAP(5SA), TAZ(S89A), or control expression vectors. Two days after transfection the cells were harvested for a western blot, and the expression of YAP, TAZ, Z, R, BMRF1, and tubulin was assessed. Results were quantitated using ImageJ software and normalized to the loading control result for each condition. The vector control or YAP(5SA) result (if vector control had no signal) was set as 1.

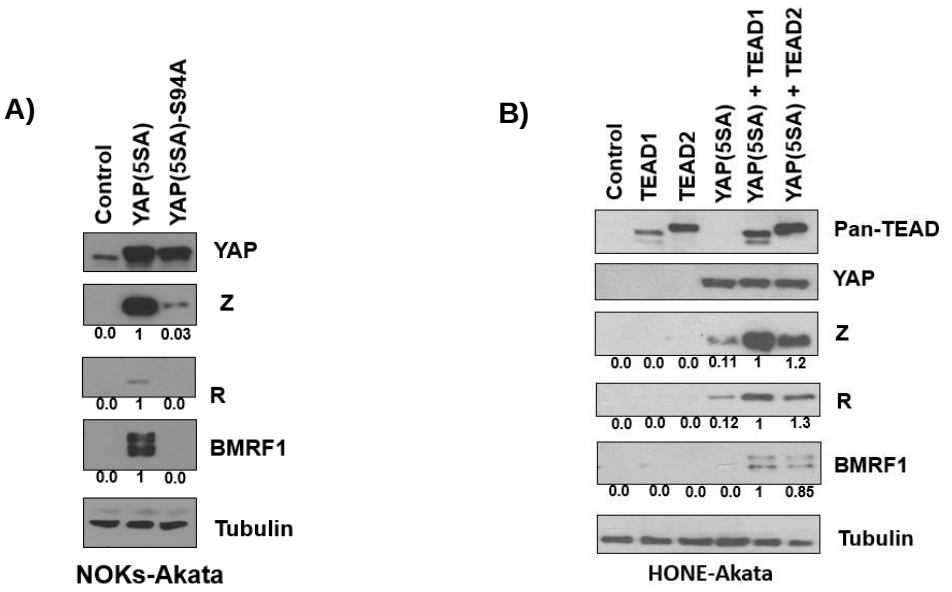
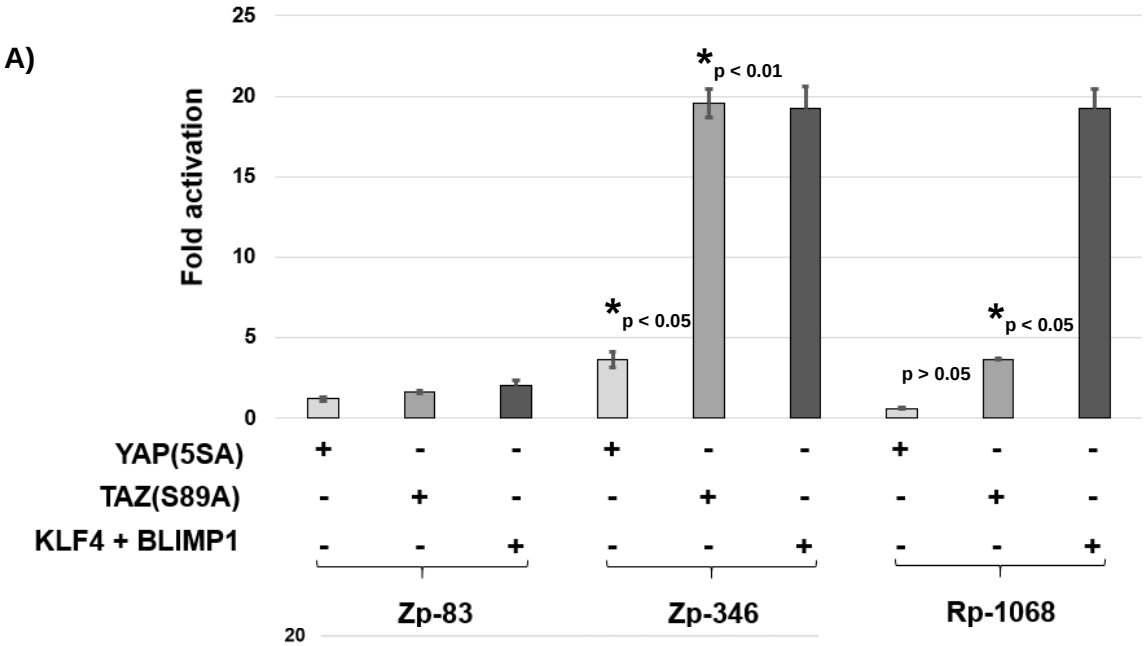
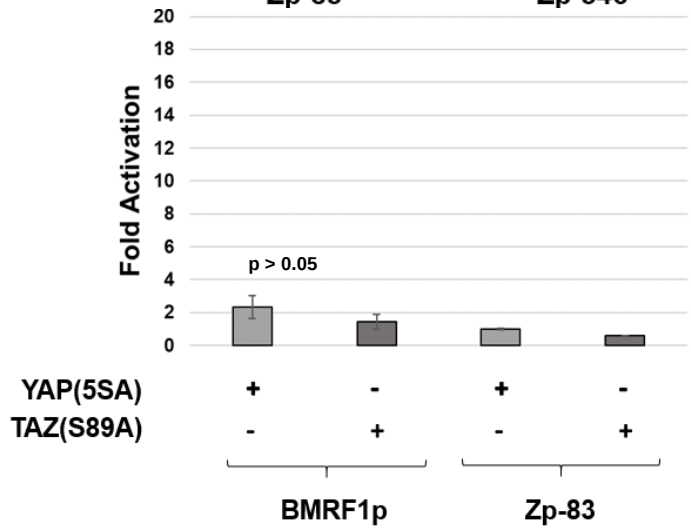


Fig 6: YAP and TAZ cooperate with TEADs to induce EBV lytic reactivation in epithelial cells. A) NOKs-Akata cells were transfected with YAP(5SA-S94A), YAP(5SA), or control vectors. After 48 hours an immunoblot was performed to detect expression of Z, R, BMRF1, YAP, and tubulin. Results were quantitated using ImageJ software and normalized to the loading control result for each condition; the YAP(5SA) vector result was set as 1. **B)** HONE-Akata cells were transfected with the YAP(5SA) expression vector with or without a TEAD1 or TEAD2 expression vector. 48 hours after transfection an immunoblot was performed to examine the expression of Z, R, BMRF1, TEADs and a tubulin loading control. Results were quantitated using ImageJ software and normalized to the loading control result for each condition. The YAP(5SA) vector plus TEAD1 result was set as 1.

A)


Fold activation
25
20
15
10
5
0
*p < 0.01
*p < 0.05
*p < 0.05
p > 0.05
YAP(5SA)
TAZ(S89A)
KLF4 + BLIMP1
Zp-83
Zp-346
Rp-1068

B)


Fold Activation
20
18
16
14
12
10
8
6
4
2
0
p > 0.05
YAP(5SA)
TAZ(S89A)
BMRF1p
Zp-83

C)


Zp-226
Zp-218
Zp-266
GAGGATTTGAATCTGGACTCCCCCCTGACCCCCGAACTTAATGAAAT
CTTGGATACATTTCTAAATGATGAATGTCTGCTGCATGCCATGCATATT
TCAACTGGGCTGTCTATTTTTGACACCAGCTTATTTTAGACACTTCTGA
AAACTGCCTCCTCCT Zp-186

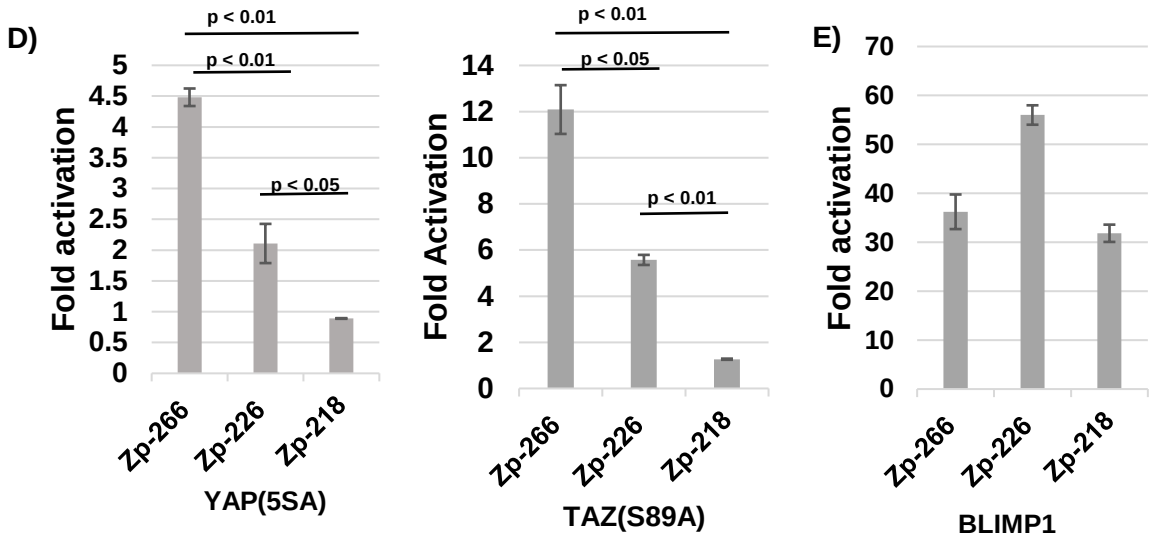
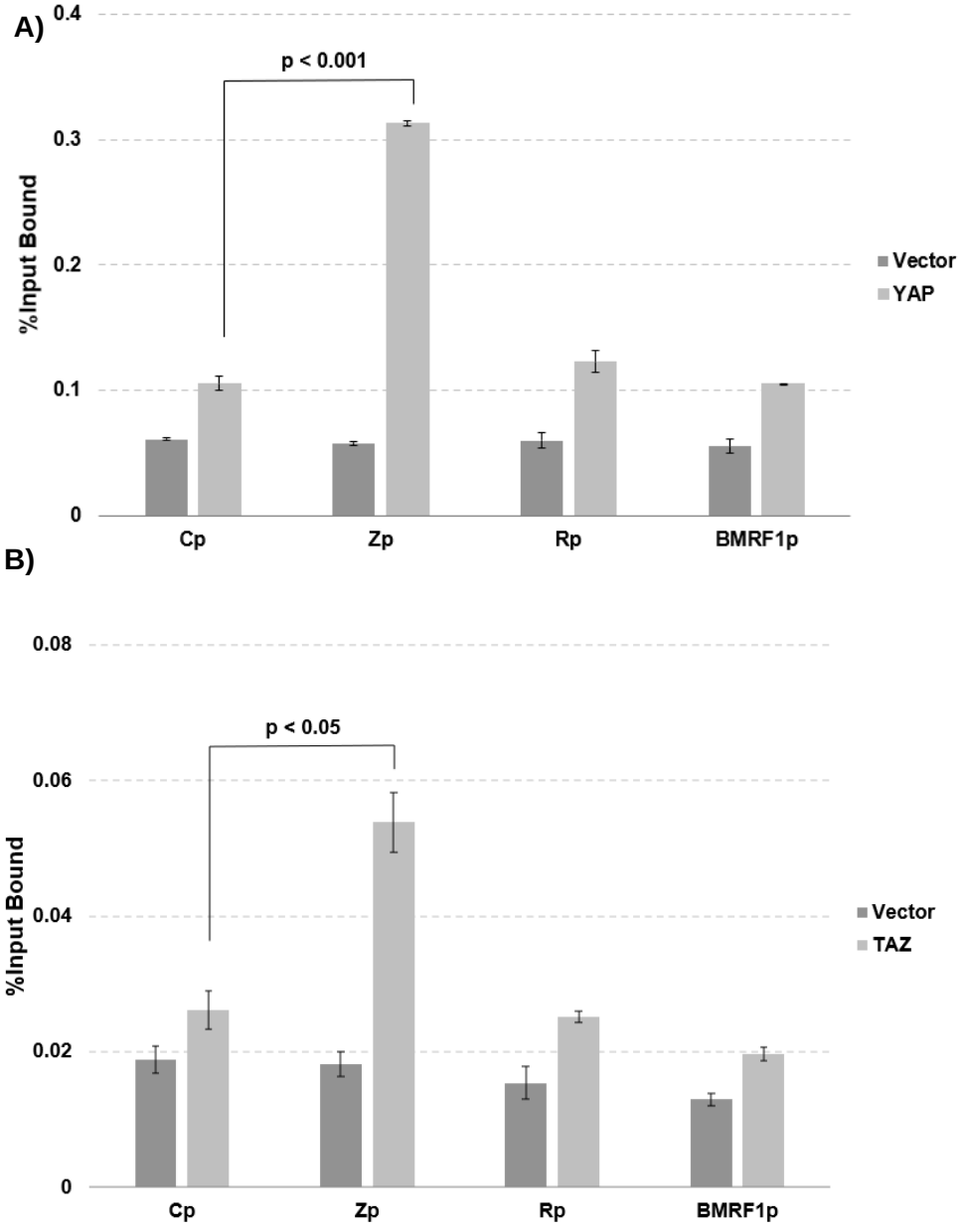


