NEEDHAM, JASON M., M.S. Cell-Specific Regulation of Immediate Early Gene BZLF1 in Epstein-Barr Virus under mTOR Inhibition. (2016)
Directed by Dr. Amy Adamson. 51pp.

Epstein-Barr Virus (EBV) is a nearly ubiquitous human herpesvirus which infects epithelial cells and B-lymphocytes. Additionally, long-term EBV latency has been associated with various forms of cancers including Burkitt's lymphoma, nasopharyngeal carcinoma, and gastric carcinoma. Rapamycin, an mTORC1 inhibitor, has been demonstrated to suppress expression of the immediate-early gene BZLF1 and subsequent lytic replication in B-lymphocytes. Paradoxically, mTOR inhibition has also been reported to increase BZLF1 levels and lytic replication in epithelial cells. In order to identify possible mechanisms for this cell-specific response, a luciferase reporter assay using the BZLF1 promoter was performed in AGS-BX1 epithelial cells under mTOR inhibition. It was found that the removal of terminal YY1-binding domains resulted in the loss of rapamycin-sensitivity of BZLF1 transcription in AGS-BX1 cells. Furthermore, while YY1 was observed to localize to the nucleus of rapamycin-treated Raji B-lymphocytes, little change was observed in YY1 localization in AGS-BX1 cells. These results, in conjunction with those of the luciferase assay, suggest YY1 may play an activating role of BZLF1 transcription in epithelial cells under mTOR inhibition while repressing viral transcription in B-lymphocytes. To test this hypothesis, both AGS-BX1 cells and Raji cells were subject to YY1 knockdown using RNAi transfection and compared using Western Blotting. BZLF1 protein levels under mTOR inhibition in YY1-knockdown AGS-BX1 cells were decreased, while significantly increased in the B-

lymphocytes under the same inhibition. These results suggest that YY1, as regulated by the mTOR pathway, may play an important role in the activation of the lytic cycle of EBV in epithelial cells, while simultaneously acting as a repressor to lytic replication in B-lymphocytes.

CELL-SPECIFIC REGULATION OF IMMEDIATE EARLY GENE BZLF1 IN EPSTEIN-BARR VIRUS UNDER MTOR INHIBITION

by

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A Thesis Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2016

Approved by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

1.1 Epstein-Barr Virus

Epstein-Barr Virus (EBV), also known as human herpesvirus 4, possesses a linear DNA genome, and is thought to currently infect over 90% of adult humans worldwide. Once transmitted to a new host, EBV is capable of entering and replicating within both epithelial cells and B-lymphocytes, potentially remaining unnoticed within the host for decades[1]. Though commonly associated with the nonlethal disease infectious mononucleosis, recent studies have associated long-term EBV infection with various malignancies such as nasopharyngeal carcinoma, endemic Burkitt's lymphoma, and gastric carcinoma[2]. Though the exact mechanisms by which EBV promotes cell transformation is unknown, the high correlation between long-term EBV infection with these cancers suggests EBV has oncogenic properties. Similar to other members of the herpesviridae family, EBV has the ability to undergo either lytic replication or latency on a cell-dependent basis; undergoing solely lytic replication in epithelial cells and both lytic replication and latency within B-lymphocytes[3]. Lytic replication, either during the initial infection or induced from latency, is initiated by the expression of immediate-early genes BZLF1 (Zta) and BRLF1 (Rta). Zta and Rta act as transcription factors for both the viral and host genome; initiating the transcription of the second phase of lytic replication and altering host gene expression to maximize viral efficiency (**Figure 1.1**)

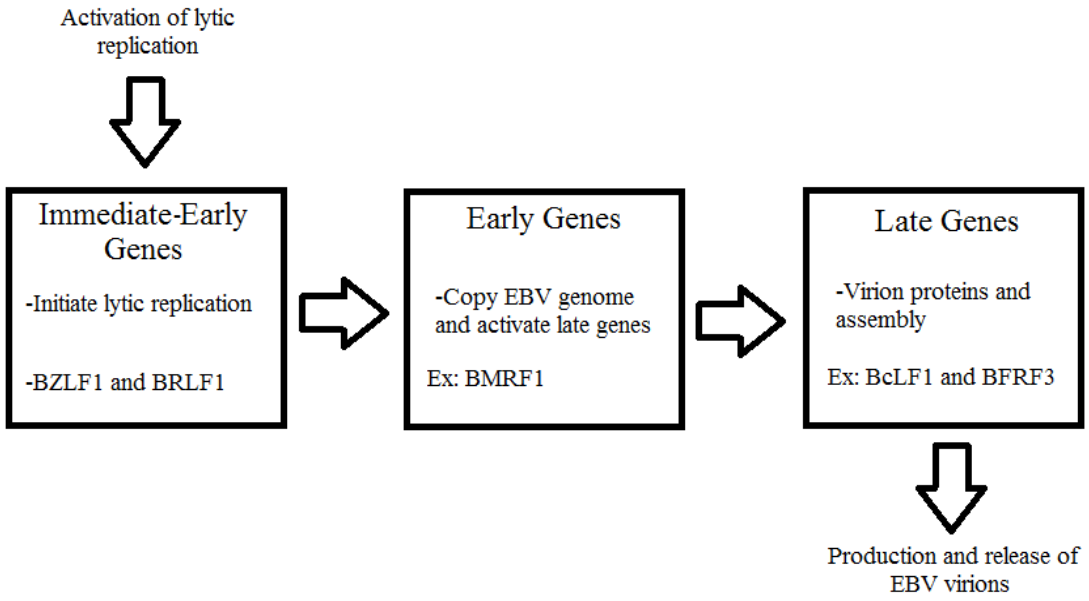

Figure 1.1. Transcriptional Cascade of EBV Lytic Replication. After activation of immediate-early genes via various external and internal stimuli, early genes are expressed. Early genes begin the mass replication of the EBV genome within the host nucleus and activate late genes. Late gene products include virion capsid proteins and virion assembly chaperones, as well as latency promoting proteins to inhibit further activation of the immediate-early genes.

Additionally, splicing of the BZLF1 and BRLF1 mRNA may result in the expression of a fusion protein called RAZ. RAZ, though retaining the DNA-binding domains and dimerization domains for Zta and Rta, lacks the transactivation domain. The expression of RAZ, therefore, inhibits Zta transactivation through the heterodimerization and sequestration of Zta; leading to the next phase of gene expression[4]. This second phase of viral gene expression, designated the early genes, initiate the replication of the viral genome and act as activators for the final wave of gene expression, late genes. Late genes encode proteins necessary for the assembly and release of newly formed, infectious viral particles. Within cells in which EBV is capable of entering latency, additional

proteins and noncoding RNAs are expressed following late gene expression. These proteins promote latency largely through the inhibition of Zta and Rta expression, as well as the epigenetic modification of the EBV genome and decrease of overall EBV gene expression. Latency proteins, such as EBNA1, may also alter host cell gene expression; leading to the downregulation of pro-apoptotic gene expression and subsequent cell immortalization[5]. In this state, EBV may remain latent for decades, only triggering lytic reactivation during the differentiation of B-cell in plasma cells[6].

1.2 mTOR and Rapamycin

The mammalian Target of Rapamycin (mTOR) is an evolutionarily conserved serine-threonine kinase that is ubiquitously expressed within mammalian cells[7]. Initiation of the mTOR pathway may be activated externally by receptor transduction cascades such as antigen receptors, Toll-like receptors, and cytokine receptors; or internally through shifts in ADP/ATP ratios or amino acids levels. Furthermore, downstream effects of mTOR activity include a wide range of functions including autophagy, cell growth, proliferation, immune responses, and cap-dependent translation (**figure 1.2**). Dysregulation of the mTOR pathway may lead to cellular abnormalities such as cancer and genetic defects[8].

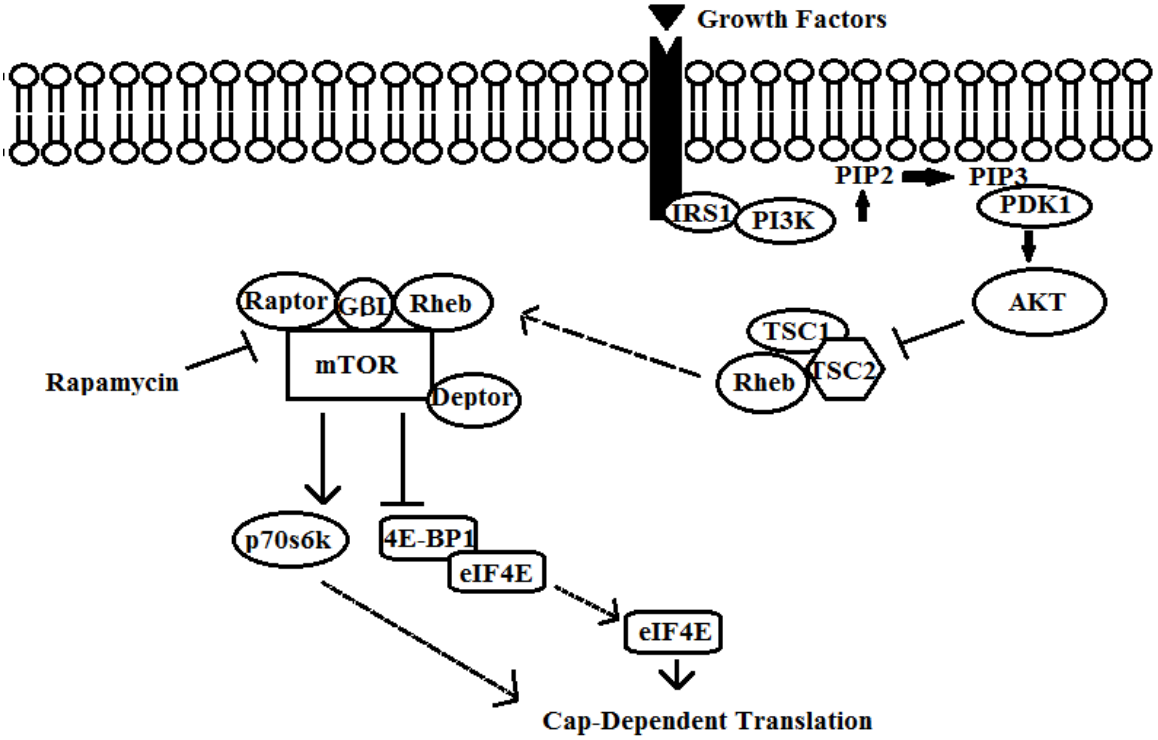

Figure 1.2. The Archetypal Activation of the mTOR Pathway. External activation of receptor tyrosine kinases by various growth factors initiate the AKT signaling pathway. AKT phosphorylation of the TSC1/2 complex releases Rheb, and allows for the interaction with and activation of mTORC1. Through regulation of the phosphorylation of downstream effectors, mTORC1 promotes a wide range of cellular changes including the initiation of cap-dependent translation.

Directly upstream, mTOR is regulated through the release of the GTPase Rheb from the inhibitive TSC1/2 complex by phosphorylation though AKT. Rheb, no longer bound to the TSC1/2 complex, is then free to associate with and activate mTOR. Activated mTOR, though capable of targeting and phosphorylating a large number of substrates, is further regulated by the formation of specific complexes with additional proteins. mTOR complex 1 (mTORC1) is composed of the stable interaction of mTOR with Rheb, Raptor, Deptor, GβL, and is the primary target of the mTOR inhibitor

rapamycin[7]. Rapamycin is a drug which was first isolated in 1975 from Streptomyces hygroscopicus, a streptomycete found in the soil of Easter island. While the drug was found to inhibit a variety of fungi, such as Candida albicans, Microsporum gypseum, and Trichophyton granulosum; the compound exhibited only a marginal inhibition of gram-positive bacterial species and no inhibition of gram-negative species. Soon after, it was found that rapamycin also displayed anti-proliferative and immunosuppressive effects on other eukaryotic cells through the inhibition of the mTOR pathway. Rapamycin specifically inhibits mTORC1 by destabilizing interactions between mTOR and Raptor, thereby reducing mTOR's ability to target and phosphorylate proteins[9]. The inhibition of mTOR has since been shown to suppress lytic activation of a variety of herpesviruses such as Kaposi's sarcoma virus, cytomegalovirus, herpes simplex virus 1, and murine gammaherpesvirus. It was also recently discovered that mTOR inhibition alters EBV's ability to enter lytic replication in a cell-specific manner. While rapamycin treatment leads to suppression of lytic replication of EBV in B-lymphocytes, lytic replication was significantly increased in epithelial cells. This increase of lytic replication was defined by increases in immediate-early gene protein levels, as well as increases in subsequent early gene protein levels[10].

