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Epstein-Barr virus (EBV) is a ubiquitous herpesvirus that infects individuals worldwide. EBV actively replicates and establishes a latent infection in epithelial cells and B cells, which can lead to diseases such as infectious mononucleosis and Burkitt's lymphoma. The reactivation of EBV from latency is contingent upon the expression of immediate-early genes BZLF1 and BRLF1. During EBV latency, BZLF1 and BRLF1 are negatively regulated by Yin-Yang 1 (YY1). YY1 is a highly expressed protein that participates in various cellular and viral processes. It has been demonstrated that the effects of rapamycin, a mechanistic target of rapamycin complex 1 (mTORC1) inhibitor, on YY1 in epithelial cells and B cells alters EBV lytic replication. Rapamycin's effects on YY1 cause it to positively regulate BZLF1 in epithelial cells and negatively regulate BZLF1 in B cells. While the effects of rapamycin on YY1 regulation of BZLF1 have been studied, the effects on BRLF1 have not. For this study, I investigated the effects of rapamycin on YY1 regulation of BRLF1 in epithelial cells and B cells during lytic replication. I wanted to determine if the effects of rapamycin on YY1 regulation of BRLF1 would be like the effects of rapamycin on YY1 regulation of BZLF1. The results of this study show that they are indeed similar. YY1 positively regulates BRLF1 in epithelial cells and negatively regulates BRLF1 in B cells during mTORC1 inhibition and lytic replication. This suggests that rapamycin alters YY1 transcriptional regulation of BRLF1 in a cell type-specific manner. Using rapamycin in combination with YY1-

targeted therapies could reduce EBV infectivity and decrease the likelihood of developing EBV-associated diseases.

YIN-YANG 1 REGULATION OF EPSTEIN-BARR VIRUS BRLF1 TRANSCRIPTION IS ALTERED DURING MTORC1 INHIBITION IN A CELL TYPE-SPECIFIC MANNER

by

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Approved by

_____
Committee Chair

Dedicated to my family for all the love and support they have always provided me.

APPROVAL PAGE

This thesis written by Steven Javier Moran has been approved by the following committee of the Faculty of The Graduate School at the University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

Epstein-Barr Virus (EBV)

Epstein-Barr virus (EBV), also known as human herpesvirus 4 (HHV4), is a gammaherpesvirus that infects over 90% of the human population [24]. EBV is primarily transmitted through oral contact with infected saliva, which leads to the shedding of the virus in high concentrations for more than 6 months and the shedding of the virus in low concentrations for the duration of the infected host's lifespan [20]. Although most individuals infected with EBV are asymptomatic, EBV can cause a myriad of diseases such as infectious mononucleosis, Burkitt's lymphoma, post-transplant lymphoproliferative disorder, and nasopharyngeal carcinoma [3, 24].

EBV Infection and Replication

Initial infection and replication of EBV in a lytic manner occurs in cells of nasopharyngeal and oropharyngeal epithelia. EBV glycoproteins gHgL and gB mediate virus attachment and virus-cell membrane fusion. The interaction between EBV viral protein BMRF-2 and host cell β1 integrins is critical for EBV infection of polarized oropharyngeal epithelial cells [40]. In B cells, EBV glycoprotein gp350 binds to complement receptor type 2 (CR2) on the host cell membrane, triggering cell signaling and endocytosis within the host cell. CR2 can then be bound by gp20, which brings the virion closer to the host cell membrane. At the host cell membrane, gp42 can interact

with HLA class II, triggering the interactions of gHgL and gB with the endosomal membrane. The virus and host cell membranes fuse, which permits the EBV nucleocapsid to enter the cytoplasm of the host cell. Nuclear pores embedded in the host cell nucleus allow the entry of the linear, double-stranded DNA EBV genome into the nucleus where it can be transcribed and replicated [8, 18, 26, 40].

While plasma cells carry lytic EBV virions that result in the production of more infectious virus particles, EBV primarily establishes a lifelong infection in B cells where the virus switches from lytic to latent phase. During latent infection, EBV immortalizes itself in B cells by expressing a small subset of genes that aid in maintaining the viral genome in multiple copies called episomes [4, 13, 24, 28]. There are 10 to 50 copies of the viral genome maintained per cell that can be duplicated during host cell division using host DNA replication machinery [26]. The reactivation of EBV into its lytic form is dependent on the activation of viral genes that encode gene products that result in the production of infectious virions [28]. EBV can switch from its latent form to its lytic form spontaneously, or through chemical induction using reagents such as tetradecanoyl phorbol acetate (TPA) and sodium butyrate. Spontaneous reactivation or chemical induction trigger the expression of immediate-early genes BZLF1 and BRLF1 that play an integral role in EBV lytic replication [12, 42, 43].