Fig 7: YAP and TAZ induce Z promoter activity through TEAD motifs located from -218 to -251. A) EBV negative HeLa cells were transfected with luciferase promoter constructs that were driven by the intact Z promoter or R promoter (containing 346 or 1068 bp, respectively, of each promoter sequence relative to the transcriptional start site), or a negative control promoter (containing only 83 bp of Zp promoter sequence), along with either a vector control, or YAP(5SA), TAZ(S89A), or KLF4+BLIMP1 expression vectors. The luciferase activity produced by each promoter was measured 48 hours post-transfection. The average fold difference in luciferase activity in conditions transfected with control vector versus YAP, TAZ or KLF4/BLIMP1 vectors is shown, along with the standard error. Statistical analysis (two-sample t-test) showed results of luciferase activity of the Zp-346 vector transfected with YAP(5SA) or TAZ(S89A) vectors, versus the vector control, were significantly different as indicated. **B)** HeLa cells were transfected with luciferase promoter constructs that were driven by the early lytic BMRF1 promoter or the negative control promoter (Zp-83) along with either a vector control, or YAP(5SA) or TAZ(S89A) vectors. The average fold difference in luciferase activity in conditions transfected with control vector versus YAP or TAZ vectors is shown, along with the standard error and p value. **C)** The sequence of the BZLF1 (Zp) promoter located between -266 and -186

(relative to the transcriptional start site) is shown; suspected TEAD binding motifs, each containing a 5/6 match to the consensus TEAD binding site (CATTCC) are outlined in green. Arrows indicate locations of the 5' Z promoter deletions. **D)** EBV negative HeLa cells were transfected with 5' luciferase Z promoter constructs with either a vector control or, the YAP(5SA) expression vector, the TAZ(S89A) expression vector, or a BLIMP1 expression vector as indicated. The average fold difference in luciferase activity in conditions transfected with control vector versus the YAP(5SA), TAZ(S89A), or BLIMP1 expression vectors is shown, along with the standard error. Statistical analysis for YAP(5SA) and TAZ(S89A) induction of the Z promoter constructs -266, -226, -218, was done with the two-sample t-test. **E)** EBV negative HeLa cells were transfected with 5' luciferase Z promoter constructs with either a vector control or a BLIMP1 expression vector as indicated.


A)
0.4
0.3
0.2
0.1
0
p < 0.001
%Input Bound
Vector
YAP
Cp
Zp
Rp
BMRF1p
B)
0.08
0.06
0.04
0.02
0
p < 0.05
%Input Bound
Vector
TAZ
Cp
Zp
Rp
BMRF1p

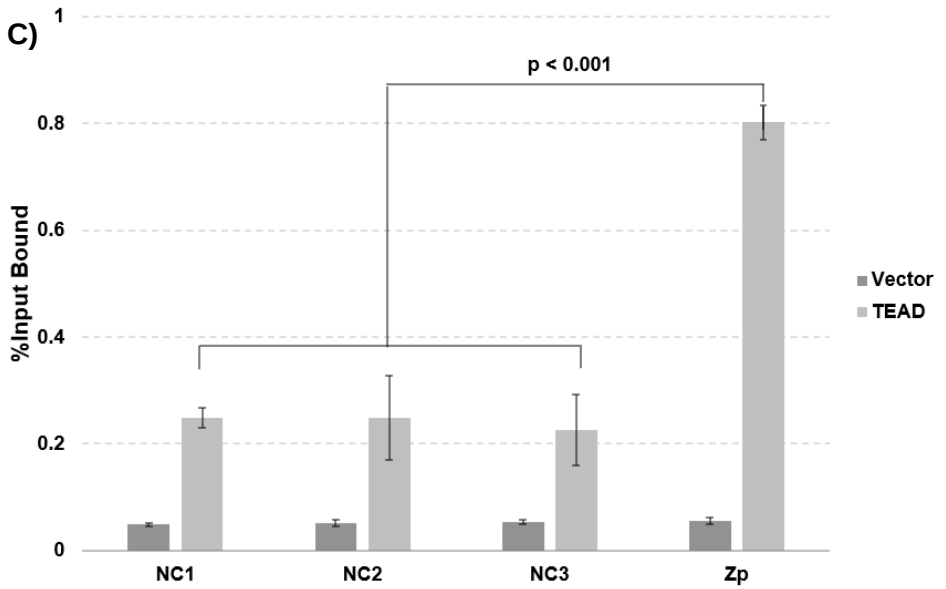


Fig 8: YAP and TAZ are complexed to the Z immediate-early promoter. HONE-Akata cells were transfected with either **A)** a vector control or co-transfected with a FLAG-tagged YAP(5SA) expression vector and TEAD1 vector, or **B)** a vector control or a FLAG-tagged TAZ(S89A) expression vector and TEAD1 vector. ChIP assays were performed 24 hours later using anti-FLAG antibody as described in the methods. Binding of FLAG-tagged YAP(5SA) and TAZ(S89A) to various parts of the EBV genome, including the lytic Z, R and BMRF1 promoters and the latent Cp promoter was determined by qPCR. **C)** HONE-Akata cells were transfected with a vector control or FLAG-tagged YAP(5SA) expression vector and myc-tagged TEAD1 vector. ChIP assays were subsequently done 24 hours post transfection with a myc antibody as described in the methods. Binding of myc-TEAD1 protein to the Z promoter, or negative control (NC) sites on the EBV genome was determined by qPCR. All experiments are representative of two independent biological replicates, and statistical significance was determined by the two-sample t-test.

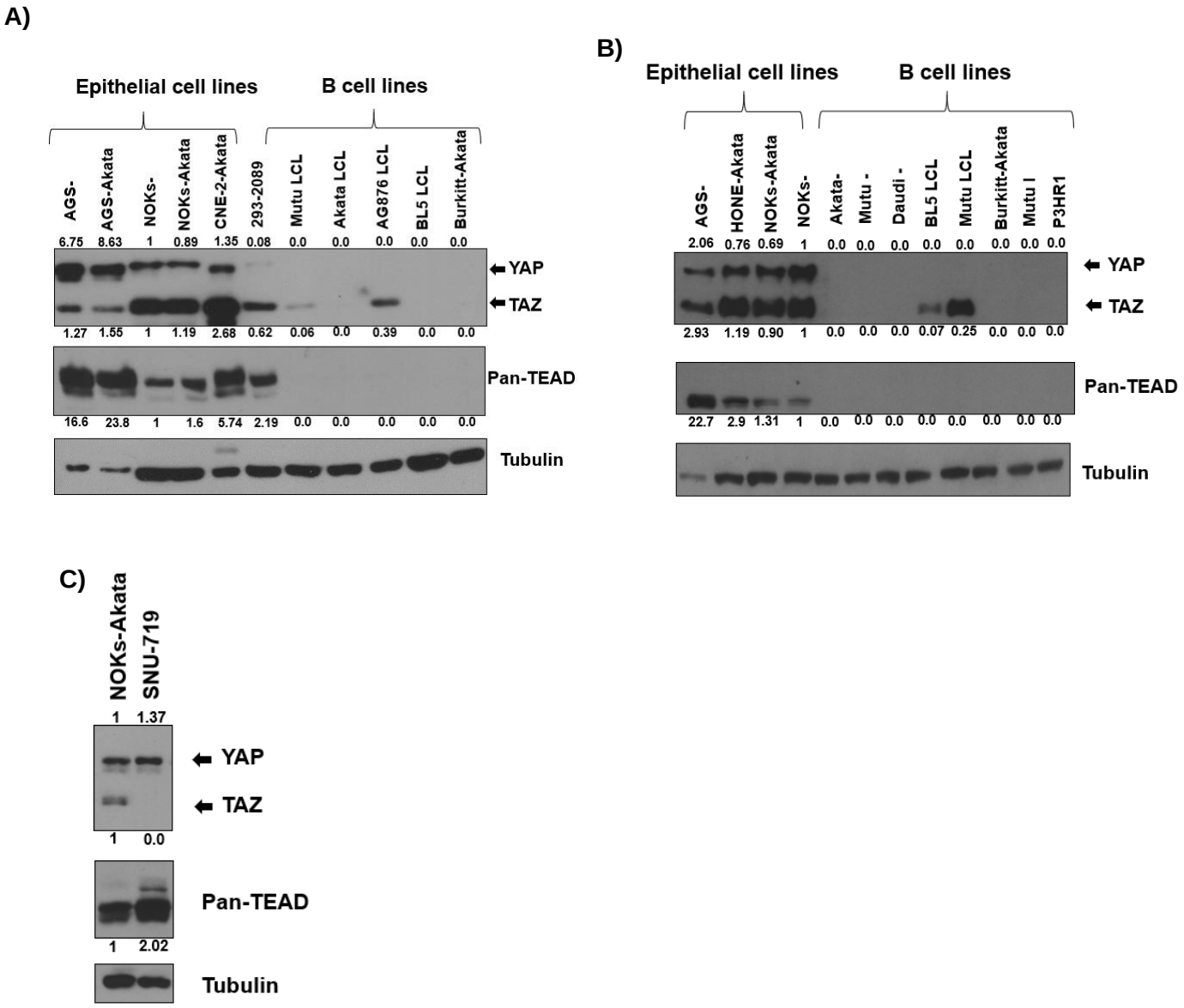


Fig 9: YAP, TAZ, and TEADs are expressed in EBV-infected gastric and oral epithelial cells but are not highly expressed in B cells. A) An immunoblot blot was performed to survey YAP/TAZ, TEADs, and tubulin expression in a number of different cell lines of either epithelial or B cell origin. Note that less protein was loaded in the AGS samples. The uninfected NOKs- cell condition result was set as 1. **B)** A repeat immunoblot experiment was performed in various epithelial cell lines and B cell lines in which expression of YAP/TAZ, TEADs, and tubulin was

assessed. Note that less protein was loaded in the AGS sample. Results were quantitated using ImageJ software and normalized to the loading control result for each condition. The uninfected NOKs- cell condition result was set as 1. **C)** YAP/TAZ and TEAD levels were compared in NOKs-Akata versus SNU-719 gastric carcinoma cells. The results were quantitated using ImageJ software and normalized to tubulin for each condition. The NOKs-Akata cell condition was set as 1.