1.3 Yin Yang 1

Yin yang 1 (YY1) is a ubiquitously expressed transcription factor known to play a crucial role in the transcription of cellular genes regulating proliferation, apoptosis, and differentiation, as well as several mitochondrial genes. Named for its ability to

simultaneously activate and repress the transcription of both cellar and viral genes, YY1's activity is highly dependent upon post-translational modifications, localization, and protein interactions[11]. While YY1 protein interactions have been well-documented, little is known about the effects of the post-translational modifications. Post-translational modifications which are known to occur on YY1 include phosphorylation of serine/threonine and tyrosine residues, glycosylation, nitrosylation, sumosylation, acetylation, and methylation. Phosphorylation of YY1 is known to occur within two of the three zinc-finger DNA-binding domains, and is thought to alter DNA-binding efficiency[12]. YY1 is of particular interest for this study as YY1 has been demonstrated to interact with mTOR and Raptor of mTORC1[13], and is known to bind to and repress the promoters of EBV's immediate-early genes, BZLF1 and BRLF1, in B-lymphocytes[14, 23]. Additionally, YY1 expression, localization, and DNA binding efficiency have been reported to vary substantially through cell cycling[15, 16].

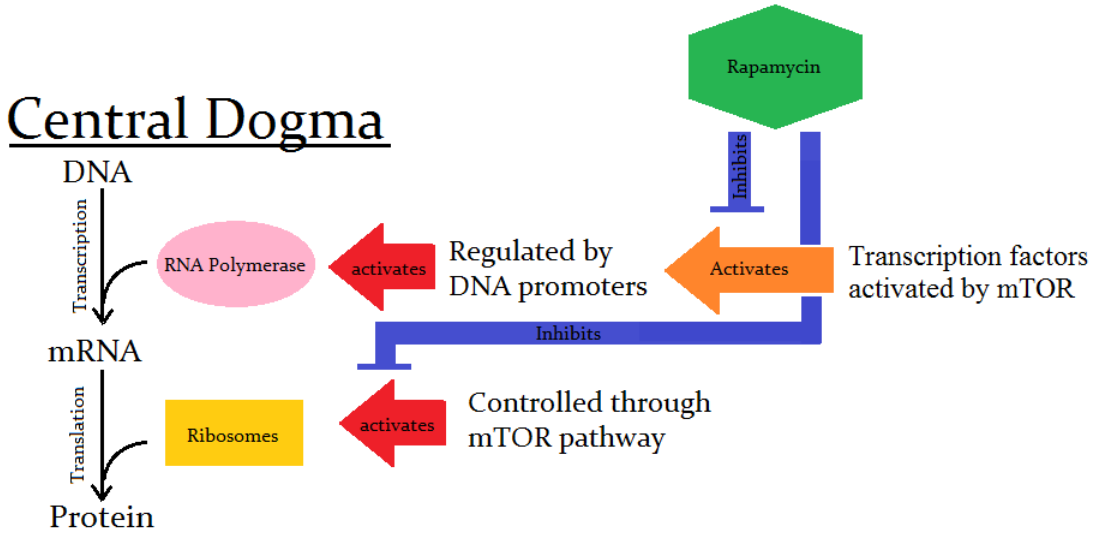

Figure 1.3. Possible Mechanisms by which Rapamycin-induced mTOR Inhibition Affects Changes in Protein Expression. Since the mTOR pathway may lead to changes in both transcription factor activity and cellular cap-dependent translation, it is unknown whether cell-specific EBV activation is the result of altered transcription or translation.

1.4 Project Overview

The mTOR pathway is a ubiquitous and vital pathway for sensing and regulating cellular processes including metabolism, growth, transcription, and cap-dependent translation[7]. While activation of Epstein-Barr Virus lytic replication is expectedly repressed by mTOR inhibition in B-lymphocytes, it is paradoxically increased in epithelial cells[10]. As a downstream target of mTOR activity, the transcription factor Yin yang 1 is a common regulator of cellular and viral gene expression and is known to bind to the promoter elements of both EBV immediate-early genes[14]. The goal of this study was to determine if the cell-specific changes of EBV lytic activation in response to mTOR inhibition is specifically mediated through the activity of YY1 (**figure 1.3**).

1.5 Specific Aims

1. Analyze the relative BZLF1 levels under chemical mTOR inhibition within epithelial cells and B-lymphocytes at various time points post-induction of EBV lytic replication.
2. Use luciferase reporter constructs to determine the relative contribution of upstream segments of the BZLF1 promoter to gene expression under chemical mTOR inhibition.
3. Elucidate the degree and direction of YY1 localization changes under mTOR inhibition in epithelial cells and B-lymphocytes.
4. Determine the effects of YY1 knockdown in epithelial cells and B-lymphocytes under chemical mTOR inhibition.

CHAPTER II

METHODS

2.1 Reagents

Raji B-lymphocytes and AGS-BX1 cells were obtained from ATCC and gifted from L. Hutt-Flechter respectively. Cells were grown in Ham's F12 supplemented with 10 fetal bovine serum and treated with penicillin, streptomycin, fungicide and 500ug/mL G418. Lytic replication was induced using 20ng/mL TPA, 3mM sodium butyrate for B-lymphocytes; and 5ng/mL TPA, 0.75mM sodium butyrate for epithelial cells (both from Sigma-Aldrich). Rapamycin in DMSO was obtained from Sigma-Aldrich. pGL3-Basic and pGL4.75 plasmids[18] were purchased from Promega, with specific primers obtained from Eurofins. Plasmid isolation and purification was performed using a Qiagen Maxi column and suspended in TE buffer (10nM Tris-HCl pH 8.0, 1mM EDTA). YY1 knockdown was performed using a Qiagen YY1 RNA probe kit, and controlled against the Qiagen All-Stars scrambled RNA reagent. Chemical transfection was achieved using Lipofectamine 2000[19] for epithelial cells, and additional 2 second electroporation at 250V, 950mA for B-lymphocytes. Luciferase reagents were purchased from Promega and mixed prior to each luciferase assay[20]. Whole protein extraction was performed by suspending cells in lysis buffer (0.25M NaCl, 0.1% NP40, 50nM HEPES, 5nM EDTA), subjecting cells to two freeze/thaw cycles, centrifugation, and collection of the resulting

supernatant. Cytosolic protein extraction was performed by treating whole cells with CE buffer + NP-40 (10mM Hepes pH 7.6, 60mM KCl, 1mM EDTA, 1mM DTT, 0.7% NP-40). After harvesting cytosolic proteins, nuclei were washed using CE buffer two times before being suspended in lysis buffer and undergoing the previously mentioned whole protein extraction protocol. Separated proteins were transferred to Immobulin (Millipore) membranes and blocked using a blocking buffer (0.25% dried milk powder with 1% Tween-20 and 1xPBS). Anti-BZLF1, anti-Tubulin, anti-YY1, and anti-TBP antibodies (Santa Cruz or Cell signaling) were used to probe Western blots using a dilution of 1:500 in 0.25% milk blocking solution. Secondary antibodies (goat anti-rabbit or rabbit anti-mouse; from Jackson Immunoresearch) were diluted at 1:5000 in 0.25% milk blocking buffer and used for visualization with Advansta reagents on a Li-Cor blot developer.

2.2 Cell Culture

As a model for epithelial cells throughout these experiments, a AGS-BX1 gastric carcinoma cell line was used. Though EBV is typically unable to undergo latency in epithelial cells, AGS-BX1 cells contain the viral genome containing a G418 resistance gene which is only expressed while the viral genes are transcriptionally silenced. By using AGS-BX1 cells, the presence of the EBV genome was not only ensured through G418 selection, but lytic replication could also be inhibited. Additionally, EBV has already demonstrated enhanced activation in AGS-BX1 under mTOR inhibition. The AGS-BX1 cell line was grown at 37^{o}c in 20mL of supplemented Ham's F12 media (Ham's F12, 10% fetal bovine serum, fungicide, streptomycin, and penicillin) in a

150cm^2 culture flask until confluent. At confluency, 2mL of the non-enzymatic cell dissociation solution Cellstripper was used to collect AGS-BX1 cells, after which cells were washed in 1x sterile phosphate buffered saline (PBS) to remove residual dissociation solution, and redistributed onto 60mm plates with 5mL of supplemented Ham's F12 and G418. Prior to rapamycin treatment, plates containing AGS-BX1 cells were washed in sterile 1x PBS to remove residual G418, and re-aliquoted 5mL of supplemented Ham's F12. Viral induction was achieved by the removal of G418 and subsequent treatment with 5ng/mL TPA, 0.75mM sodium butyrate.

Raji Burkitt's lymphoma cells were selected as the model cell line for B-lymphocytes due to Raji cells possessing a latent EBV genome and were previously identified to display reduced lytic activation under mTOR inhibition. Raji cells were grown at 37^{o}c in a 150cm^2 culture flask with supplemented RPMI media (RPMI, 10% fetal bovine serum, fungicide, streptomycin, and penicillin) until confluent. Unlike AGS-BX1 cells, Raji cells are neither adherent nor require G418 selection to maintain latency of the EBV genome. As such, Raji cells were collected and washed in 1x sterile PBS without the use of a dissociation solution, and then aliquoted into 25cm^2 culture flasks containing only 5mL of the supplemented RPMI. In order to induce lytic replication, cells were treated with 20ng/mL TPA, 3mM sodium butyrate.

2.3 Western Blot for BZLF1, YY1, and Tubulin

Protein extraction of harvested AGS-BX1 and Raji cells was performed by washing cells in sterile 1mL of 1x PBS, followed by suspension in 30uL of lysis buffer (0.25M NaCl, 0.1% NP40, 50nM HEPES, 5nM EDTA) with protease/phosphatase inhibitors. This suspension then underwent two freeze/thaw cycles at -80^{o}c and 37^{o}c respectively, and spun down at 12000rpms for 10 minutes prior to harvesting the resulting supernatant. Protein extracts was then quantified using an Eppendorf Bio Photometer. For each sample, the appropriate volume of extract to contain 20ug of protein was then aliquoted into an equal volume of protein dye and boiled at 100^{o}c for 2 minutes. Prepared protein extracts were separated using 10% SDS-PAGE at 200V and transferred to Immobulin PVDF at 100mA overnight. The resulting western blots were blocked in a 0.25% milk solution (0.5g powdered milk, 0.1% Tween 20, 1x PBA) using the SNAP I.D. 2.0 vacuum system. Immunostaining was then performed by incubating blocked blots with Santa Cruz (BZLF1, Tubulin, YY1) or Cell signaling (TBP) primary antibodies in a 1:500 dilutions of 0.25% milk solution. Incubated blots were then washed three times with Western wash (0.1% Tween 20, 1x PBS) and incubated with secondary antibodies (Jackson Immunoresearch rabbit anti-mouse or goat anti-rabbit) diluted at 1:5000 for 10 minutes. After three washes, the probed blots were placed in Li-Cor WesternSure ECL substrate, developed using a Li-Cor blot developer at high exposure, and stored at 0^{o}c. The detected protein bands were quantified using Image Studio Digital software and recorded for data analysis.

2.4 Luciferase Reporter Plasmid Construction

To construct the luciferase reporter plasmids, a pGL-3 Basic expression vector containing a luciferase gene, an ampicillin resistance gene, and cloning site was obtained from Promega and amplified in DH5-α E. coli in 200mL of ampicillin containing Lim broth (LB). The amplified plasmid was purified using a Qiagen Plasmid Maxi extraction column and analyzed for purity using a NanoDrop ND-1000 Spectrophotometer. Promoter inserts were generated by PCR of the BZLF1 promoter using forward and reverse primers containing Bgl11 and Hind111 restriction endonuclease sites respectively. Five individual forward primers and a single reverse primer corresponding to the transcriptional start site were used to generate 509, 409, 359, 309, and 259bp lengths of the BZLF1 promoter (**figure 2.1**). The template EBV genome was extracted by using a modified Hirt procedure for extrachromosomal DNA purification[21] from untreated Raji B-lymphocytes.

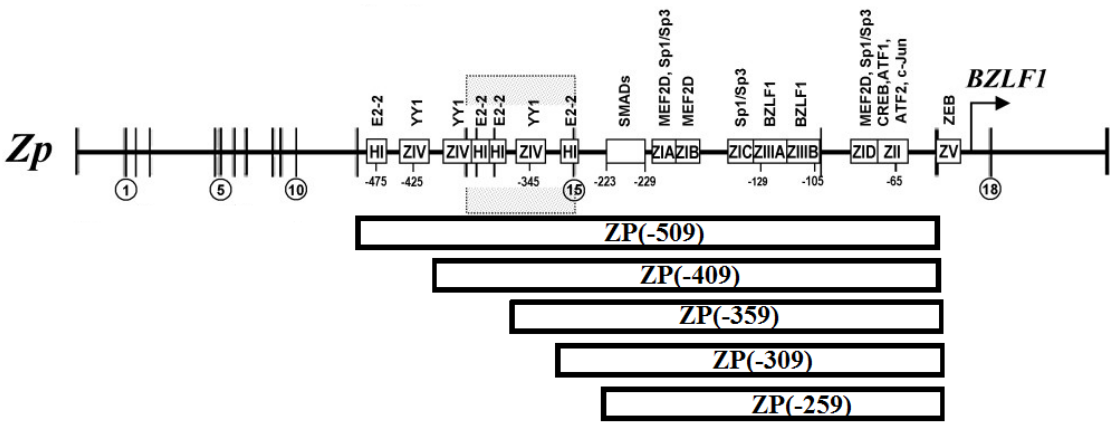

Figure 2.1. BZLF1 Promoter and Luciferase Reporter Construct Inserts. Each insert designation corresponds to the length of the insert starting at the transcriptional start site and moving in the upstream direction.