EBV Immediate-Early Genes

BZLF1 and BRFL1 both encode viral proteins (Z and R) that function as transcriptional activators that synergistically activate one another's promoters to propagate lytic gene expression [2, 12, 14, 34]. Both Z and R mRNA are primarily

derived from their respective promoters with small amounts of Z transcript also coming from the BRLF1 promoter due to the promoter's ability to bicistronically transcribe Z mRNA [42, 43]. The extensively studied BZLF1 promoter is known to contain both positive and negative regulatory elements that interact with chemical agents such as TPA, and transcription factors that modulate transcriptional activity (Figure 1) [42].

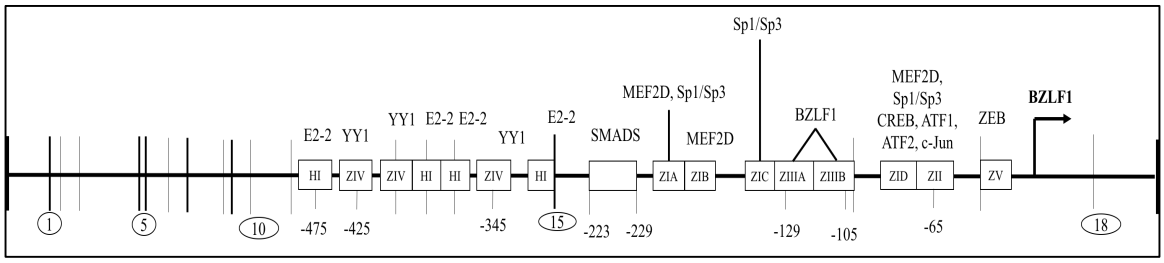


Figure 1. Epstein-Barr Virus (EBV) BZLF1 Promoter. The BZLF1 promoter codes for Z protein, which is critical for the initiation of EBV lytic replication. It contains various binding sites for its own encoded protein, and for other transcription factors that participate in EBV lytic replication and latency.

BZLF1 can be divided into two promoters: P1 and P2. The P1 promoter transcribes 1-kb monocistronic mRNA while the P2 promoter transcribes 2.8-kb bicistronic mRNA, which encodes Z and R [30, 42]. The P1 promoter contains most of the positive and negative regulatory elements that mediate BZLF1 expression and eventual lytic replication. Z encoded by BZLF1 binds to AP1 and CREB-like binding sites present in EBV promoters such as the BRLF1 promoter, which upregulates BRLF1 expression. The mechanism for how BRLF1 activates BZLF1 expression has yet to be fully elucidated [2, 12, 28, 34, 42, 43].

The BRLF1 promoter is an EBV promoter of interest for future study (Figure 2). The BRLF1 promoter contains Sp1 and Zif268 binding sites that are important for EBV constitutive activity and TPA-induced stimulation, respectively [43].

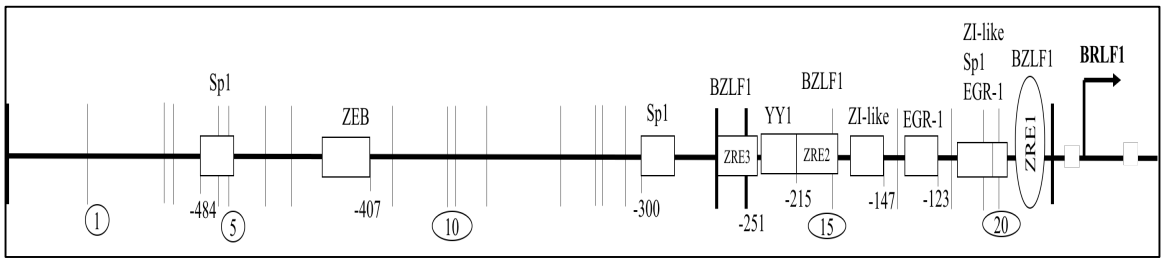


Figure 2. Epstein-Barr Virus (EBV) BRLF1 Promoter. Like the BZLF1 promoter, the BRLF1 promoter codes for its respective protein, and contains various binding sites that aid in EBV lytic replication and latency. Unlike BZLF1, BRLF1 is not always needed to initiate EBV lytic replication.