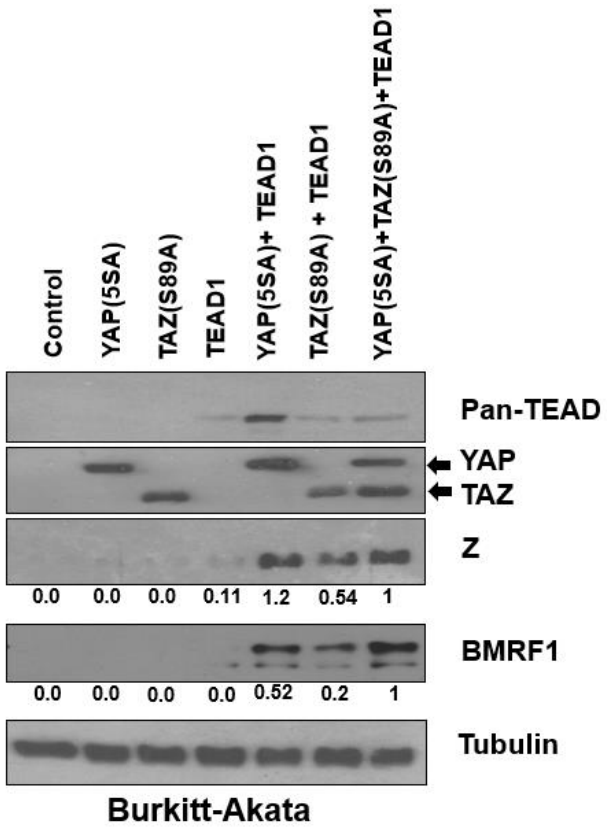


Fig 10: YAP and TAZ cooperate with TEADs to induce EBV lytic reactivation in B cell lines. Akata Burkitt-lymphoma cells were transfected with YAP(5SA), TAZ(S89A), and TEAD1, alone or in combination with each other, along with a vector control. After 48 hours post-transfection the cells were harvested and immunoblot performed to detect expression of YAP, TAZ, TEADs, Z, BMRF1 and the loading control tubulin. Results were quantitated using ImageJ software and normalized to the loading control result for each condition. The result in cells transfected with YAP(5SA), TAZ(S89A), and TEAD1 was set as 1.

Fig 11: EBV infection of NOKs cells increases YAP and TAZ activity. EBV-negative and EBV-positive NOKs-Akata cells were grown in sub-confluent conditions in KSFM media without growth factors for 24 hours. The cells were then harvested for an immunoblot to examine expression of total YAP, TAZ, LATS1, YAP phosphorylated at serine reside 127, TAZ phosphorylated at serine residue 89, and LATS1 phosphorylated at serine 909. Tubulin expression was examined as a loading control. Results were quantitated using ImageJ software and normalized to the loading control result for each condition. The uninfected NOKs cell condition shown in lane 2 was set as 1. Black line indicates where irrelevant lane(s) in the western blot were removed.

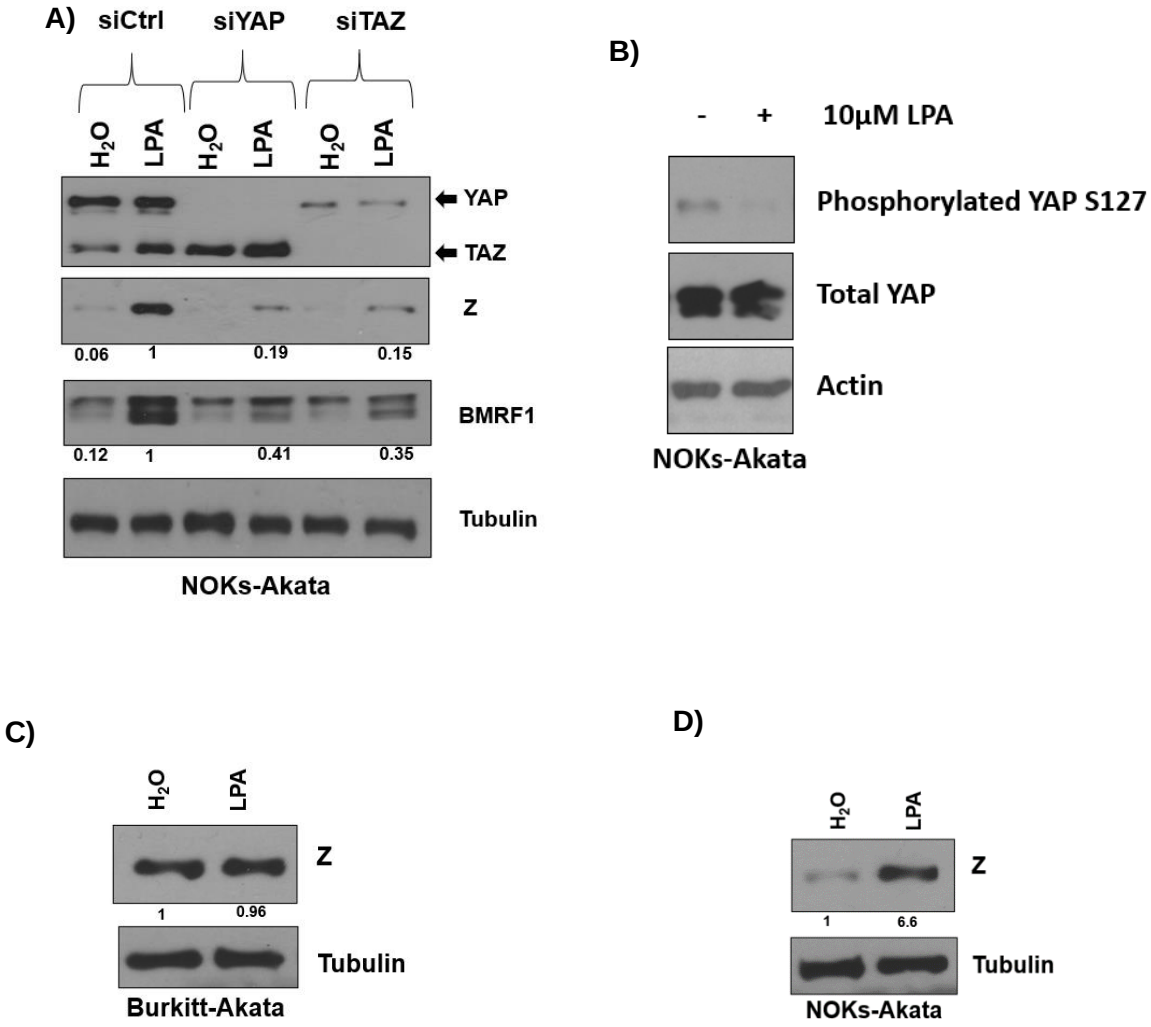
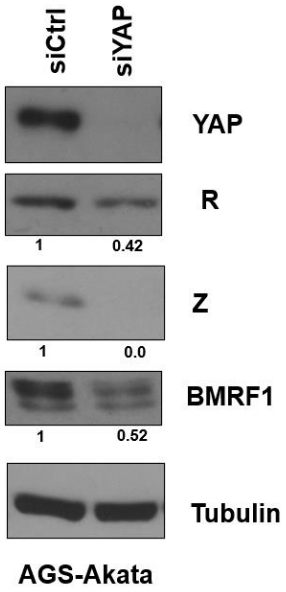


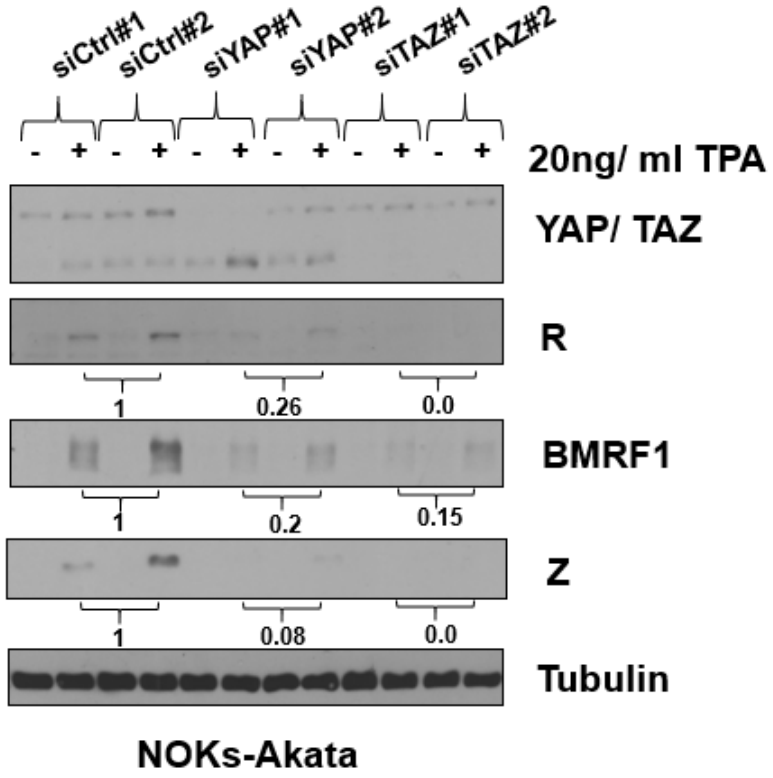
Fig 12: LPA induces EBV lytic reactivation through activating either YAP or TAZ in epithelial cells. A) NOKs-Akata cells were transfected with control siRNA or siRNAs targeting YAP or TAZ for 24 hours and then treated with 10 μM LPA for 24 hours before harvesting for a western blot. The expression of EBV lytic genes Z and BMRF1 was assessed, as well as the expression of YAP/TAZ, and tubulin for loading control. Results were quantitated using ImageJ software; and then normalized to the loading control result for each condition. The siRNA control result was set as 1. **B)** NOKs-Akata cells were treated with 10 μM LPA for 1 hour before harvesting for a western blot to assess the levels of total YAP versus YAP phosphorylated at

serine residue 127. Actin level was assessed as a loading control. Results were quantitated using ImageJ software and then normalized to the loading control result for each condition. LPA treated NOKs-Akata cell results were set as 1. **C)** Burkitt-Akata cells were treated with 10 μM LPA for 24 hours before harvesting with lysis buffer to perform immunoblot analysis, where the expression of Z and the loading control tubulin was assessed. **D)** NOKs-Akata cells were treated with 10 μM LPA for 24 hours before harvesting for an immunoblot where the expression of Z and the loading control tubulin was assessed. Results were quantitated using ImageJ software and then normalized to the loading control result for each condition. The untreated cell results in each cell type were set as 1.