Following amplification of these promoter inserts, both the pGL3-Basic plasmids and inserts were digested with Bgl11 and Hind111 restriction endonucleases for three hours at 37^{o}c and separated on a 0.8% agarose gel. The DNA bands corresponding to the digested plasmid and insert were purified from the agarose gel, mixed at a 1:2 ratio in the presence of T4 DNA ligase, and incubated at room temperature overnight. The constructed plasmids then transformed into chemically-competent DH5-α E. coli, which were plated onto ampicillin-containing LB agarose plates and incubated overnight at 37^{o}c. Individual colonies were isolated and grown on separate ampicillin LB agar plates and confirmed as containing the specific reporter plasmid through PCR. Colonies which tested positive for the desired promoter length were then amplified in 200mL of ampicillin LB broth, harvested, and purified for the reporter plasmid in TE buffer. Each reporter plasmid purification was then analyzed for optical density and labelled according to its promoter length. Additionally, each purified constructed reporter plasmid sample was sent for DNA sequencing to Eurofins (**figure 2.2**). The resulting DNA sequences were analyzed using BLAST nucleotide to ensure the presence of the intended viral promoter and lack of procedural mutations in the promoter sequences.

ZP(-509):

CGAGGCAAGTCATCTGTTGGAGGACCCTGATGAAGAAACCAGTCAGGCCGTGAAGGCCCTAAGGGAGATGGCTGACACTGTTATTCCCCA
GAAGGAGGAAGCAGCCATATGTGGACAGATGGACCTGAGCCACCCGCCCCCTCGTGGCCATTTGGACGAACTGACCACAACACTAGAGTCC
ATGACAGAGGATTTGAATCTGGACTCCCCCCTGACCCCCGAACTTAATGAAATCTTGGATACATTTCTAAATGATGAATGTCTGCTGCA
TGCCATGCATATTTCAACTGGGCTGTCTATTTTTGACACCAGCTTATTTTAGACACTTCTGAAAACTGCCTCCTCCTCTTTTAGAAACT
ATGCATGAGCCACAGGCATTGCTAATGTACCTCATAGACACACCTAAATTTAGCACGTCCCAAACCATGACATCACAGAGGAGGCTGGTG
CCTTGGCTTTAAAGGGGAGATGTTAGACAGGTAACTCACTAAACATTGCACCTTGCCG

ZP(-409):

AAGCAGCCATATGTGGACAGATGGACCTGAGCCACCCGCCCCCTCGTGGCCATTTGGACGAACTGACCACAACACTAGAGTCCATGACAGA
GGATTTGAATCTGGACTCCCCCCTGACCCCCGAACTTAATGAAATCTTGGATACATTTCTAAATGATGAATGTCTGCTGCATGCCATGC
ATATTTCAACTGGGCTGTCTATTTTTGACACCAGCTTATTTTAGACACTTCTGAAAACTGCCTCCTCCTCTTTTAGAAACTATGCATG
AGCCACAGGCATTGCTAATGTACCTCATAGACACACCTAAATTTAGCACGTCCCAAACCATGACATCACAGAGGAGGCTGGTGCCTTGGC
TTTAAAGGGGAGATGTTAGACAGGTAACTCACTAAACATTGCACCTTGCCG

ZP(-359):

CATTTGGACGAACTGACCACAACACTAGAGTCCATGACAGAGGATTTGAATCTGGACTCCCCCCTGACCCCCGAACTTAATGAAATCTTG
GATACATTTCTAAATGATGAATGTCTGCTGCATGCCATGCATATTTCAACTGGGCTGTCTATTTTTGACACCAGCTTATTTTAGACAC
TTCTGAAAACTGCCTCCTCCTCTTTTAGAAACTATGCATGAGCCACAGGCATTGCTAATGTACCTCATAGACACACCTAAATTTAGCAC
GTCCCAAACCATGACATCACAGAGGAGGCTGGTGCCTTGGCTTTAAAGGGGAGATGTTAGACAGGTAACTCACTAAACATTGCACCTTGC
CG

ZP(-309):

TCTGGACTCCCCCCTGACCCCCGAACTTAATGAAATCTTGGATACATTTCTAAATGATGAATGTCTGCTGCATGCCATGCATATTTCAA
CTGGGCTGTCTATTTTTGACACCAGCTTATTTTAGACACTTCTGAAAACTGCCTCCTCCTCTTTTAGAAACTATGCATGAGCCACAGGC
ATTGCTAATGTACCTCATAGACACACCTAAATTTAGCACGTCCCAAACCATGACATCACAGAGGAGGCTGGTGCCTTGGCTTTAAAGGG
GAGATGTTAGACAGGTAACTCACTAAACATTGCACCTTGCCG

ZP(-259):

TAAATGATGAATGTCTGCTGCATGCCATGCATATTTCAACTGGGCTGTCTATTTTTGACACCAGCTTATTTTAGACACTTCTGAAAAC
TGCCTCCTCCTCTTTTAGAAACTATGCATGAGCCACAGGCATTGCTAATGTACCTCATAGACACACCTAAATTTAGCACGTCCCAAACC
ATGACATCACAGAGGAGGCTGGTGCCTTGGCTTTAAAGGGGAGATGTTAGACAGGTAACTCACTAAACATTGCACCTTGCCG

Figure 2.2. DNA Sequence of BZLF1 Promoter Inserts. Each insert name corresponds to the each of the promoter insert, with ZP(-509) representing the full-length BZLF1 promoter. BLAST nucleotide analysis of the sequenced promoter inserts had a 100% match to known EBV BZLF1 sequences.

2.5 Transfection of Nucleic Acids

For transfection of nucleic acids into epithelial cells, 60mm plates of AGS-BX1 were grown to confluency in supplemented Ham's F12 media under G418 suppression. Immediately prior to transfection, cells were washed in sterile 1x PBS and placed in 5mL of Ham's F12 media. Transfection media for the luciferase reporter assay was prepared using 3ug of BZLF1 promoter pGL-3 plasmid and 3ug of the constitutively active renilla

reporter plasmid pGL-4.75 in 300uL of Ham's F12 media. The transfection media for the YY1 knockdown, alternatively, used either 3uL of each of the four Qiagen YY1 siRNAs (12uL in total) or 12uL of Qiagen All-Star scrambled RNA suspended in 300uL of Ham's F12 media. Concurrently, 6uL of Lipofectamine 2000 transfection reagent was added to aliquots of 300uL Ham's F12 for each nucleic acid suspension. The nucleic acid and Lipofectamine suspensions were then mixed to a volume of 600uL and incubated at room temperature for 20 minutes. Each 60mm plate of AGS-BX1 cell was then treated with 100uL of the transfection media for 24-hours, after which media was washed off using sterile 1x PBS and replaced with supplemented Ham's F12 media. AGS-BX1 cells transfected with luciferase reporter plasmids were treated immediately following removal of transfection media while YY1 knockdown AGS-BX1 cells were treated with G418 and incubated an additional 4 days prior to the removal of G418 media, washing with sterile 1x PBS, addition of supplemented Ham's F12 media, and subsequent experimentation.

Raji B-lymphocytes were transfected using a novel combination of Lipofectamine 2000 chemical transfection and electroporation which produced higher transfection rates than either method individually. The transfection media used for electroporation of the four Qiagen YY1 siRNAs in Raji cells was prepared with the same protocol used for AGS-BX1 transfection media but using RPMI in lieu of Ham's F12. At confluency, 6mL of Raji cells in enriched RPMI were collected, washed in sterile 1x PBS, and suspended in 3mL of cold RPMI media. 500uL of the Raji suspension was then pipetted into electroporation cuvettes along with 100uL of the transfection media, and left on ice for 10 minutes prior to electroporation. Each sample was then electroporated at 950uF, 250V

for 2 seconds and incubated on ice for an additional 10 minutes. Electroporated Raji cells were then added to individual 25cm^2 culture flasks containing 5mL of supplemented RPMI, and incubated at 37^{o}c for 4 days prior to rapamycin treatment. The Raji electroporation protocol was then confirmed using pGL4.75 renilla luciferase as a marker for transfection. All luciferase reporter assays were performed on a Biotek Synergy 2 plate reader in accordance with the luciferase reporter assay protocol.

2.6 Luciferase Reporter Assay

AGS-BX1 cells transfected with luciferase reporter plasmids were treated with 0nM, 1nM, 5nM, or 10nM rapamycin immediately following removal of transfection media. After 24 hours of rapamycin treatment, EBV lytic replication was induced using 5ng/mL TPA, 0.75mM sodium butyrate for an additional 24 hours before cells were harvested. Cells were scraped from their individual plates, washed in sterile 1xPBS, suspended in 75uL of 1xPBS on an optically clear 96-well plate, and treated with the Promega DualGlo Luciferase Assay kit reagents. After a 10-minute incubation period at room temperature, expression of the BZLF1-controlled luciferase reporter plasmid and constitutively-active renilla luciferase plasmid were separately quantified using a BioTek Synergy 2 spectrophotometric plate reader. Luciferase expression of the BZLF1 promoter constructs and renilla luciferase plasmids were expressed as relative light units (RLUs) and recorded for data analysis. Five biological replicates and two technical replicates were collected and analyzed for each of the five constructed plasmids.

2.7 YY1 Localization

To determine if rapamycin treatment and/or EBV induction resulted in shifts in YY1 localization, nuclear and cytosolic extracts were collected for individual analysis. AGS-BX1 and Raji cells were grown to confluency in supplemented Ham's F12 and RPMI media respectively, treated with 0nM, 1nM, 5nM, and 10nM rapamycin for 24 hours, and induced with TPA/NaBut for an additional 24 hours. To collect the cytosolic extract, cells were harvested and suspended in CE buffer (10mM HEPES, 60mM KCl, 1mM EDTA, 1mM DTT) + 0.7% NP-40 with protease/phosphatase inhibitors, and incubated on ice for 5 minutes. The resulting mixtures were spun down at 8000rpm for 4 minutes prior to the collection of the supernatant, labelled cytosolic extract. The resulting pellet was then washed twice in CE buffer and suspended in 100uL of CE + 0.7% NP-40 with protease/phosphatase inhibitors. The suspension was subject to two freeze/thaw cycles and centrifuged at 10,000rpm for 10 minutes. The resulting supernatant for each sample was collected and labeled as the nuclear extract. Both the cytosolic and nuclear extracts were analyzed for optical density using the NanoDrop ND-1000 Spectrophotometer and separated using 10% SDS-PAGE using 20ug of protein per well. The separated proteins were transferred to Immobulin PVDF membranes using Western Blot, and probed for YY1. Standardization of YY1 quantifications were performed using either tubulin or TATA-binding protein (TBP) for the cytosolic and nuclear extracts respectively. Normalized YY1 quantifications were then standardized to the untreated, uninduced sample, and expressed as a ratio of the normalized nuclear extract to the normalized cytosolic extract.

2.8 YY1 Knockdown

In order to measure the potential role of YY1 in BZLF1 expression under mTOR inhibition, YY1 knockdown studies were performed in AGS-BX1 and Raji cells. Both AGS-BX1 and Raji cells were first transfected with four Qiagen YY1 siRNAs using their respective transfection methods. Initial experimentation of YY1 knockdown determined that the greatest level of YY1 depression occurred on day six post-transfection (**Figure 3.13**). As such, both transfected cell lines were treated with 0nM, 1nM, 5nM, or 10nM rapamycin on day four, induced with TPA/NaBut on day five, and harvested on day six. Cells were washed in sterile 1x PBS and subject to whole cell protein extraction using ELB lysis buffer with protease and phosphatase inhibitors. 20ug of each protein extract was then separated using 10% SDS-PAGE and compared with Western Blotting. The resulting blots were immunostained for BZLF1 and tubulin, and developed using the Li-Cor Western blot developer. BZLF1 band quantifications were normalized using tubulin band quantifications, and held relative to the normalized untreated induced value.

2.9 Data Analysis

For Western blot immunostaining, BZLF1 and YY1 quantification values were normalized using either tubulin or TBP as a loading control, with normalized values held relative to the untreated, induced sample. YY1 localization was further expressed as a ratio of the normalized, relative values of the nuclear extract to the cytosolic extract. Standard error was calculated by dividing the standard deviation of all normalized,

relative quantifications by the square of the number of samples. Significance was tested using a heteroscedastic student two-tailed T-test, with $p<0.05$ considered significant.

Results of the luciferase reporter assay were analyzed by dividing the RLUs of the BZLF1 promoter construct by those of the renilla luciferase. This allowed for the variation in sample transfection efficiency, as well global, non-specific changes of transcription, to be controlled. These normalized RLU values were then held relative to the untreated, induced condition within each replicate. Standard error was calculated by dividing the standard deviation of all normalized, relative values by the square of the number of samples. Significance was tested using a heteroscedastic student two-tailed T-test, with $p<0.05$ considered significant.