Its own gene product R can also activate and regulate BRLF1 promoter activity. R can activate certain EBV lytic promoters such as SM, BMRF1, and BHRF1 by directly binding to GC-rich sequences known as BRLF1-responsive elements (RREs) [34]. When R directly binds to RREs present in promoters, it can strongly activate promoters due to its alternate function as an enhancer factor when bound to RREs. Although there are direct mechanisms for how R binds to the promoters of other EBV viral genes, the direct mechanism for how R activates BZLF1 expression remains unknown [2, 12, 34].

BZLF1 expression alone is enough to disrupt EBV latency in both epithelial cells and B cells. While BRLF1 expression is capable of inducing EBV lytic replication in epithelial cells, it is unable to induce EBV lytic replication in B cells, such as those of the Raji cell line (an EBV-positive Burkitt's lymphoma cell line), due to its inability to activate BZLF1 expression from the endogenous viral genome [1, 12, 42]. For BRLF1

expression to occur in B cells, BZLF1 expression must first occur [1]. It has been suggested that R may only be able to indirectly activate BZLF1 expression by enhancing signal transduction cascades mediated by MAP kinases (i.e. JNK, ERK, and p38 kinases) that induce the phosphorylation of transcription factors such as c-Jun/ATF2 that can bind to the BZLF1 promoter [2, 12, 34, 42]. The ability of Z and R to initiate and promote viral replication by activating cellular pathways makes them and their respective promoters key targets for potential inhibition of EBV lytic replication.

Mechanistic Target of Rapamycin (mTOR) Pathway

A major cell-signaling pathway that mediates various activities throughout the cell is the mechanistic target of rapamycin (mTOR) pathway (Figure 3). The protein at the center of the pathway, mTOR, is an evolutionarily conserved serine-threonine kinase that interacts with several other proteins to form two different protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [3, 15, 27]. Of the two protein complexes, mTORC1 is the best characterized. mTORC1 can receive a variety of intracellular and extracellular cues such as growth factors, cell stress, and energy status to control major cellular processes that include macromolecule biosynthesis, cell cycle progression, cell growth, and mitochondrial metabolism [3, 27].

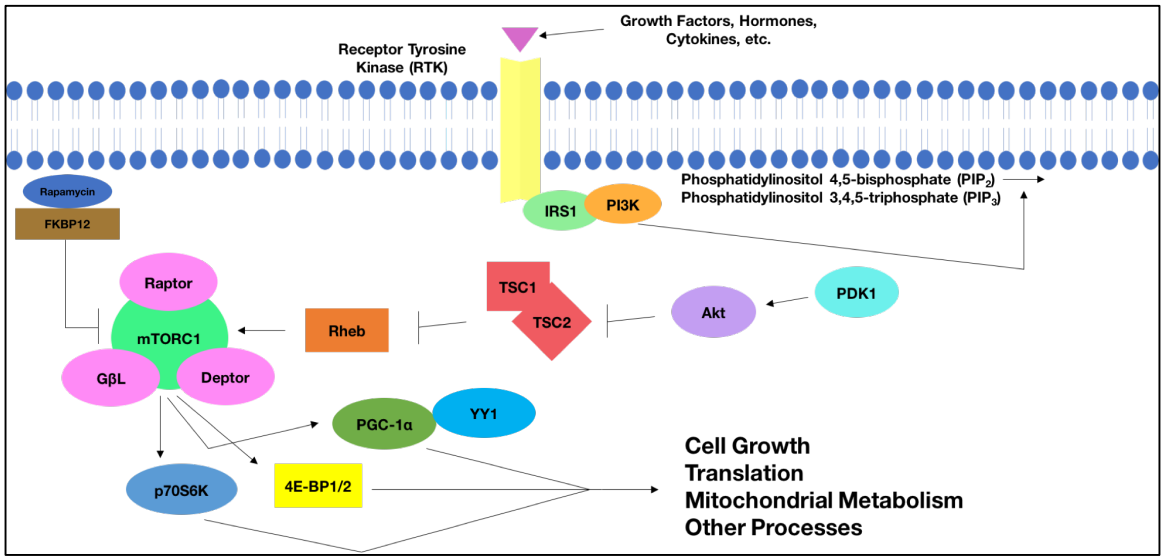


Figure 3. Overview of the mTOR Pathway. A stimulus binds to a receptor tyrosine kinase (RTK), and ultimately leads to the recruitment of phosphoinositide-dependent kinase-1 (PDK1), which will phosphorylate and activate Akt. Akt phosphorylates tuberous sclerosis 1 (TSC1) and TSC2 (TSC 1/2), which releases Ras homolog enriched in brain (Rheb) of its inhibition and allows Rheb to bind GTP. GTP-bound Rheb then activates mechanistic target of rapamycin complex 1 (mTORC1), which phosphorylates downstream proteins to enact various cellular responses.