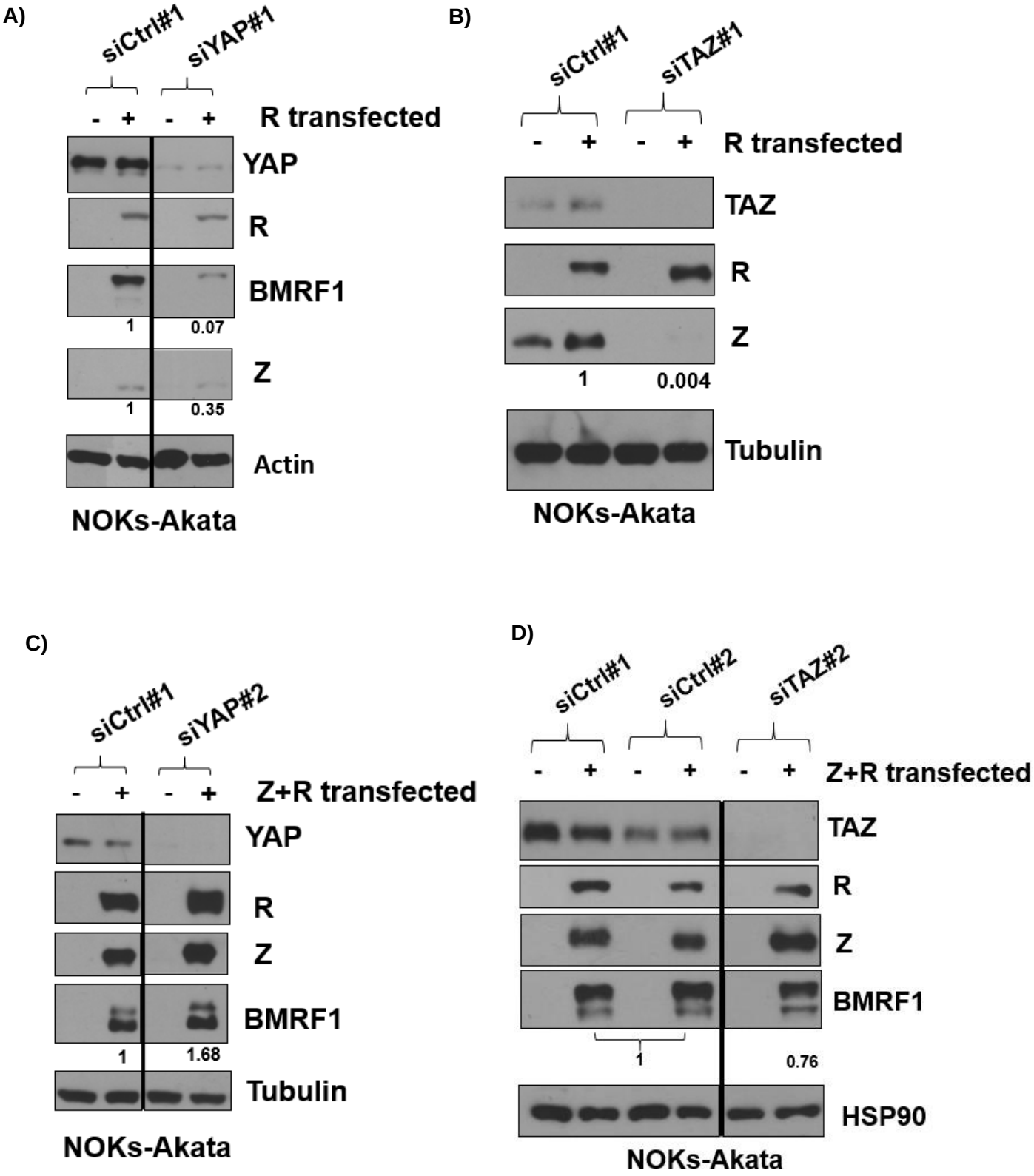
Supplemental Figure Legends.



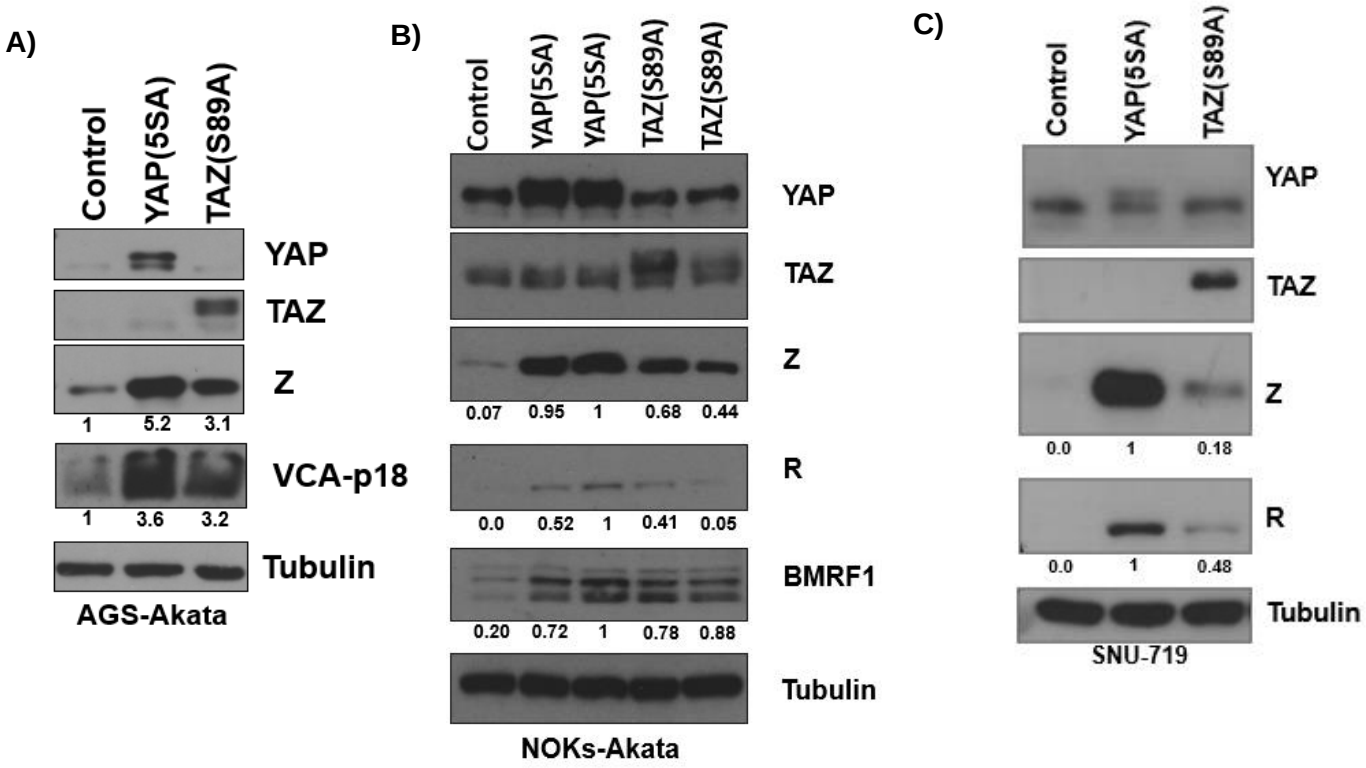
Supplemental. Fig.1. Depletion of YAP decreases constitutive lytic protein expression in AGS-Akata cells. Pooled siRNAs targeting YAP or a control sequence were transfected into AGS-Akata cells. Cells were harvested after two days and immunoblotted for Z, R, BMRF1, YAP, and tubulin (loading control). Results were quantitated using ImageJ software; results were normalized to the tubulin result for each condition. The siRNA control result was set as 1.



Supplemental Fig 2: YAP and TAZ expression are both essential for efficient TPA-induced EBV lytic reactivation in NOKs cells. NOKs-Akata cells were treated with either 20 pM of two different control siRNAs, or 20 pM of two different siRNAs against TAZ or YAP. 24 hours post-siRNA treatment, the cells were dosed with 20 ng/ml TPA. After a subsequent 24 hours the cells were harvested for immunoblots, where the expression of YAP, TAZ, Z, R and BMRF1 was determined. Tubulin was used as a loading control. Results were quantitated using ImageJ software, with the results for each set of siRNAs averaged and then normalized to the tubulin result for each condition. The average siRNA control result was set as 1.

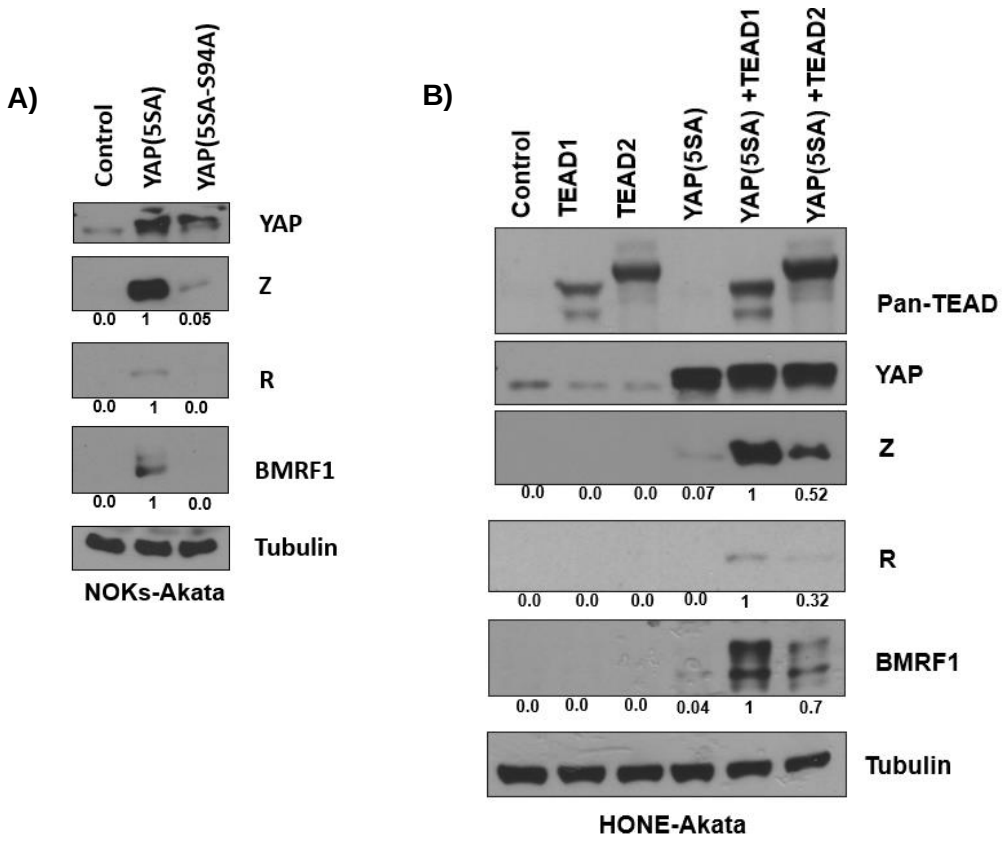

A)
siCtrl#1
siYAP#1
- + - +
R transfected
YAP
R
BMRF1
1
0.07
Z
1
0.35
Actin
NOKs-Akata
B)
siCtrl#1
siTAZ#1
- + - +
R transfected
TAZ
R
Z
1
0.004
Tubulin
NOKs-Akata
C)
siCtrl#1
siYAP#2
- + - +
Z+R transfected
YAP
R
Z
BMRF1
1
1.68
Tubulin
NOKs-Akata
D)
siCtrl#1
siCtrl#2
siTAZ#2
- + - + - +
Z+R transfected
TAZ
R
Z
BMRF1
1
0.76
HSP90
NOKs-Akata

Supplemental Fig. 3. Efficient R-induced lytic reactivation requires expression of YAP and TAZ. A) NOKS-Akata cells were transfected with control siRNA or siRNA against YAP, and then 24 hours cells were transfected with an R expression vector or control vector. After another 24 hours an immunoblot was performed to assess the levels of R, YAP, Z, BMRF1, and actin. **B**) NOKS-Akata cells were transfected with control siRNA or siRNA against TAZ, and 24 hours later were transfected with R expression or control vectors. A western blot was performed to determine expression levels of TAZ, Z, R, BMRF1 and tubulin. **C**) NOKS-Akata cells were transfected with control siRNA or siRNAs against YAP, and were transfected 24 hours later with both Z and R expression vectors. The cells were harvested after 24 hours for immunoblot analysis where the expression of YAP, R, Z, BMRF1, and the loading control tubulin was determined. **D**) NOKS-Akata cells were transfected with control siRNA or siRNA against TAZ, and were then transfected 24 hours later with Z and R expression vectors. The cells were harvested after 24 hours for western blot analysis to detect expression of TAZ, R, Z, BMRF1, and HSP90. Results were quantitated using ImageJ software (with the results for each set of siRNA controls averaged) and normalized to the loading control result for each condition. The siRNA control result was set as 1. Black lines indicate where irrelevant lanes in the western blot were removed.