CHAPTER III

RESULTS

3.1 Changes in Expression Patterns of BZLF1 in AGS-BX1 and Raji Cells Under mTOR Inhibition

As predicted from the literature, levels of BZLF1 in untreated Raji B-lymphocytes increased from undetectable levels at the 0 hour, to a maximum at 16 hours post-induction before decreasing[3]. While protein levels appeared to increase again at 24 hours post-induction, these levels do not reach those seen at 16 hours. BZLF1 levels in the 5nM rapamycin-treated Raji cells, however, remained greatly reduced relative to the untreated group at every hour post-induction (**figure 3.1**). Additionally, instead of forming two expressive peaks at 16 hours and 24 hours post-induction, BZLF1 levels in rapamycin-treated Raji cells formed only a single, long peak at 20 hours post-induction.

A similar pattern of BZLF1 expression was seen in the untreated AGS-BX1 cells as found in the Raji cells. Protein levels of BZLF1 increased after induction to a maximum at 16 hours post-induction before decreasing between 18-20 hours post induction (**figure 3.2**). At 24 hours post-induction, however, BZLF1 levels increased again to levels similarly expressed at 16 hours. BZLF1 levels in rapamycin-treated AGS-BX1 cells displayed a single peak of expression at 18 hours post-induction, representing a 20% increase in BZLF1 protein relative to the untreated cells.

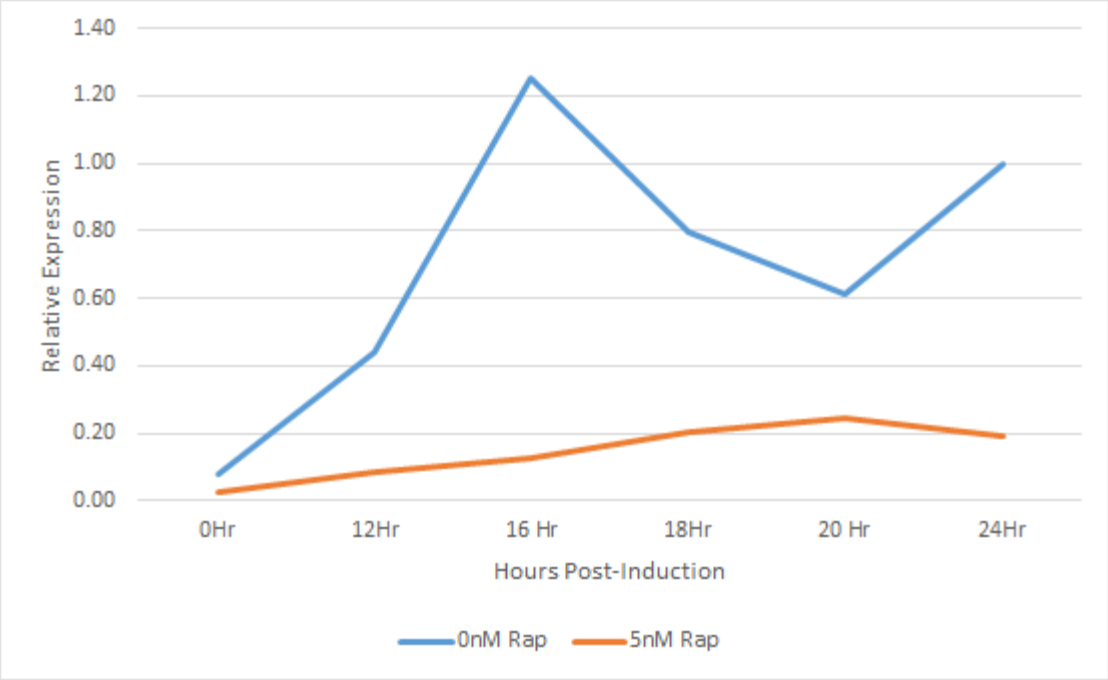

Figure 3.1. Relative Expression of BZL1 in Raji B-lymphocytes Over Time. While BZLF1 in untreated Raji cells formed peaks at 16 and 24 hours post-induction, BZLF1 levels in cells treated with 5nM rapamycin did not substantially increase in expression relative to the untreated group. Data set represents two biological replicates, each with two technical replicates.

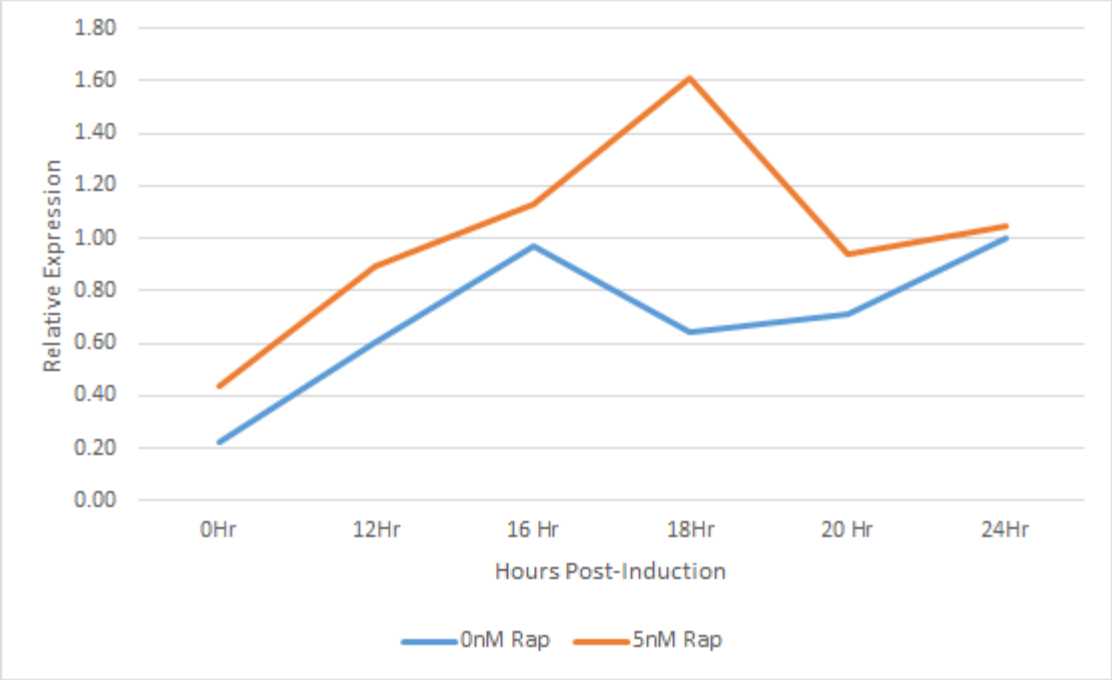

Figure 3.2. Relative Expression of BZLF1 in AGS-BX1 Epithelial Cells Over Time. While the rate of increase in BZLF1 expression appeared consistent between the treated and untreated groups, BZFL1 levels continued to increase in the 5nm rapamycin treated group; forming an expressive peak at 18 hours post-induction. Data set represents two biological replicates, each with two technical replicates.

3.2 Relative Contribution of YY1 Binding Domains on BZLF1 Expression in AGS-BX1 Cells

In AGS-BX1 cells, each luciferase reporter plasmid was assayed to look for changes in rapamycin sensitivity, as well as general changes in expression relative to the untreated, uninduced control (**Figure 3.3)**. ZP(-509), which contained the entirety of the known BZLF1 promoter, displayed an expression pattern similar to that of the BZLF1 protein levels: luciferase expression positively correlated with increases in rapamycin treatment, and decreased spontaneous activation of luciferase expression with rapamycin treatment (**Figure 3.4**). Unlike the BZLF1 protein levels, however, luciferase levels in the

rapamycin-treated, induced cells were reduced relative to the untreated, induced cells. The deletion of the first 100bp of the BZLF1 promoter appears to result in both rapamycin insensitivity as no significant differences ($p \geq 0.75$) were noted between the untreated and treated induced samples (**Figure 3.5**). Additionally, loss of rapamycin-associated suppression of spontaneous transcription was also seen in the ZP(-409) reporter plasmid. This loss of the rapamycin-associated spontaneous expression, and even the partial increase in spontaneous expression, was further seen in the ZP(-359), ZP(309), and ZP(-259) plasmids. Positively correlated rapamycin sensitivity was seen in ZP(-359) plasmids, and at a greater degree of enhancement than in ZP(-509) (**Figure 3.6**). This enhancement, however, was lost in both ZP(-309) and ZP(-259) plasmids with the deletion of the final YY1 domain (**Figure 3.7** and **Figure 3.8**). In addition, luciferase expression of ZP(-259) in all rapamycin treated, induced cells were reduced relative to the untreated, induced standard. In comparison to the full length ZP(-509) promoter, only ZP(-409) reporter plasmid expressed reduced relative expression of luciferase activity (**Figure 3.9**) as determined by standardizing to the untreated, uninduced control. The other three plasmids increasingly expressed elevated luciferase expression which inversely correlated with promoter length.

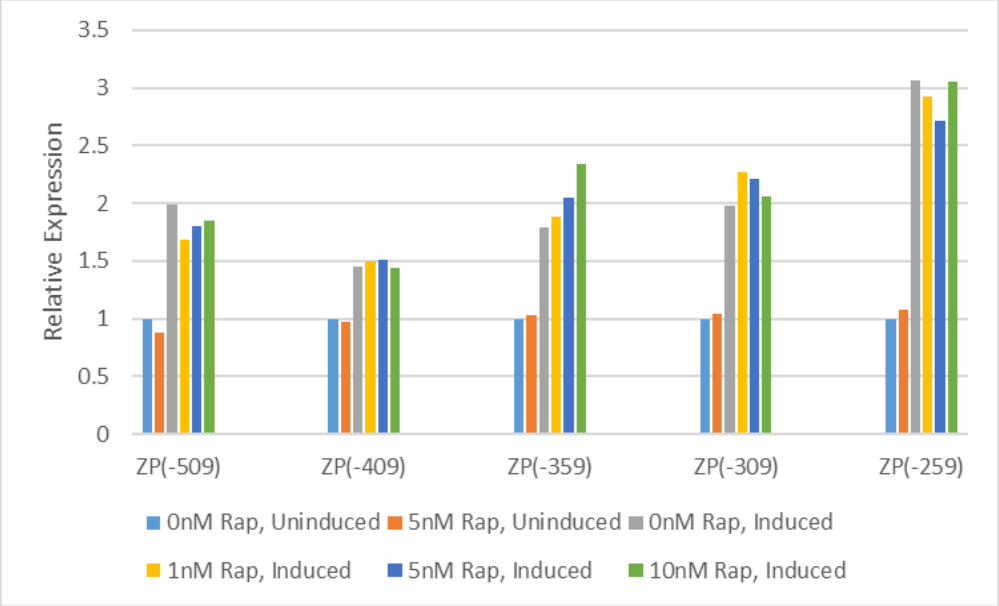

Figure 3.3. BZLF1 Promoter Luciferase Reporter Assay. Results are standardized to the untreated, uninduced sample in each plasmid. While ZP(-409) expressed lower levels of luciferase expression than ZP(-509), the other three plasmids consistantly expressed elevated levels of luciferase compared to the full-length BZLF1 promoter. Data sets represent five biological replicates with two technical replicates.

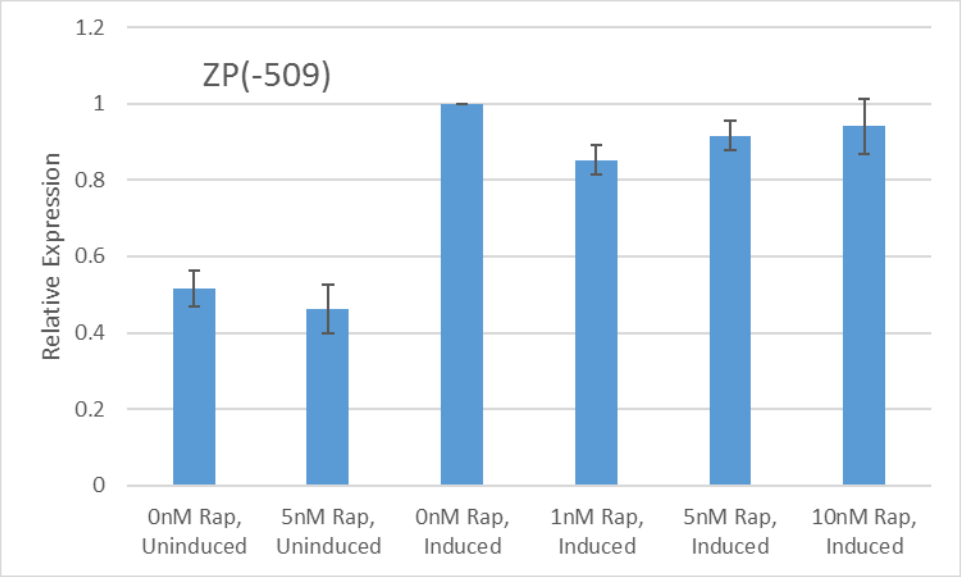

Figure 3.4. Luciferase Reporter Assay Results of the ZP(-509) Plasmid. These results mimic with the observed patterns in BZLF1 protein expression such as positive correlation between expression and rapamycin concentration, and rapamycin suppression of leaky activation in the treated, uninduced sample. Data set represent five biological replicates with two technical replicates.