A key upstream regulator of mTORC1 activity is a heterodimer complex composed of tuberous sclerosis 1 (TSC1) and TSC2. TSC1/2 acts as a GTPase-activating protein (GAP) for Ras homolog enriched in brain (Rheb), which can directly interact with mTORC1 and enhance its kinase activity when bound by GTP. TSC1/2 transmits many of the upstream signals that act on mTORC1 such as growth factors like insulin and insulin-like growth factor 1 (IGF1) that stimulate the phosphoinositide 3-kinase (PI3K) and Ras pathways [27]. Kinases within the pathway—protein kinase B (Akt/PKB) and extracellular-signal-related kinase 1/2 (ERK 1/2)—directly phosphorylate TSC1/2, which inactivates it and activates mTORC1. The catalytic mTOR subunit of the mTORC1 complex can phosphorylate downstream effectors such as eukaryotic initiation factor 4E

(eIF4E) binding protein (4E-BP1) and p70 S6 kinase (p70S6K) to promote cap-dependent translation and regulate cellular machinery necessary for protein biosynthesis. 4E-BP1 and p70S6K are two of many proteins that viruses such as EBV can utilize to ensure the translation of their own viral proteins [3, 27, 31].

In cells infected with either adenovirus or human cytomegalovirus (HMCV), cellular synthesis of viral particles and cell growth increased in conjunction with increases in mTOR activity [9]. In airway epithelial cells infected with adenoviruses, there was an increase in mTOR activation under varying nutrient conditions and mTOR activation was a significant step in progressing infected cells through to the S phase of the cell cycle. Heart-transplant patients treated with mTOR inhibitors such as RAD001 and cyclosporine for immunosuppression showed a lower incidence of HMCV infection and vasculopathy compared to other patients treated with cyclosporine and azathioprine [9]. Both studies suggest that inhibiting mTOR activity could potentially decrease or inhibit viral activity within infected cells. A well-known inhibitor of mTOR that is of clinical relevance for the treatment of cancer and other diseases is rapamycin.

Rapamycin

Rapamycin is an active anti-fungal compound derived from the soil bacterium *Streptomyces hygroscopicus* found on Easter Island (Rapa Nui). When administered to mammals, rapamycin inhibits the proliferation of mammalian cells—particularly B and T cells [15]. The mechanism for how rapamycin inhibits mTOR involves the formation of an inhibitory complex, composed of rapamycin and intracellular 12-kDa FK506-binding protein (FKBP12), that directly interacts with and inhibits the mTOR unit of mTORC1

[27]. It is unknown how the binding of rapamycin-FKBP12 to mTOR inhibits mTOR activity. Alterations within the structural integrity of mTORC1 and reduced kinase domain activity could explain rapamycin-induced mTOR inhibition [15, 27].

Yin-Yang 1 (YY1)

Yin-Yang 1 (YY1) is a GLI-Krüppel class of zinc-finger protein that is a part of the Polycomb protein group (PcG)—a group of proteins that regulate developmental processes such as stem-cell development, and maintain lineage-specific gene expression programs in various organisms [7]. It is a highly expressed protein that is evolutionarily conserved throughout vertebrates. There have been YY1 consensus binding sites found in more than 7% of vertebrate genes, which suggests that it plays a crucial role in regulating gene expression [25, 44]. YY1 was first identified as a transcriptional repressor that could function as an activator in the presence of adenovirus E1A protein; however, YY1 is now classified as a transcriptional activator, repressor, and initiator binding protein [6, 25, 30, 39]. Some of the cellular processes YY1 mediates on a transcriptional level include cell proliferation, induction of apoptosis, DNA repair, and the recruitment of proteins required for epigenetic modifications [37]. YY1 has also been implicated in tumorigenesis and repression of viral gene expression [6, 39, 44].

YY1 transcriptional activity can vary depending on its interactions with other proteins, localization within the cell, and post-translational modifications [11, 32, 38]. YY1 can interact with a variety of proteins in different cell signaling pathways. In the mTOR pathway, YY1 can interact with mTOR and other proteins to regulate mitochondrial metabolism [11]. YY1 can localize in different areas of the cell contingent

upon phases of the cell cycle. During the transition from G1 to S phase, YY1 translocates from the cytoplasm to the nucleus to aid in DNA synthesis [32]. Post-translational modifications of YY1, such as phosphorylation, glycosylation, and acetylation/deacetylation, can make YY1 act either as an activator or repressor of transcription, or even alter its DNA-binding ability [38].