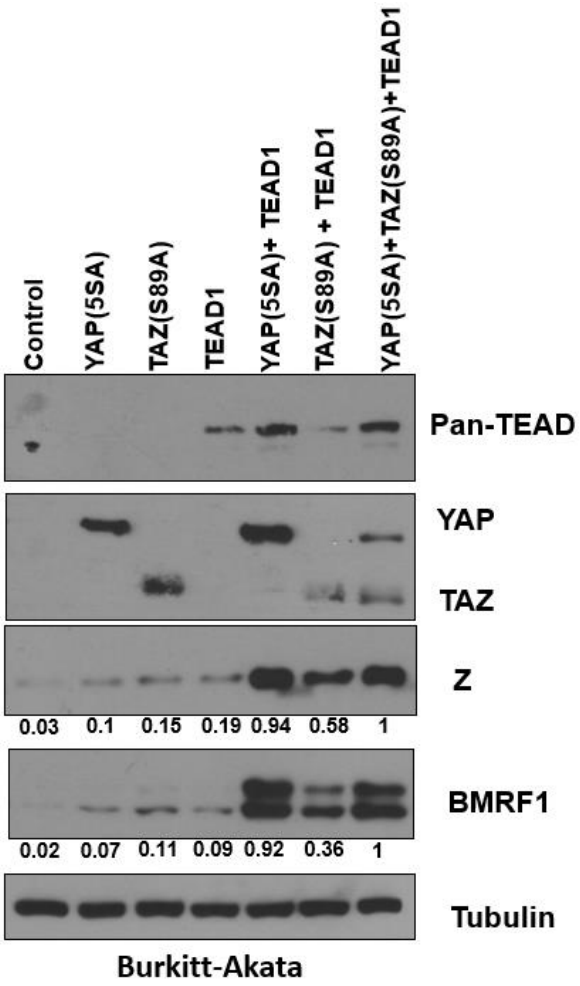


Supplemental Fig 4: Constitutively activated YAP and TAZ induce lytic EBV reactivation in epithelial cell lines. A) AGS-Akata cells were transfected with constitutively active YAP (YAP(5SA)), or TAZ (TAZ(S89A)) expression vectors, or a vector control. Two days after transfection, the cells were harvested for a western blot and the expression levels of YAP, TAZ, Z, VCA-p18 and tubulin (loading control) was examined. Results were quantitated using ImageJ software and normalized to the loading control result for each condition. The vector control result was set as 1. **B)** NOKs-Akata cells were transfected with constitutively active YAP(5SA), TAZ(S89A), or a vector control. Three days after transfection the cells were harvested for an immunoblot where the expression of YAP, TAZ, Z, R, BMRF1, and tubulin was determined. Results were quantitated using ImageJ software and normalized to the loading control result for each condition. The second YAP(5SA) result was set as 1. **C)** SNU-719 cells were transfected with YAP(5SA) or TAZ(S89A) expression vectors, or a vector control, and immunoblot was performed to detect expression of transfected proteins YAP and TAZ, and Z, R, or tubulin

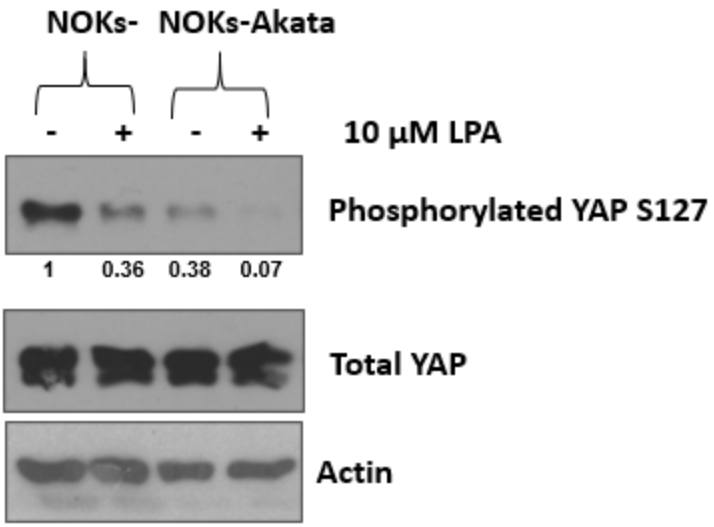
(loading control). Results were quantitated using ImageJ software and normalized to the loading control result for each condition. The vector control result was set as 1.



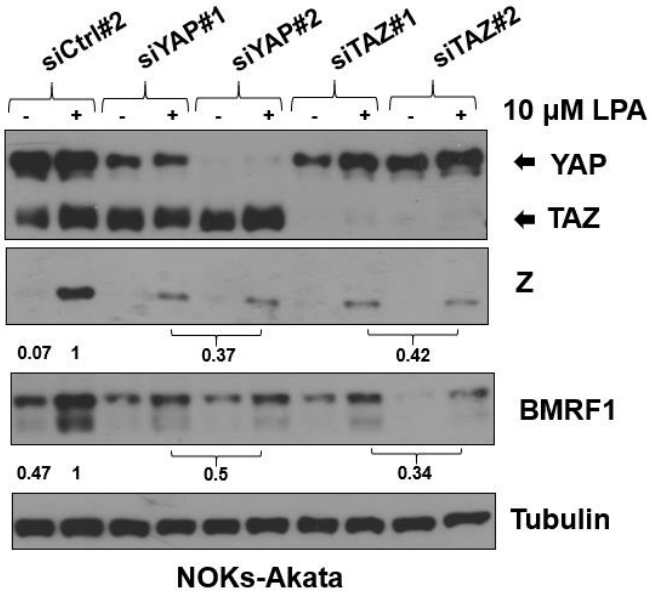
Supplemental Fig. 5: YAP and TAZ cooperate with TEADs to induce EBV lytic reactivation in epithelial cells. A) NOKs-Akata cells were transfected with YAP(5SA-S94A), YAP(5SA), or control vectors. After 48 hours an immunoblot was performed to detect expression of Z, R, BMRF1, YAP, and tubulin. Results were quantitated using ImageJ software and normalized to the loading control result for each condition. The YAP(5SA) vector result was set as 1. **B)** HONE-Akata cells were transfected with the YAP(5SA) expression vector with or without a TEAD1 or TEAD2 expression vectors. 48 hours after transfection an immunoblot was performed to examine the expression of Z, R, BMRF1, TEADs and a tubulin loading control. Results were quantitated using ImageJ software and normalized to the loading control result for each condition. The YAP(5SA) vector plus TEAD1 result was set as 1.



Supplemental Figure 6. YAP and TAZ cooperate with TEADs to induce EBV lytic reactivation in B cells. Akata Burkitt-lymphoma cells were transfected with YAP(5SA), TAZ(S89A), and TEAD1, alone or in combination with each other, along with a vector control. After 48 hours post-transfection the cells were harvested and immunoblot performed to detect expression of YAP, TAZ, TEADs, Z, BMRF1 and loading control tubulin. Results were quantitated using ImageJ software and normalized to the loading control result for each condition. The result in cells transfected with YAP(5SA), TAZ(S89A), and TEAD1 was set as 1.



Supplemental Figure 7: EBV infection of NOKs cells increases YAP activity. EBV-negative and EBV-positive NOKs-Akata cells were grown in sub-confluent conditions in KSFM media without growth factors, in the presence or absence of LPA for 24 hours. The cells were then harvested for an immunoblot to examine expression of total YAP, YAP phosphorylated at serine reside 127 and tubulin. Results were quantitated using ImageJ software and normalized to the loading control result for each condition. The untreated uninfected NOKs cell condition shown in lane 1 was set as 1. Note that lanes 3 and 4 in this figure were also the source for phosphorylated and total YAP blot in Figure 12B.



Supplemental Figure 8: **LPA induces EBV lytic reactivation in epithelial cells in a YAP/TAZ dependent manner.** NOKs-Akata cells were transfected with control siRNA or siRNAs targeting YAP or TAZ for 24 hours and then treated with 10 μM LPA for 24 hours before harvesting for a western blot. The expression of EBV lytic proteins Z and BMRF1 was assessed, as well as the expression of YAP/TAZ, and tubulin for a loading control. Results were quantitated using ImageJ software and then normalized to the loading control result for each condition. The siRNA control result was set as 1.

Chapter 4

Discussion

Summary

EBV lytic reactivation occurs in response to many cellular processes. These inducers include differentiation of B cells and epithelial cells, hypoxia, apoptosis, B-cell receptor engagement, DNA damage, inflammasome response, and TGF-β signaling (1–8). The study of EBV lytic reactivation in B cells has been extensive because of the relative ease of developing latently infected cell cultures and animal models in this cell type (9). In contrast, the study of the regulation of EBV lytic reactivation in “normal” latently infected epithelial cells only emerged after it became clear that telomerase-immortalized epithelial cells could support a latent infection of EBV compared to refractory primary epithelial cells (10,11). This discovery provided the opportunity to study how latent and lytic EBV infection is regulated in a non-transformed oral epithelial cell line such as NOKs, studies performed in such cells are biologically relevant as the oral epithelial tract is thought to be the source of virus circulating in saliva (12,13). Additionally, studying EBV in NOKs cells has provided a chance to examine how the virus modulates the host environment to favor cell survival and proliferation, tilting the cell fate towards malignancy. It has been clear for some time that differentiating epithelial cells undergo EBV lytic reactivation. This is based on studies of oral hairy leukoplakia, and studies of organotypic raft cultures (1,14–17). However, many crucial aspects regarding the regulation EBV lytic reactivation in epithelial cells remain to be determined. For example why don’t the majority of undifferentiated epithelial cells undergo lytic reactivation in cases of EBV-associated epithelial cancers such as NPC, even though these cell populations support extensive EBV infection (18,19). Also of great interest is determining how epithelial cell-specific signaling pathways in addition to differentiation facilitate the transition into lytic reactivation.