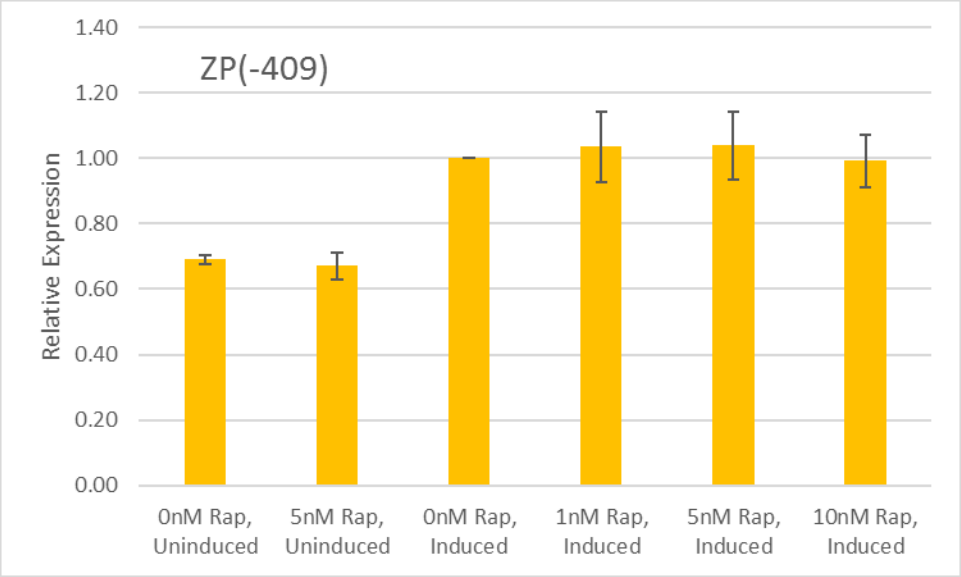

Figure 3.5. Luciferase Reporter Assay Results of the ZP(-409) Plasmid. Loss of rapamycin sensitivity in both induced and uninduced groups were seen. Data set represent five biological replicates with two technical replicates.

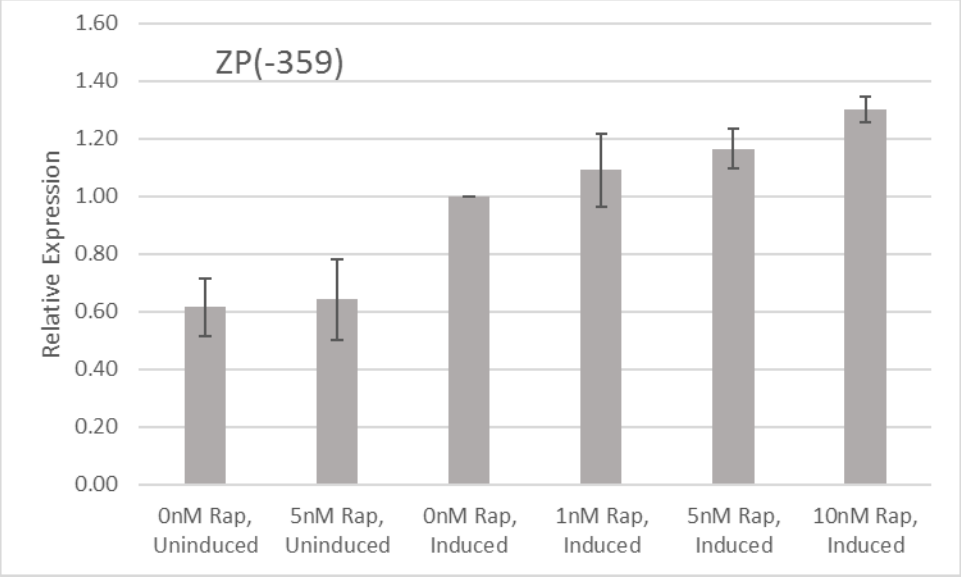

Figure 3.6. Luciferase Reporter Assay Results of the ZP(-359) Plasmid. Though luciferase expression was positively correlated with increasing rapamycin concentrations in induced samples, insensitivity to rapamycin in the 5nM, uninduced sample was observed. Data set represent five biological replicates with two technical replicates.

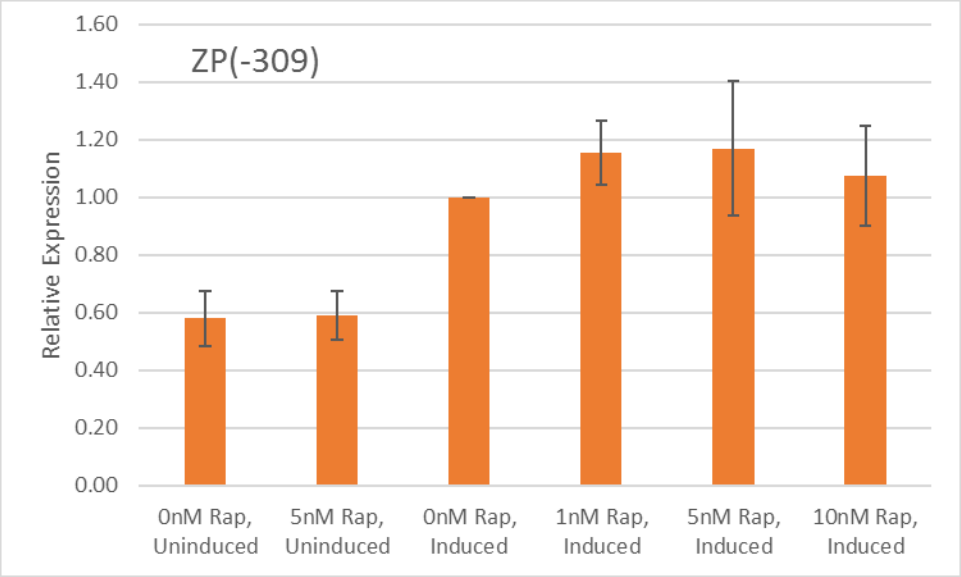

Figure 3.7. Luciferase Reporter Assay Results of the ZP(-309) Plasmid. Rapamycin insensitivity was observed the 5nM, uninduced sample. Additionally, an inverse correlation between luciferase expression and rapamycin concentration appears in the treated, induced groups. Data set represent five biological replicates with two technical replicates.

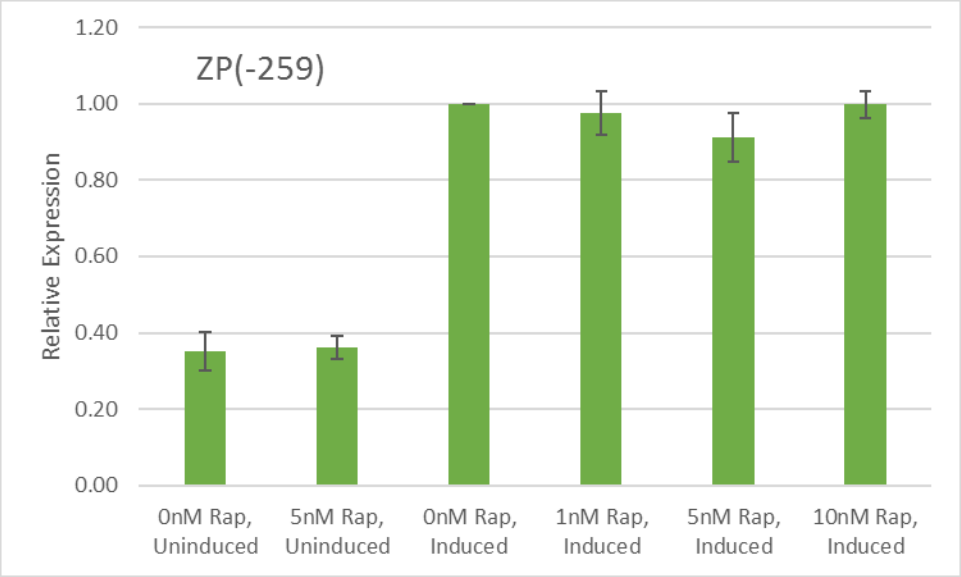

Figure 3.8. Luciferase Reporter Assay Results of the ZP(-259) Plasmid. Rapamycin sensitivity appears to be lost in both induced and uninduced cells. Additionally, luciferase levels are reduced relative to the untreated, induced standard. Data set represent five biological replicates with two technical replicates.

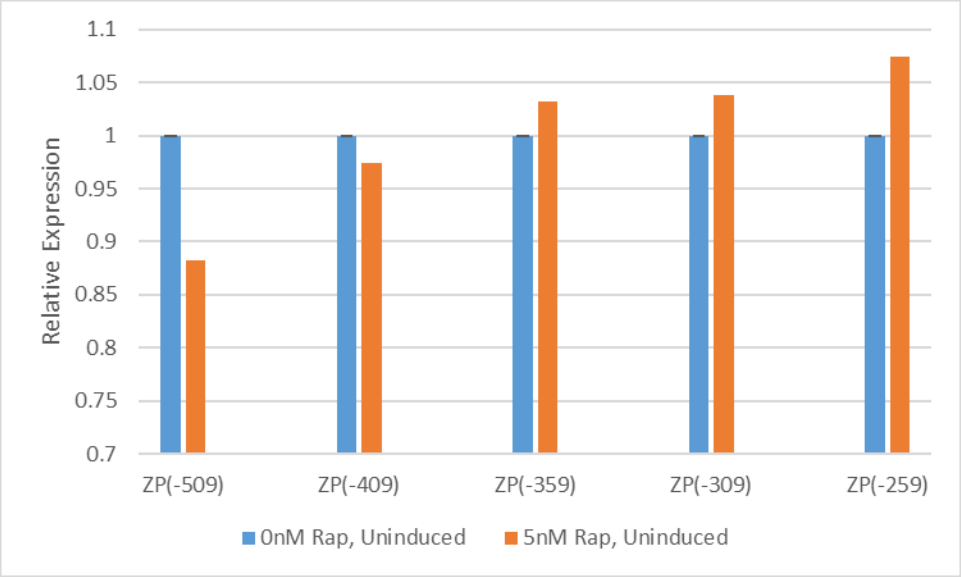

Figure 3.9. BZLF1 Promoter Reporter Assay of Untreated and Treated Uninduced AGS-BX1 Cells. After the deletion of the -509bp to -410bp section of the promoter, rapamycin suppression of leaky transcription is reduced. Further deletions of the promoter result in increases in leaky transcription. Data set represent five biological replicates with two technical replicates.

3.3 Shifts in YY1 Localization Due to mTOR Inhibition and Viral Induction in AGS-BX1 and Raji Cells

After quantification of YY1 protein bands in both the nuclear extract (NE) and the cytosolic extract (CE), the ratio NE/CE was used to generalize YY1 localization in each condition (**Figure 3.10**). NE/CE values were then standardized to the untreated, uninduced sample. Samples in which the NE/CE ratio was greater than 1 were considered to have YY1 localization to the nucleus; while samples with a NE/CE ratio less than one were considered to have YY1 localization to the cytosol. The Raji B-lymphocytes consistently demonstrated YY1 localization to the nucleus, with the highest nuclear localization occurring the 5nM treated, induced sample (**Figure 3.11**). Interestingly,

while YY1 localization appears to compound with viral induction and rapamycin treatment in the 1nM and 5nM groups, the 10nM treated, induced group displayed a YY1 localization ratio similar to the 5nM treated, uninduced group. AGS-BX1 epithelial cells demonstrated only a slight localization to the cytosol which did not correlate with increasing concentrations of rapamycin or induction state (**Figure 3.12**).

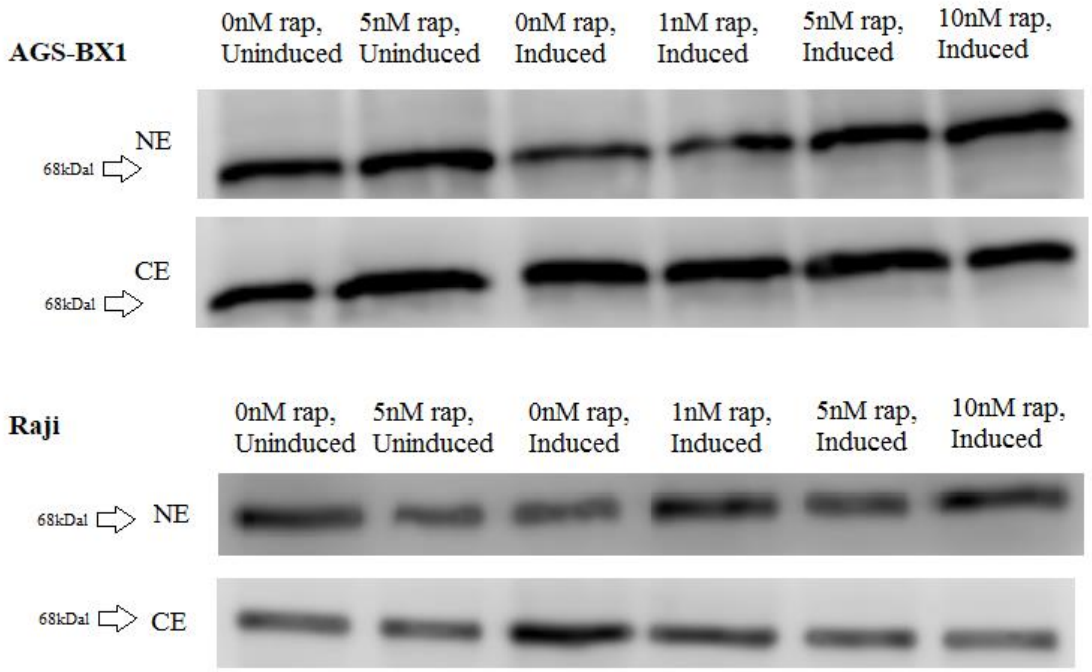

Figure 3.10. Cytosolic Localization Versus Nuclear Localization of YY1 in AGS-BX1 Epithelial Cells and Raji B-lymphocytes. Cytosolic extract (CE) YY1 quantifications were normalized to tubulin and nuclear extract (NE) YY1 quantifications were normalized to Tata-Binding Protein. Localization was determined by taking a ratio of normalized NE to CE YY1 quantifications.

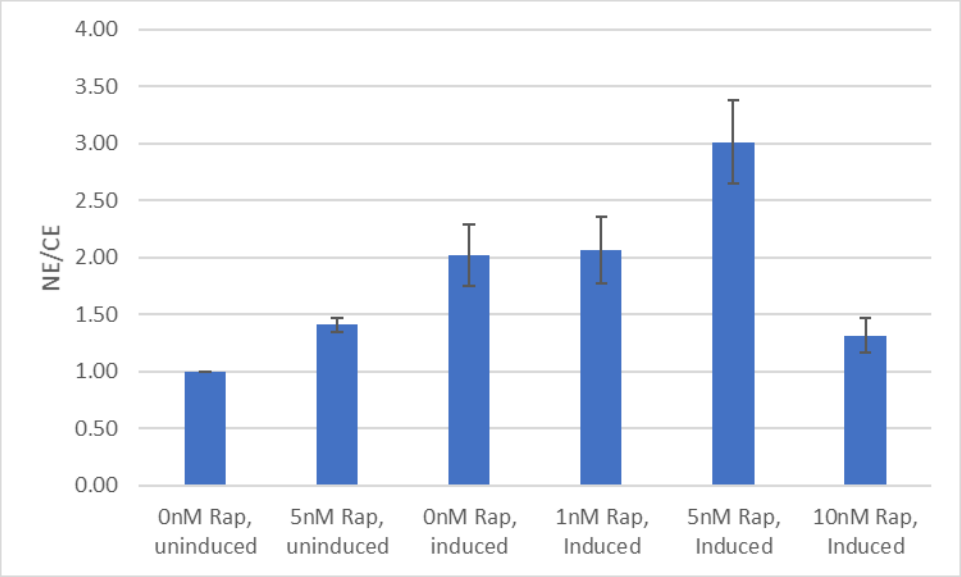

Figure 3.11. YY1 Localization in Raji B-lymphocytes. NE/CE ratios greater than 1 correlate to nuclear localization of YY1, while values less than 1 correlate to cytosolic localization of YY1. Relative to the untreated, uninduced condition, YY1 appeared to shift into the nucleus in every condition. This may be the result of combinatorial effects of mTOR inhibition and initiation of EBV latency. Data set represents three biological replicates with two technical replicates each.

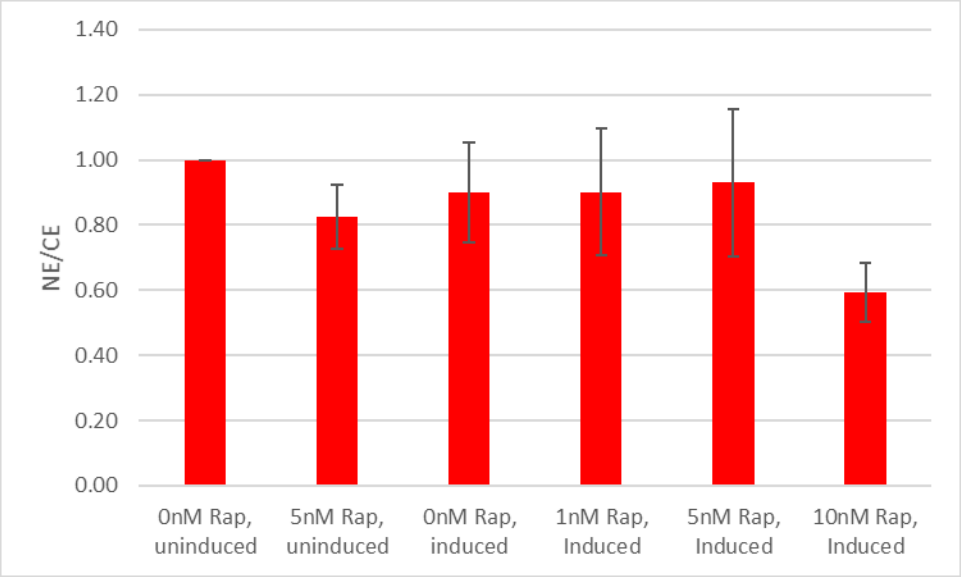

Figure 3.12. YY1 Localization in AGS-BX1 Epithelial Cells. NE/CE ratios greater than 1 correlate to nuclear localization of YY1, while values less than 1 correlate to cytosolic localization of YY1. YY1 did not appear to undergo significant localization shifts, with the exception of the 10nM rapamycin treated, induced sample, in which YY1 is localized to the cyosol. Data set represents three biological replicates with two technical replicates each.

3.4 RNAi Mediated Knockdown of YY1 in AGS-BX1 and Raji Cells Alters Expression of BZLF1

Though BZLF1 levels in the YY1 knockdown treated, induced AGS-BX1 samples were still elevated relative to the untreated control, the degree of increase in protein expression was substantially reduced compared to samples transfected with only scrambled RNA (**Figure 3.15** and **Figure 3.17**). This effect was most pronounced at the 1nM and 10nM rapamycin treated, induced cells and, overall, demonstrated a loss in rapamycin-sensitivty. In contrast, 1nM and 5nM treated, induced YY1 knockdown Raji cells exhibited an increase in BZLF1 levels (**Figure 3.14** and **Figure 3.16**). This increase was both in comparison to the scrambled RNA transfected control and the YY1

knockdown untreated, induced control. Despite this, BZLF1 levels still displayed a negative relationship with increasing concentrations of rapamycin.

Overall changes in BZLF1 expression was also analyzed for AGS-BX1 cells by standardizing normalized BZLF1 levels to the untreated, uninduced control. In the YY1 knockdown AGS-BX1 cells, BZLF1 protein levels increased relative to those in the scrambled control the 5nM, uninduced and 0nM, induced samples (**Figure 3.18**). Substantial changes were not detected in BZLF1 levels of the 1nM and 5nM treated, induced groups, though a large decrease in relative protein levels was found in the 10nM, induced group.

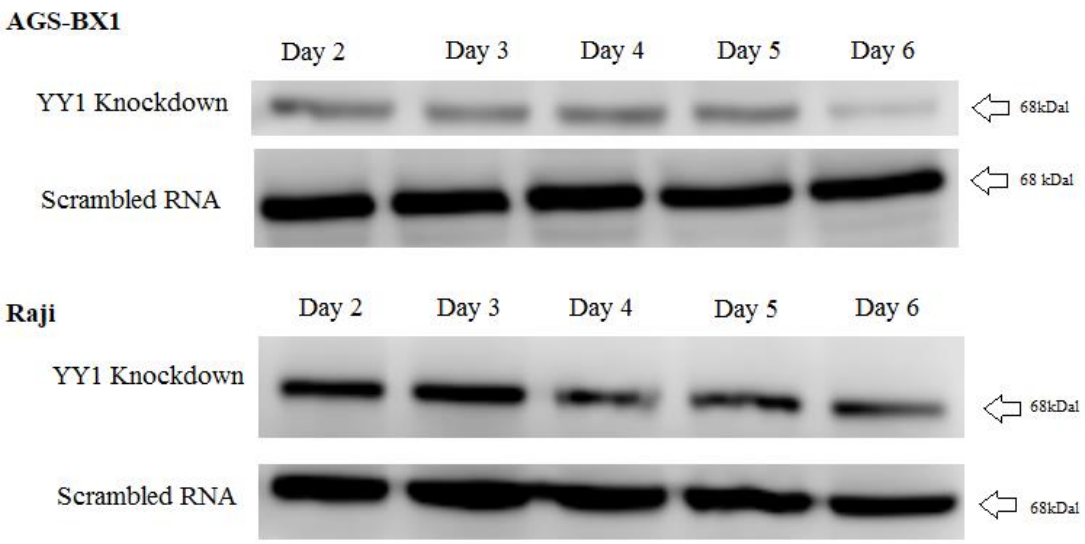

Figure 3.13. YY1 Knockdown in AGS-BX1 Epithelial Cells and Raji B-lymphocytes. The average YY1 knockdown on day six in AGS-BX1 cells after normalization with tubulin was 39%, while the average YY1 knockdown on day six in Raji cells after normalization with tubulin was 20%. YY1 knockdown and scrambled RNA control were repeated twice in each cell line with two technical replicates per repetition.

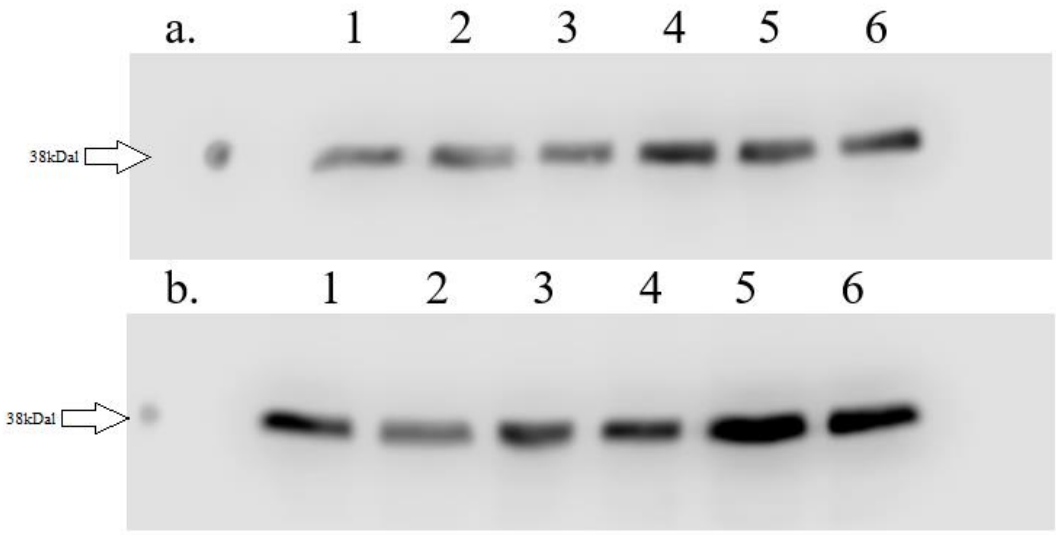

Figure 3.14. BZLF1 Expression of Negative Control and YY1 Knockdown AGS-BX1 Epithelial Cells. The negative control (a) was transfected with scrambled RNA and subject to the same treatment protocol as the YY1 knockdown group (b). Lane 1 – 0nM rapamycin, uninduced, lane 2 - 5nM rapamycin, uninduced, lane 3 – 0nM rapamycin, induced, lane 4 – 1nM rapamycin, induced, lane 5 – 5nM rapamycin, induced, lane 6 – 10nM rapamycin, induced.

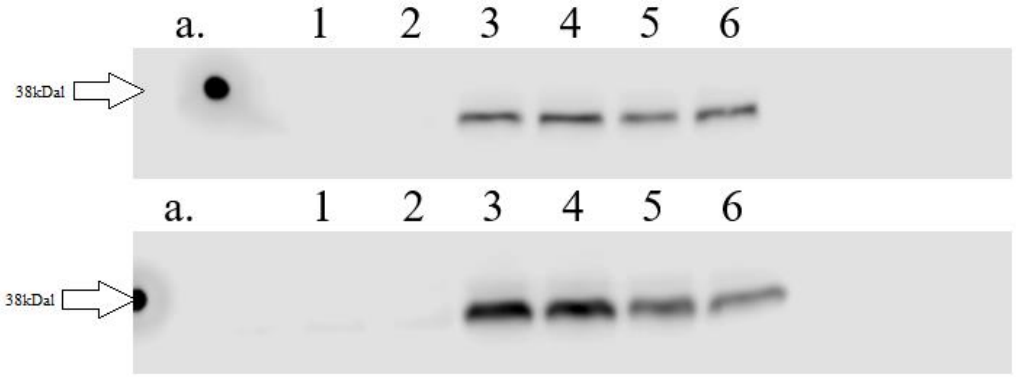

Figure 3.15. BZLF1 Expression of Negative Control and YY1 Knockdown in Raji B-lymphocytes. The negative control (a) was transfected with scrambled RNA and subject to the same treatment protocol as the YY1 knockdown group (b). Lane 1 – 0nM rapamycin, uninduced, lane 2 - 5nM rapamycin, uninduced, lane 3 – 0nM rapamycin, induced, lane 4 – 1nM rapamycin, induced, lane 5 – 5nM rapamycin, induced, lane 6 – 10nM rapamycin, induced.