My aims in this thesis were

i) Identify cellular transcription factors that inhibit lytic reactivation in epithelial cells.

ii) Determine what cellular transcription factors induce lytic reactivation in epithelial cells and explore the conditions where these cellular transcription factors are active.

In this thesis, the main conclusions of my work are

i) p63 keeps EBV in latency in both B cells and epithelial cells by multiple mechanisms.

ii) YAP and TAZ both induce lytic reactivation in epithelial cells.

iii) TEADs can bind to the EBV genome and are required for YAP and TAZ to induce lytic reactivation in epithelial cells.

The identification of ΔNp63α as a repressor of EBV lytic reactivation.

In Chapter 2 of this thesis, I demonstrated that p63α isoforms inhibit lytic reactivation in both B cells (TAp63α) and epithelial cells (ΔNp63α). Additionally, I showed that ΔNp63α represses EBV lytic reactivation in the context of epithelial cancer cell lines that cannot differentiate and in a normal epithelial cell line that remains capable of differentiation. The biological implications of these discoveries are broad as ΔNp63α and TAp63α expression occur in all undifferentiated oral-pharyngeal epithelial cells (both in a normal context and in NPC) and many EBV+ lymphomas, respectively (20–23).

First, ΔNp63α prevents EBV lytic reactivation in epithelial cells until the epithelial cells stratify and ΔNp63α expression is lost. Loss of ΔNp63α expression is likely at least part of the reason why EBV is observed lytically infecting the most differentiated parts of the tongue, but not the undifferentiated tissues in oral hairy leukoplakia. Furthermore, ΔNp63α and TAp63α both suppress EBV lytic reactivation in EBV-positive malignancies such as NPC and Burkitt's lymphoma. The expression of these transcription factor isoforms maintains viral latency, perpetuates the cell population's proliferative capacity, and ensures that the tumor cells do not

induce apoptosis due to their inhibition of p53 function (24). However, numerous additional interesting experiments could be performed in the future to explore further how ΔNp63α inhibits various aspects of EBV infection.

Does ΔNp63α also prevent EBV infection of keratinocytes by upregulating interferon-K?

While I have demonstrated that ΔNp63α and TAp63α prevent lytic reactivation, one possibility that I have not tested is whether ΔNp63α expression prevents EBV primary infection in epithelial cells. In studies of stratified primary epithelial tissue and OHL lesions, primary EBV infection was not detected in the undifferentiated basal tissue despite widespread lytic replication in the differentiated layers (18,19,25). These observations suggest that there may be a potent restriction factor that is resident in these untransformed normal tissues that could be inhibiting EBV infection. Furthermore, ΔNp63α expression positively regulates interferon-Kappa (K), a type of interferon that is constitutively expressed in oral epithelial cells (26) and that has recently been reported to inhibit the replication of HPV by inducing the expression of the Sp100 proteins (27). The role of interferon-K during EBV infection of epithelial cells has not been investigated, but interferon-K may potentially be a factor that inhibits EBV infection.

Recently, type I interferon responses were demonstrated in latently EBV-infected epithelial cells to prevent lytic reactivation induced by TPA (28). Given that interferon-K signals through the type I interferon pathway, it is a distinct possibility that interferon-K could also act as an impediment to EBV lytic infection. I would test this hypothesis by over-expressing ΔNp63α with a lentivirus in an EBV-uninfected oral keratinocyte cell and confirming if ΔNp63α increases interferon-K expression. I would also be infecting these cell lines with EBV subsequently. Then, by quantifying infection success by either GFP+ flow sorting or crystal violet staining of infected cell colonies (after G418 selection), it would be possible to determine if ΔNp63α (perhaps through interferon-K) inhibits EBV primary infection. If there are fewer GFP+ cells or cell colonies, this would indicate that ΔNp63α is inhibiting the ability of EBV to infect epithelial cells

successfully. However, no change in the number of infected cells would indicate that ΔNp63α is not an inhibitor of primary EBV infection. Additionally, by knocking down interferon-K expression with shRNAs in ΔNp63α over-expressing NOKs-Akata cells compared to a knockdown of interferon-K in with a control lentivirus, it would be possible to evaluate if interferon-K is a specific mechanism by which ΔNp63α inhibits EBV infection.

Does ΔNp63α alter the chromatin conformation of the EBV genome to inhibit lytic reactivation?

In Chapter 2, I determined that ΔNp63α increases the expression of c-myc, which binds to the *orilyts* of the EBV genome to inhibit lytic replication (29). While c-myc can repressively alter the EBV 3D genome conformation (29), it is as yet unknown whether ΔNp63α itself likewise induces a change in the EBV genome's chromatin structure. ΔNp63α has previously been reported to mediate chromosomal conformation alterations with several intermediates such as BMP4 and the SWI/SWF component BRG1 (30,31). EBV gene expression is regulated by chromatin looping, and this could be another potential mechanism by which ΔNp63α keeps EBV in latency (32,33). While I failed to see ΔNp63α binding to the EBV IE gene promoters, this finding does not exclude the possibility that ΔNp63α may alter the chromatin landscape of the virus indirectly through a recruited intermediate, making it difficult for the virus to undergo lytic replication.

This hypothesis could be tested using chromatin conformation capture assays (3C), which can determine interactions between genomic loci. Given the repressive effect of ΔNp63α on the Z promoter, it is possible that ΔNp63α mediates a repressive chromatin conformation on the Z promoter in a fashion similar to c-myc. By depleting ΔNp63α in NOKs-Akata with siRNAs or through a differentiation-stimuli and then performing 3C assays at multiple time points, it would be possible to determine if the Z promoter’s chromatin conformation changes during ΔNp63α depletion, as well as identify other interacting elements such as the *orilyts*. If we

observed that there was an increased interaction between the *orilyts* and the BZLF1 promoter between 2 and 24 hours in the control NOKs-Akata compared to the ΔNp63α over-expressing NOKs-Akata (the time frame others have used for this assay), that would indicate that ΔNp63α is also inhibiting lytic reactivation by maintaining a repressive chromatin landscape. A caveat to this experiment is that a comparison to a cell line with c-myc knocked down with shRNAs or deleted by CRISPR is necessary to ensure that any chromatin conformation changes were not simply due to the simultaneous loss of c-myc and ΔNp63α.

What effect do other isoforms of p53 and p63 have in EBV lytic reactivation, and does EBV modulate their expression?

In Chapter 2, I found that the C-terminal domain of the ΔNp63α and TAp63α isoforms is required to inhibit EBV lytic reactivation. However, I did not determine what effect ΔNp63β or other ΔNp63 isoforms may have on lytic reactivation, although I showed that they do not repress EBV lytic reactivation in transient transfection assays. While ΔNp63α is the dominant ΔNp63 isoform in epithelial cells, some ΔNp63β expression is present in many epithelial cell lines (22). Despite the structural similarity, ΔNp63 isoforms have differential effects on cellular transcription (24). ΔNp63α is a known competitive inhibitor of p53 functions such as apoptosis and senescence, while ΔNp63β and ΔNp63γ actually can induce p53 transcriptional targets (24,34). The Kenney lab, Mertz lab, and other research groups have shown that the expression of p53 positively regulates EBV lytic reactivation (6,35). In contexts such as DNA damage, would these other isoforms of p63 promote EBV lytic reactivation similar to p53? Furthermore, in the past decade, there have been additional isoforms of p53 discovered, which are sometimes expressed in cancers (36). Are these other p53 isoforms important for the ability of EBV to reactivate, or do they have different roles during lytic reactivation (37)? Additionally, does EBV infection during either latency or lytic reactivation modulate the expression of either p53 or p63 isoforms? ΔNp63α expression increases in both NPC tumors and cells that overexpress LMP2A

(20,38). The Kenney lab is in a unique position to test this hypothesis, as we are in the process of developing EBV mutant viruses in which their latency genes such as LMP1 and LMP2A have been deleted and have put these mutant viruses into NOKs cells. The Kenney lab has already begun the process of RNAseq on some of these mutant-infected NOKs cells so this question could be answered relatively expediently.

Does the C-terminal domain of ΔNp63α and TAp63α interact with inducers of lytic reactivation?

In Chapter 2, I found that ΔNp63α and TAp63α expression seriously compromised R function independent of R's ability to bind to EBV lytic promoters. R is known to physically interact with crucial mediators of lytic reactivation such as p300/CBP to enhance lytic reactivation (39). It remains a possibility that ΔNp63α and TAp63α also inhibit lytic reactivation by competitively binding to these protein complexes and sequestering them away from EBV genomes where they would typically bind in a mechanism that is reminiscent of LF2 (40). Relatedly, I have not investigated the possibility that ΔNp63α and TAp63α may physically interact with inducers of lytic reactivation such as KLF4 and BLIMP1 to prevent lytic reactivation by preventing their activation of the Z and R promoters. While I have demonstrated that ΔNp63α over-expression does not prevent the terminal differentiation of epithelial cells (as KLF4 and BLIMP1 were expressed to a similar degree), I did not determine if the functionality of transcription factors KLF4 or BLIMP1 to induce lytic reactivation was compromised. While ΔNp63α is known to bind to and inhibit the KLF4 promoter (41), this event remains a possibility, as EBV-infected epithelial cells are already deficient at differentiating. This impairment could obscure the loss of KLF4 or BLIMP1 function as transcription factors. To test this hypothesis, I could transfect KLF4 or BLIMP1 in the presence or absence of ΔNp63α and perform a ChIP assay to assess KLF4 or BLIMP1 occupancy of the Z or R promoters to determine if ΔNp63α expression inhibits transfected KLF4 or BLIMP1 binding to the IE promoters. In the event that

KLF4 or BLIMP1 were inhibited in their binding by ΔNp63α, I would expect a reduction in occupancy of these transcription factors of the Z and R promoters for the ChIP assay. However, ΔNp63α may inhibit the activity of these transcription factors (possibly by reducing activating post-translational modifications), and no decrease in binding of the Z and R promoters would be observed.

The requirement of active p38 to induce lytic reactivation indicates that better characterization of the p38 downstream pathways is necessary.

In Chapter 2, I showed that ΔNp63α over-expression decreases the activity of p38 MAPK. Furthermore, siRNA depletion and chemical inhibition demonstrated that functionality and expression of p38 MAPK14, was required for efficient lytic reactivation by R. While numerous groups have shown that the p38 MAPK is essential for the ability of many lytic stimuli to induce EBV lytic reactivation (42–47), the substrates that p38 MAPK phosphorylates to induce lytic reactivation still need to be better characterized. Indeed, p38 MAPK can induce the activity of numerous positive regulators of EBV lytic reactivation such as HIF-1α, p53, ATF2, and c-jun (48). It is likely that uncharacterized inducers of EBV lytic reactivation are downstream kinase targets of p38 MAPK. Additionally, while I determined that inhibition of the p38 MAPK target MAPKAK2 and the ZFP36-mediated mRNA stability pathway are not likely to be required for the ability of R to induce lytic reactivation, there are still many other regulatory modules of p38 MAPK that could be important for EBV lytic reactivation.