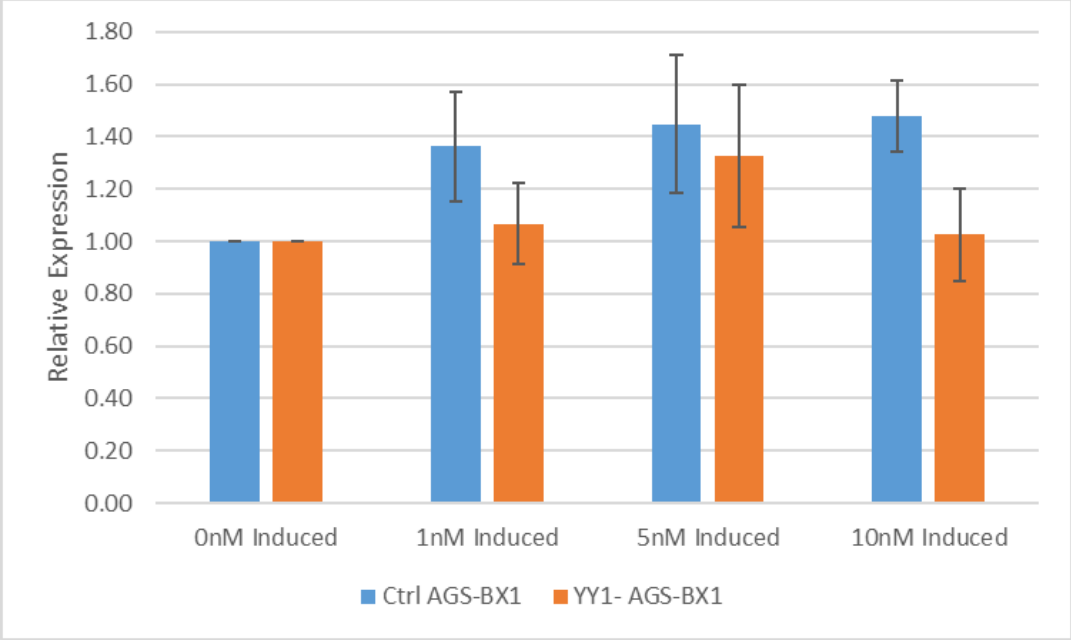

Figure 3.16. Comparison of Relative Expression of BZLF1 Between YY1 Knockdown AGS-BX1 Epithelial Cells and the Negative Control. Decreases in BZLF1 were seen in the rapamycin treated, induced groups of YY1 knockdown cells compared to the scrambled RNA negative control. Data set represents four biological replicates with two technical replicates each.

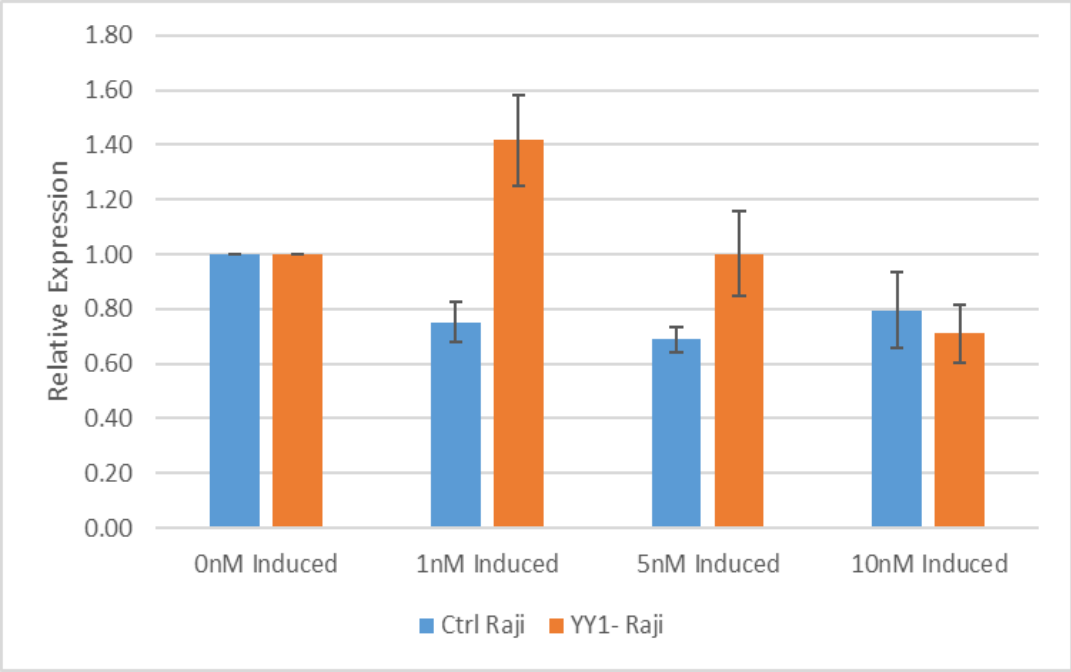

Figure 3.17. Comparison of Relative Expression of BZLF1 Between YY1 Knockdown Raji B-lymphocytes and the Negative Control. BZLF1 levels were significantly increased in rapamycin treated, induced YY1 knockdown Raji cells compared to both the scrambled negative control and the untreated, induced standard at 1nM. Protein levels were also increased at 5nM in YY1 knockdown Raji cells, but are depressed at 10nM rapamycin treatment. Data set represents four biological replicates with two technical replicates each.

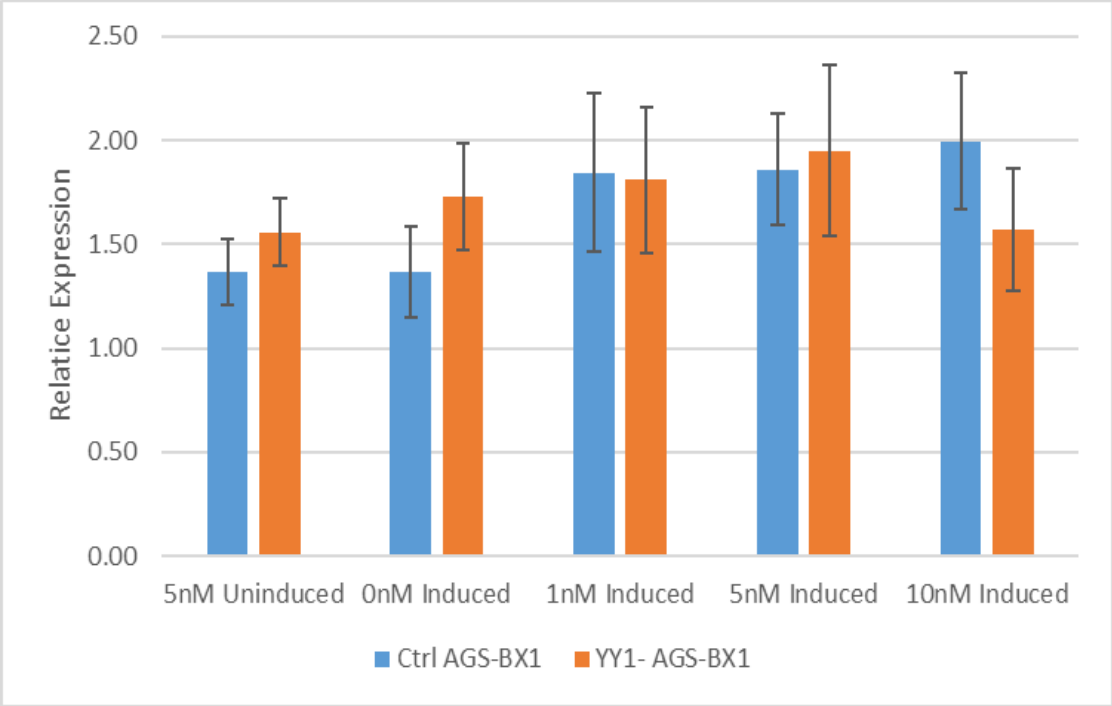

Figure 3.18. Overall Expression of BZLF1 in YY1 Knockdown and Scrambled RNA Transfected AGS-BX1 Epithelial Cells. Though overall BZLF1 levels are not substantially altered in the treated, induced groups, protein levels were elevated in both the 5nM, uninduced and 0nM, induced YY1 knockdown cells. Also, BZLF1 in the 10nM, induced YY1 knockdown cells were decreased compared to the scrambled control. Data set represents four biological replicates with two technical replicates each.

CHAPTER IV

DISCUSSION

4.1 Changes in Expression Patterns of BZLF1 in AGS-BX1 and Raji Cells Under mTOR Inhibition

The highest peak of expression of BZLF1 was at 16 hours post-induction in the untreated Raji B-lymphocytes after which BZLF1 levels decreased though still remained elevated compared to the 5nM rapamycin treated cells. At 24 hour post-induction, BZLF1 levels began to increase again, though not to the levels seen at the 16 hour point. This suggests that there may be multiple waves of initiation of immediate-early gene activation, culminating with elevated levels of BZLF1 at 16 hours and at 24 hours. Though BZLF1 levels did increase in the 5nM rapamycin treated Raji cells, protein levels peaked at the 20 hours post-induction; producing only 20% of untreated 24 hour levels. The immediate disparity between protein levels of the treated and untreated groups suggest that rapamycin suppression of BZLF1 occurs during the initial activation of BZLF1, rather than the premature initiation of latency. By inhibiting the initial expression of BZLF1, insufficient levels of the immediate-early gene are transcribed to produce normal lytic replication. Additionally, since the pattern of expression in the treated Raji cells only a single long peak compared to the double peak seen in the untreated cells, it supports the hypothesis that mTOR inhibition is affecting BZLF1 expression at a transcriptional level rather than simple decreasing translation efficiency.

BZLF1 levels in untreated epithelial cells also demonstrated a maximum peak at 16 hours post-induction. Unlike the expression pattern seen in the untreated B-lymphocytes, BZLF1 levels further increased at the secondary peak at 24 hour post-induction time point. This suggests that, while multiple rounds of expression may occur in untreated, induced AGS-BX1 and Raji cells, the secondary waves are decreased in Raji cells and increased in AGS-BX1 cells. This is supported by the observation that EBV is able to undergo latency in B-lymphocytes but not in epithelial cells. It may be that latency associated products, such as EBNA1, are produced following late-gene expression, thereby decreasing secondary waves of lytic expression in B-lymphocytes. EBV does not produce EBNA1 in epithelial cells, so lytic replication is allowed to continue in an increasing cycle of expression.

Epithelial cells treated with 5nM rapamycin support the hypothesis that mTOR inhibition increases BZLF1 expression in EBV induced epithelial cells by prolonging BZLF1 expression. While the pattern of BZLF1 expression between the treated and untreated groups was initial similar, BZLF1 expression continued to increase after 16 hours; forming an initial peak at 18 hours post-induction. While BZLF1 levels then fell, mirroring the expression pattern seen in untreated cells, the secondary peak of BZLF1 at 24 hours remained elevated compared to the untreated cells. Since the early expression patterns between the treated and untreated groups were similar, and differences only arose later, initial activation of BZLF1 appeared unaffected in the treated epithelial cells. This suggests that BZLF1 expression in epithelial cells, under mTOR inhibition, is subject to prolonged activation. Additionally, since the expressive patterns between the

two groups were initial similar, this evidence again supports the theory that rapamycin-associated activation of lytic replication in epithelial cells is a result of transcriptional upregulation.

4.2 Relative Contribution of YY1 Binding Domains on BZLF1 Expression in AGS-BX1 Cells

The results of the full-length BZLF1 promoter containing plasmid ZP(-509) supports the hypothesis that mTOR inhibition mechanistically affects BZLF1 transcriptional activation in epithelial cells. While luciferase activity was partially repressed by rapamycin treatment relative to the 0nM, induced control, increasing concentrations of rapamycin positively correlated with increases in luciferase activity. The impact of upstream deletions on luciferase activity was compared against the ZP(-509) plasmid for the other reporter constructs. ZP(-409), which was the result of a YY1 domain and E2-2 domain deletion, saw immediate loss of rapamycin sensitivity in both induced and uninduced samples, and an overall decrease in expression relative to the untreated, uninduced control. This suggests that elements in the -509 to -409bp region of the BZLF1 promoter are activating, and responsive to mTOR inhibition. The overall repressive result of this 100bp deletion is likely due to the loss of the terminal E2-2 domain, as earlier work has demonstrated that site-directed mutagenesis of this region shows a similar repression[22]. In order to determine if the loss of rapamycin sensitivity was due to E2-2 or YY1 deletions, ZP(-359), ZP(-309), and ZP(-259) were also compared. The ZP(-359) plasmid displays both a dramatic increase in expression relative

to the untreated, uninduced control, and the reemergence of rapamycin-sensitivity. Since two E2-2 and a single YY1 domain were deleted to produce the ZP(-359) plasmid, it was still uncertain if these changes were attributable to YY1 or E2-2 deletions. In ZP(-309), which maintains a E2-2 domain bute deletes the final YY1 domain, rapamycin sensitivity is lost and no change in overall protein expression relative to the untreated, uninduced was observed. The final 50bp deletion in ZP(-259) removed the final E2-2 domain but did not alter the rapamycin sensitivity. Interestingly, however, overall luciferase expression was increased in the induced cells (**Figure 3.3**). These results suggest that the YY1 domains are principally involved in the rapamycin sensitivity of BZLF1 transcription in epithelial cells. While E2-2 domains may play a role in the overall responsiveness of the BZLF1 promoter to lytic activation, these increases are rapamycin insensitive. Furthermore, the upstream E2-2 domain appears to be important in rapamycin-associated suppression of spontaneous transcription, as luciferase activity in 5nM treated, uninduced cells were undifferentiable from untreated, uninduced levels. Spontaneous expression of luciferase under mTOR inhibition continued to increase in the truncated promoters, but only following E2-2 deletions.