P38 MAPK14 is reported to be an inhibitor of the nonsense-mediated decay (NMD) pathway, a cellular process known to inhibit gamma-herpesvirus lytic reactivation by targeting R mRNA transcripts for degradation (49,50). Determining if p38 MAPK14 inhibits NMD during lytic reactivation as part of a pro-lytic program could be done by knocking down p38 MAPK and assaying for NMD targets such ATF3 mRNA transcripts via qPCR (49). If ATF3 mRNA expression is decreased during p38 MAPK14 knockdown, that would indicate that NMD is more

active during instances when EBV is probably latent, consistent with what other groups have observed. In addition, while p38 MAPK is notable for directly phosphorylating many transcription factor proteins, p38 MAPK is also capable of phosphorylating chromatin to modulate gene expression (51). Given that the epigenetic status of the EBV genome dictates susceptibility to lytic reactivation, this possibility is of great interest. The hypothesis that p38 MAPK alters the histone marks on the EBV genome could be tested by depleting p38 MAPK expression with siRNAs (or deleting with CRISPR) and performing ChIP assays of the EBV IE promoters using antibodies that recognize the histone marks p38 MAPK is known to modulate such as tri-methylated H3K4 or phosphorylated H3S28. If the presence of these marks on the EBV genome is decreased during p38 MAPK14 depletion, that would suggest that these marks may also be a mechanism by which p38 MAPK14 positively regulates lytic reactivation.

Single-cell RNA-seq of EBV-infected epithelial cells overexpressing ΔNp63α may allow for the identification of novel inducers and repressors of lytic reactivation.

In Chapter 2, I demonstrated that ΔNp63α could induce c-myc expression and decrease p38 phosphorylation as at least two means of inhibiting lytic reactivation. Because ΔNp63α is a master regulator of many genes both transcriptionally and post-transcriptionally, it is possible that ΔNp63α expression also creates a cellular environment that is nonpermissive for lytic reactivation by upregulating repressors of lytic reactivation, and downregulating lytic activators. With the increasingly commonplace usage of single-cell RNA sequencing, it is possible to observe different populations of cells undergoing distinct cellular events. A fault with assessing gene expression with western blots or bulk RNAseq is that this technique summarizes gene expression patterns in a population of cells, but does not distinguish between specific subgroups that the population's overall trend may mask. The Kenney group has previously conducted single-cell RNA sequencing comparing type I EBV LCLs against type II EBV LCLs.

We confirmed that type II LCLs are more lytic than type I, which validates that the read depth can detect distinct cell populations undergoing lytic replication.

So far, only one study of EBV lytic reactivation in the context of epithelial cells with single-cell RNA sequencing has been published (52). This study used primary cells and only confirmed lytic infection in one of the samples, suggesting that using EBV-infected NOKs cells could be a more informative approach for the regulation of EBV infection in epithelial cells. Fortunately, the prior study already demonstrated that EBV-infected epithelial cells differentiated on a collagen membrane filter could be viable samples for single-cell RNAseq, so comparing this stimuli to undifferentiated cells is a valid approach. Alternatively, since ΔNp63α inhibits and dampens the ability of the virus to undergo reactivation through at least several different mechanisms, using cells that are either over-expressing ΔNp63α with a lentivirus (which would be uniformly expressed in all cells) or a control empty vector would allow us to determine which genes are differentially affected by the ΔNp63α over-expression compared to the control. Given that ΔNp63α is a master regulator of epithelial cell gene expression, the use of single-cell RNA sequencing could segregate and identify groups of novel genes that regulate EBV lytic reactivation that are under the regulation of ΔNp63α.

YAP and TAZ induce EBV lytic reactivation, and the TEAD gene family mediates this process.

In this thesis, I demonstrated that the Hippo signaling effectors YAP and TAZ induce EBV lytic reactivation. I also showed that the coactivator TEADs, which are bound to the Z promoter, mediate YAP/TAZ induction of lytic reactivation. YAP and TAZ induce numerous genes essential for cell proliferation, organ size, and survival (53,54). What is notable about these two transcription coactivators is the number of biological processes that regulate their transcriptional activities, such as mechanotransduction, cell density, cell polarity, and differentiation (55).

Future studies to characterize upstream YAP and TAZ regulators.

While I have demonstrated that the signaling effectors YAP and TAZ induce lytic EBV reactivation when active, additional inquiry into the upstream Hippo regulatory pathway and how it impacts EBV lytic reactivation is necessary. Of note, the study conducted in Chapter 3 did not determine the impact that the core regulators of Hippo signaling, LATS1/2 and MST1/2, may have on the induction of lytic reactivation. Although I did not see a decrease in LATS phosphorylation with latent EBV infection, the inhibitory function of these kinases on YAP and TAZ transcriptional activities likely makes them potent inhibitors of EBV lytic reactivation (55).

The Hippo signaling pathway is complex. For example, MST1/2 is not necessarily required to inhibit YAP/TAZ function in certain contexts (56). The roles of these inhibitors in the latent to lytic switch could be tested by over-expressing them, which has been reported in the literature to dampen YAP and TAZ transcriptional activity, or by depleting their expression with siRNAs (57). If lytic reactivation is either increased or decreased when the Hippo kinases are depleted or over-expressed, respectively, this would suggest that the Hippo kinase cascade also is an inhibitor of lytic reactivation. A better understanding of how Hippo signaling impacts EBV lytic reactivation would also provide insight how or if EBV modulates this pathway to regulate its latent-lytic switch. Over-expressed LMP1 outside of the context of the intact virus has been reported to decrease the phosphorylation of LATS1/2 (58). However, we did not see this result in the context of an EBV-infected cell. This difference may be a reflection of differences between non-physiologic and physiologic levels of LMP1 expression. Whether other EBV latency proteins such as LMP2A or EBNA1 can also modulate the post-translational modifications of this pathway also remains to be determined.

Does YAP and/or TAZ utilize additional DNA binding partners during lytic reactivation?

In Chapter 3, I demonstrated that YAP and TAZ required TEAD proteins as a DNA binding partner for their induction of EBV lytic reactivation, but it remains possible that these transcriptional effectors also access the EBV genome using other factors in addition to the TEAD proteins. YAP and TAZ have been reported in the literature to interact with numerous transcription factors to induce their transcriptional targets, including SMADs, AP-1, KLF4, RUNX2 (59–64). Several of these coactivators have been shown to induce lytic reactivation (1,8,65). Thus, YAP and TAZ may be previously unappreciated mediators of these known lytic reactivation processes. To test the essentiality of YAP and TAZ expression for the ability of these coactivators to induce lytic reactivation, we could deplete YAP or TAZ with siRNAs and then transfect the coactivator of interest such as KLF4. Since these coactivators are known to bind to the EBV genome, if we determine that YAP and TAZ are crucial for KLF4 (or another of these coactivators) mediated reactivation, I would follow up by determining if binding to the EBV Z promoter by these coactivators is decreased when YAP and TAZ are absent through ChIP assays.

Does competition for TEADs inhibit YAP/TAZ induction of lytic reactivation?

I have not yet investigated if other TEAD-interacting proteins compete with YAP and TAZ for TEAD binding as a mechanism that could keep EBV in latency. One already known TEAD-interacting protein is VGLL4, a member of the VGLL protein family. In both gastric and breast cancer, VGLL4 and VGLL4-like peptides can repress YAP signaling by competitively binding to TEADs, stymying growth of these heavily YAP-dependent carcinomas (66–69). VGLL4 is expressed in our epithelial cell lines based on our NOKs RNAseq data and online databases (17,70). By use of a VGLL4 expression vector and siRNAs against VGLL4, it would be possible to begin to study if VGLL4 inhibits EBV lytic reactivation in the context of these epithelial cell lines. An immunoblot comparing lytic reactivation of cells that over-expressed VGLL4 compared

to the control in the constitutively lytic AGS-Akata cell line would test the hypothesis that VGLL4 expression can antagonize YAP induction of lytic reactivation. If VGLL4 over-expression decreased lytic reactivation in this cell line, that would indicate that VGLL4 could be a repressor of lytic reactivation via the YAP-TEAD axis. Given that YAP-TEAD interaction antagonism by VGLL4 peptides as well as by the drug verteporfin have been proposed as therapeutic options for YAP over-expressing carcinomas, this study could not only establish the importance of Hippo signaling in the regulation of EBV lytic reactivation, but also provide some additional treatment options for EBV-associated malignancies in the future (17,70,71).

The function of YAP and TAZ during differentiation of EBV-infected epithelial cells remains to be explored.

I demonstrated that YAP and TAZ were required for EBV to reactivate during differentiation. This interesting observation was counter-intuitive given that the expression and nuclear localization of YAP and TAZ is thought to inhibit differentiation of most epithelial cell types, including in oral keratinocytes (72,73). Conversely, YAP and TAZ activation of target genes is critical for colon and lung epithelial cells to differentiate fully (59,74). During the collagen filter experiment shown in Figure 3 of Chapter 3, we did not find that YAP or TAZ depletion reduced epithelial cell differentiation during EBV infection; however, we also did not observe a noticeable decrease in the expression of YAP and TAZ during this process as expected. Nevertheless, differentiation of keratinocytes also inhibits YAP/TAZ function by increasing its phosphorylation and preventing its nuclear localization, which we did not examine. Our previous studies showed continued abnormal expression of proliferation markers in EBV-infected differentiated NOKS cells (17), suggesting functional YAP/TAZ activity. Thus, it remains possible that EBV infection enhances YAP and TAZ function during epithelial cell differentiation. Another possibility is that YAP and TAZ are transiently activated during the initiation of differentiation through the addition of serum given that serum induces rapid dephosphorylation

of YAP and TAZ (75). Also, this process might allow for the induction of lytic reactivation even if YAP and TAZ were shuttled out of the nucleus during the later stages of differentiation.

Characterizing the role of GPCR signaling during EBV lytic reactivation is of great interest.

In Chapter 3, I showed that LPA which acts via several GPCRs can induce lytic reactivation in a YAP/TAZ-specific manner. One physiologic situation where this ligand may induce EBV lytic reactivation is within the oral epithelium, where LPA present in the saliva can enhance the terminal differentiation of these cells (76). Additionally, the expression of LPA in the saliva drastically increases during periodontitis, a disease of the gums in which EBV has been implicated to play a role (77–79). My findings suggest a mechanism by which this EBV-associated pathology could potentially occur. Furthermore, a study by Yu *et al.* broadly implicates many GPCR ligands as either positive (such as LPA and S1P) or negative (such as glucagon and epinephrine) regulators of EBV lytic reactivation (75). Drugs targeting GPCRs are 34% of all FDA-approved drugs (80), and my findings presented in Chapter 3 suggests the possibility that some of these drugs could be repurposed to induce lytic reactivation in EBV positive tumors if they can regulate YAP or TAZ activity (i.e., “lytic induction” therapy).

Does the EBV-encoded GPCR BILF1 induce the activation of YAP and TAZ?

In Chapter 3 I showed that latent EBV infection induced the dephosphorylation of both YAP and TAZ, and I hypothesized that the EBV early gene BILF1, which encodes a constitutively active GPCR, could further stimulate YAP and TAZ activity (81). Viral GPCRs in other herpesviruses are important for efficient viral replication (82–84). If we were to find that the EBV-encoded GPCR induces YAP and TAZ activity, one could also ask if all the herpesvirus GPCRs share the ability to activate YAP and TAZ to induce their replication. It is already known that the KSHV vGCPR can induce YAP expression, and KSHV vGPCR expression is known to

enhance lytic reactivation (85). However, this study did not investigate if KSHV lytic reactivation was induced by the expression of YAP (86). To begin to test the hypothesis that BILF1 helps to reactivate EBV by enhancing YAP/TAZ function, one could clone BILF1 into an expression vector and transfect it into cells to study lytic reactivation and the phosphorylation status of YAP/TAZ. If an immunoblot confirmed that BILF1 expression increased lytic protein levels, as well as decreased inhibitory YAP/TAZ phosphorylation, this would suggest that BILF1 contributes to lytic reactivation through additional enhancement of YAP and TAZ activity. Conversely, one could delete BILF1 from the EBV genome through either bacmid cloning or CRISPR-Cas9 mutagenesis to determine if the loss of the vGPCR has deleterious effects on lytic reactivation similar to other herpesviruses. This could be tested by comparing constitutive lytic activity between the ΔBILF1 virus and a wildtype virus in the context of AGS cells for example. If ΔBILF1 virus had less lytic gene expression, and more phosphorylated YAP and TAZ, than the wildtype virus, this would reflect that BILF1 increases lytic reactivation in a YAP or TAZ dependent manner.

Does LMP1-induced lytic reactivation during epithelial cell differentiation require YAP and TAZ?

In Chapter 3, we found that EBV infection induced YAP and TAZ dephosphorylation in NOKs cells; however, we did not observe a decrease in the expression of phosphorylated LATS1, a known kinase of YAP and TAZ. One possibility is that this observation is due to the low levels of LMP1 expressed in the infected basal epithelium and that increased levels of LMP1 expression during epithelial cell differentiation may dampen LATS kinase activity in this setting (87). If so, the activated YAP and TAZ may inhibit further differentiation (88). Additionally, YAP and TAZ have been previously reported to initiate tumor development (89). LMP1 is not expressed in most NPCs; however, LMP1 is commonly expressed in premalignant tissue of NPC (90). This leads us to hypothesize that LMP1 may increase YAP or TAZ

expression or activity as part of its oncogenic program during the progression of a premalignant lesion into a tumor. While we do not yet have an animal model of NPC to test this hypothesis, it is an interesting question to pose for future studies once such a model system is developed. Furthermore, LMP1 is considered an oncogene and can induce EMT when expressed in NPCs (90,91). In breast cancer, EMT induces TEAD2 signaling (92). While LMP1 stabilizes TAZ expression, it would be of great interest if LMP1 also increased the expression of the TEAD gene family as well.

Why are both YAP and TAZ required for lytic reactivation?

An unaddressed question in Chapter 3 was the specific roles of YAP versus TAZ in our EBV-infected cells. YAP and TAZ in some biological contexts are functionally redundant. Indeed, when both YAP and TAZ were transfected together into Burkitt-Akata cells along with the TEADs, the amount of lytic reactivation that was induced was not synergistic. The lack of synergy between these two effectors indicates that the mechanism by which YAP and TAZ induce EBV lytic reactivation is similar, such as a competition for binding to the same limiting coactivator such as the TEADs. However, loss of either YAP or TAZ expression appears to be sufficient to inhibit lytic reactivation in several contexts, including epithelial cell differentiation and treatment of NOKs-Akata cells with TPA**.**

These differences may be due to an unappreciated difference in functionality between these two proteins. For example, maybe YAP, but not TAZ, induces acetylation or other activating epigenetic marks on the EBV genome to enhance lytic reactivation. This hypothesis is supported by recent work by several groups indicating YAP activity induces the accumulation of global acetylation marks (62,93). The work of the Kenney lab and others has reported acetylation of the EBV genome as a vital part of the lytic reactivation cascade (94). The idea that YAP or TAZ may be responsible for inducing epigenetic changes on the EBV genome to facilitate lytic reactivation is worth investigating. It could be done by depleting cells of either YAP

or TAZ expression and conducting RNAseq analysis to look for changes in the cellular and viral gene expression patterns. By identifying loss of cellular gene expression that is specific to either YAP or TAZ (such as CTGF, CYR61, or ANKRD1), it might also be possible to identify novel inducers of lytic reactivation that specifically work in tandem with either YAP or TAZ.

Conclusions

Epstein-Barr virus infection can either be latent or lytic. EBV is kept in latency in EBV-associated cancers such as nasopharyngeal carcinoma and Burkitt's lymphoma. The regulation of the latent-to-lytic switch by cellular factors remains open to novel findings that allow us to better understand, not only viral replication, but also the process of tumorigenesis in EBV-associated malignancies. In Chapter 2, I demonstrated that p63 maintains EBV latency in both epithelial cells and B cell lymphomas. In this study, I showed that p63 isoforms ΔNp63α and TAp63α repress R-mediated lytic reactivation, and this repression of lytic reactivation is independent of ΔNp63α's known role as a repressor of differentiation. I found that ΔNp63α expression induces the expression of the lytic repressor c-myc, though c-myc is not solely responsible for the ability of p63 to inhibit lytic reactivation. Finally, I determined that ΔNp63α expression decreases the phosphorylation of p38 MAPK, and that p38 MAPK is required for R-mediated lytic reactivation. In Chapter 3, I showed that the Hippo signaling effectors YAP and TAZ induce lytic reactivation. I found that these two transcription effectors' abilities to bind to the Z promoter require the TEAD family of proteins. Additionally, I found that this lytic induction system is epithelial-specific, as YAP and TEADs are not expressed in B cells, and only a minority express TAZ. Finally, I showed that LPA, a GPCR ligand commonly found in saliva, can induce YAP and TAZ activation and subsequently induce lytic reactivation. In **Figure 1** and **Figure 2**, I present my models of how ΔNp63α, YAP/TAZ, and TEADs, respectively, impact EBV lytic reactivation. My work indicates that these transcription factors have critical roles in the latent-to-lytic switch in normal tissue and EBV-infected tumors.

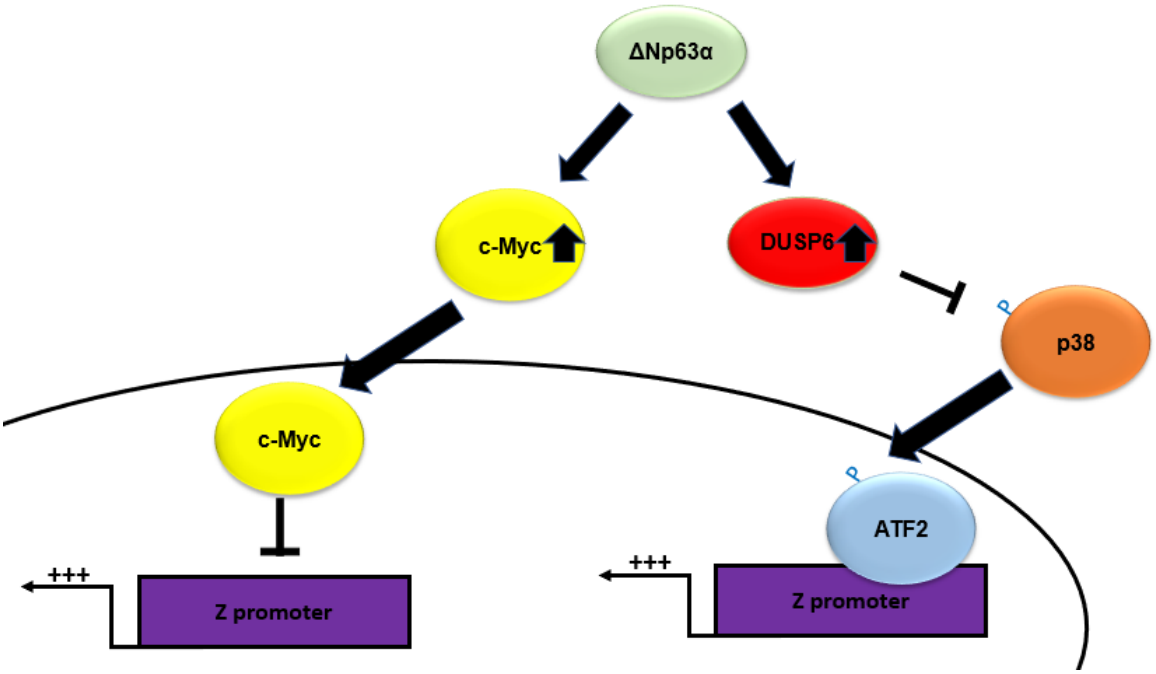


Figure 1: Proposed model for ΔNp63α repression of EBV lytic reactivation. ΔNp63α is present in undifferentiated epithelial tissue, and TAp63α is present in some lymphoma types. ΔNp63α and TAp63α both inhibit EBV lytic reactivation by upregulating the expression of c-myc by interacting with MM1 and reducing the activating phosphorylation of p38 MAPK by increasing the expression of the phosphatase DUSP6. These events ultimately decrease Z promoter activity by repressive chromatin looping in the case of c-myc, or reduced ATF2 activation of the Z promoter.

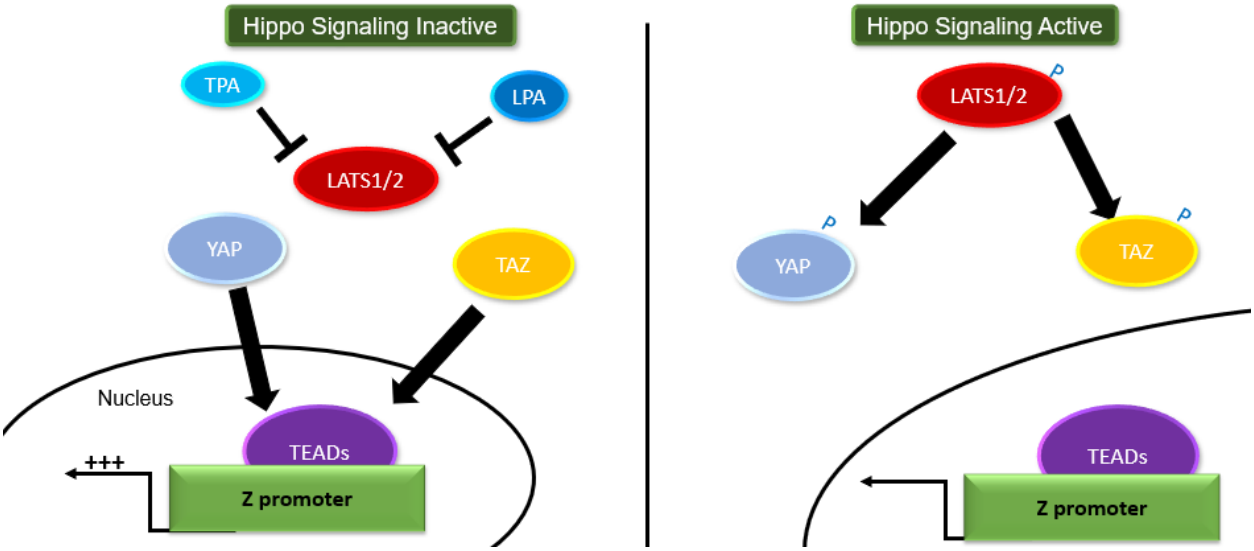


Figure 2: Proposed model for how YAP/TAZ induces lytic reactivation through the TEADs. YAP/TAZ are activated via LPA-specific GPCRs or TPA by preventing the phosphorylation of the LATS1/2 and are ultimately shuttled to the nucleus. In the nucleus, YAP/TAZ binds to the EBV Z promoter via the TEADs to induce EBV lytic reactivation.

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