4.3 Shifts in YY1 Localization Due to mTOR Inhibition and Viral Induction in AGS-BX1 and Raji Cells

Since YY1 localization may be impacted through the phosphorylation of its serine/threonine residues, it was hypothesized that inhibition of mTOR would alter localization differently in each cell type. The results support this hypothesis as YY1 was

observed localizing to the nucleus under mTOR inhibition in Raji B-lymphocytes in both treated, uninduced and treated, induced cells. Interestingly, YY1 nuclear localization was also observed in the untreated, induced Raji cells. This may be due to YY1's assumed role in B-lymphocytes as a promoter of latency, with EBV entering latency at the time cells were harvested (24 hours post induction). The treatment of rapamycin at 1nM and 5nM in induced Raji cells seemed to compound these effects, with YY1 nuclear localization increasing threefold in 5nM treated, induced cells. Treatment of induced Raji cells with 10nM rapamycin, however, resulted in the same localization value as reported in the 5nM, uninduced samples. It is possible that treatment with 10nM rapamycin suppressed EBV lytic replication sufficiently as to no longer trigger latency-associated YY1 localization.

In contrast, mTOR inhibition was not observed to substantially alter YY1 localization within AGS-BX1 cells. While there was a slight shift into the cytosol, this shift was independent of rapamycin treated or induction state. This suggests that, while YY1 localization may alter slightly under general stress-induced pathways within epithelial cells, it is not the principle cause behind rapamycin-associated enhancement of viral activity. Instead, the potential change in phosphorylation state of YY1 in epithelial cells may lead to changes in activation efficiency independent of localization. This may be due to changes in DNA binding, as well as protein interactions.

4.4 RNAi Mediated Knockdown of YY1 in AGS-BX1 and Raji Cells Alters Expression of BZLF1

Knockdown of YY1 in AGS-BX1 cells decreased the activating effects of mTOR inhibition on BZLF1 expression in treated, induced cells. This effect was most pronounced at 1nM and 10nM rapamycin, in which BZLF1 levels were indistinguishable from the untreated, induced samples. Additionally, when protein levels were standardized to the untreated, uninduced control, it was observed that BZLF1 levels were elevated in the 0nM, induced sample compared to the scrambled control. This is similar to the expression pattern seen in the ZP(-309) luciferase assay, in which luciferase activity decreased relative to the untreated, induced sample but increased relative to the untreated, uninduced sample. Furthermore, YY1 knockdown AGS-BX1 cells demonstrated a loss in rapamycin-associated repression of spontaneous EBV activation. These results support the earlier findings of this projects that YY1 in epithelial cells plays an activating role of BZLF1 under mTOR inhibition (**Figure 4.1**). According to this hypothesis, mTOR inhibition leads to a decrease in YY1 phosphorylation, which prolongs the activating effect of YY1 on BZLF1 expression. This leads to a more robust expression of BZLF1 and subsequent increase in activation of downstream lytic proteins. It is interesting that, while the luciferase data suggested E2-2 as an enhancer of overall lytic enhancement of BZLF1, the knockdown of YY1 produced similar results. This suggests that YY1 interactions with E2-2 may play a crucial role in modulating over expression levels of the immediate-early gene BZLF1.

Knockdown in Raji B-lymphocytes resulted in the significant increase in BZLF1 expression under 1nM rapamycin treatment, and general increase with 5nM rapamycin treatment. This is supportive of the hypothesis that YY1 plays a repressive role to BZLF1 in Raji cells and, under inhibition of mTOR mediated phosphorylation, localizes to the nucleus (**Figure 4.2**). By decreasing YY1 levels through RNAi, less YY1 is localized to the nucleus and BZLF1 is transcribed at a rate similar to the untreated samples. While no change was seen in BZLF1 expression in the knockdown cells at 10nM treatment and a negative trend between BZLF1 expression and rapamycin concentration remained, this may be due to an insufficient level of YY1 knockdown to overcome higher rapamycin treatment. Expression changes between the experimental conditions and an untreated, uninduced standard were unable to be performed do to the undetectable levels of BZLF1 within the untreated, uninduced samples. It is hypothesized, however, that these overall levels would be increased, as YY1 is only known to be a repressor of lytic replication in B-lymphocytes.

4.5 Conclusion

In this project, it has been suggested that the cell-specific effects of mTOR inhibition are mediated through the activities of the transcription factor YY1. In epithelial cells, YY1 binding to the BZLF1 promoter under mTOR inhibition activates expression. This activating role on the BZLF1 promoter was seen in both the luciferase assay and the YY1 knockdown assay. The activating effect may be due to epithelial-specific post-translational modification of YY1 such as phosphorylation, alternate protein interactions,

or a combination thereof. Given previous studies[22] which observed the activating effects of E2-2 on the BZLF1 promoter in epithelial cells, YY1 may interact with or affect the binding of E2-2. This is further supported by the close proximity of many of the YY1 and E2-2 domains on the upstream BZLF1 promoter.

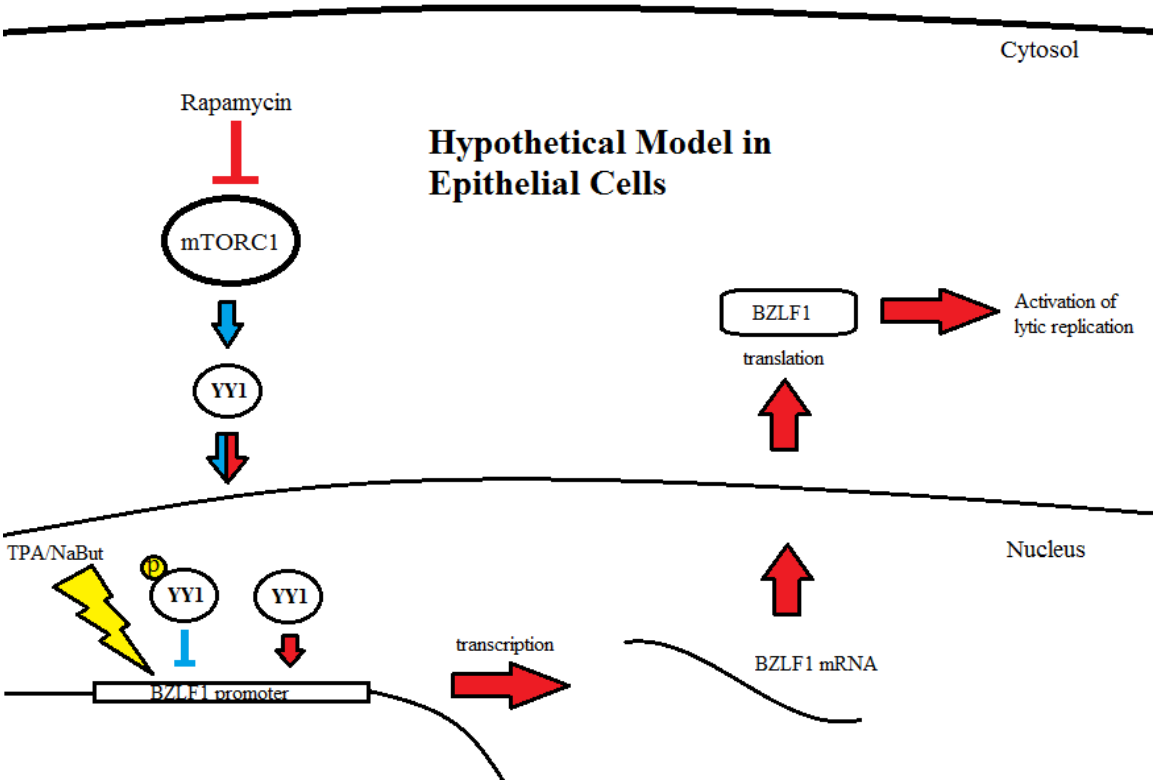

Figure 4.1. Hypothetical Model of BZLF1 Enhancement through mTOR Inhibition in Epithelial Cells. While mTOR activity did not alter YY1 localization to the nucleus, YY1 appears to increase BZLF1 activation under mTOR inhibition. Rapamycin inhibition of mTOR increases YY1's activating role, thereby leading to an increase in viral replication.

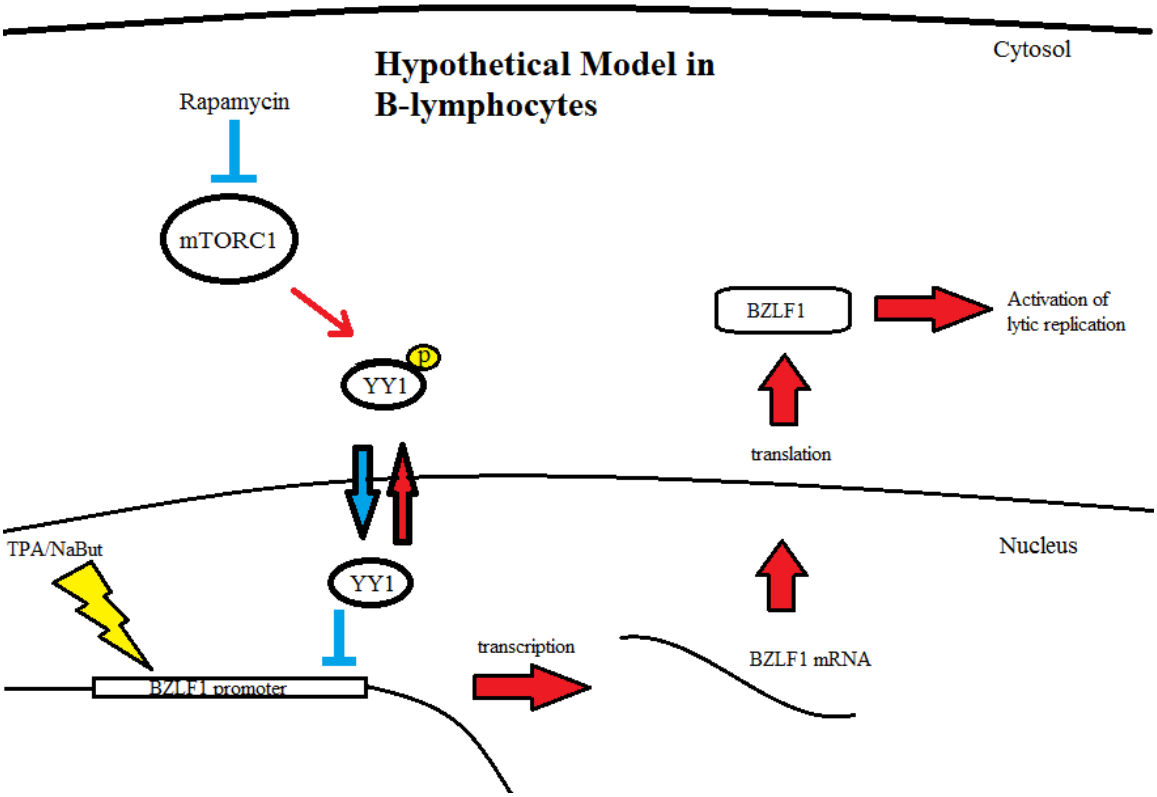

Figure 4.2. Hypothetical Model of BZLF1 Repression through mTOR Inhibition in B-lymphocytes. Activated mTOR phosphorylates YY1, leading to the cytosolic localization of YY1. Under mTOR inhibition, YY1 accumulates in the nucleus, acting a repressor to BZLF1 expression. Without BZLF1 expression, the whole lytic replication cascade is dramatically reduced.

Conversely, YY1 maintains a repressive role of BZLF1 activation in B-lymphocytes. Rapamycin sensitivity appears to be mediated by increased nuclear localization of YY1 under mTOR inhibition, suggesting that mTOR may play a role in YY1 localization through its kinase activity. Alternatively, a compensatory pathway may alter YY1 localization through any number of other post-translational modifications. This inhibition of transcription of the BZLF1 gene leads to a dramatic decrease in protein levels and, therefore, a reduced lytic cascade.

This research reveals a potentially novel mechanism by which YY1 regulates the expression of a viral gene. While YY1 has largely been attributed as a repressor of multiple viral promoters, is appears to play an activating role in the BZLF1 expression of epithelial cells. Here YY1 is predictably repressive of YY1 in B-lymphocytes, but unpredictably activating of BZLF1 expression in epithelial cells. Additionally, this research suggests that the regulation of YY1 activity between epithelial cells and B-lymphocytes may not only be responsible for this cell-specific behavior of EBV, but that this regulation is itself cell-specific. This knowledge may have transformative impacts on the potential use of rapamycin as a chemotherapeutic drug, as well as elucidate the hidden interactions between herpesviruses and their host cells.

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