YY1 and Cancer

YY1 overexpression has been observed in various cancer types such as prostate cancer, breast cancer, and B cell cancer [44]. It has been suggested that YY1 plays a role in tumorigenesis by interacting with tumor suppressor proteins and oncogenes and either positively or negatively regulating their activity [39, 44]. In studies that have looked at the interplay between YY1 and p53, a notable tumor suppressor protein, it was demonstrated that YY1 negatively regulated p53 activity by blocking p53 acetylation, promoting p53 ubiquitination and degradation, and inhibiting p53-mediated transcription [17, 36, 41, 44]. The antagonism between YY1 and p53 demonstrates how overexpressed YY1 can target tumor suppressor proteins in cancer cells.

While infection from viruses such as EBV can contribute to Burkitt's lymphoma formation, YY1 itself has been implicated in its formation through its interactions with oncogenes. It has been demonstrated that YY1 can positively regulate *c-myc*—a proto-oncogene that modulates cell proliferation and differentiation [5, 44]. YY1 can bind to an immunoglobulin heavy-chain gene HS3 enhancer and recruit CREB-binding protein (CBP) to increase c-myc promoter histone acetylation and positively regulate *c-myc* gene

expression [18, 44]. Overexpression of *c-myc* dramatically increases cell proliferation, which can aid in Burkitt's lymphoma progression [33].

YY1 and Repression of Viral Gene Expression

YY1 mainly acts as a repressor of viral gene expression. YY1 can recruit co-factors, such as histone deacetylases (HDACs), to human immunodeficiency virus type 1 (HIV-1) promoters to negatively regulate transcription (Figure 4) [10]. YY1 is also known to activate promoters of herpes simplex virus type 1 (HSV-1); however, current literature commonly supports YY1 acting as a viral gene repressor [30].

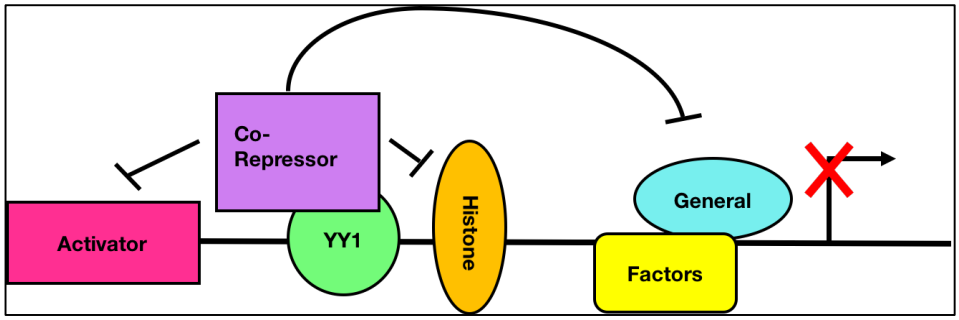


Figure 4. Model of YY1 Repression Through Co-Repressor Recruitment. In a model proposed by Thomas and Seto (1999), YY1 can interact with co-repressors such as histone deacetylases (HDACs) to repress gene expression.

In association with EBV, YY1 promotes EBV latency by negatively regulating BZLF1 and BRLF1 [6, 43]. It has been demonstrated that YY1 negatively regulates BZLF1 activity by binding to YY1 binding sites present on the P1 promoter of BZLF1, and overexpression of YY1 further downregulates P1 promoter activity [30]. YY1 can also bind to the BRLF1 promoter and negatively regulate its activity [43].

Aims of Thesis Research

The *Drosophila* homolog of mTOR (*Tor*) has been identified as a modifier of EBV Z and R activity [3]. It was further demonstrated that mTORC1 inhibition via rapamycin caused an increase in EBV lytic replication in epithelial cells, and a decrease in EBV lytic replication in B cells [3]. The repressive effects of rapamycin on mTORC1 could also extend to YY1—a transcription factor known for its roles in tumorigenesis, EBV latency, and modulating cellular processes in major cell signaling pathways like the mTOR pathway. While the effects of rapamycin on YY1 on BZLF1 have been studied, those same interactions with BRLF1 have not been investigated. This thesis research project aimed to investigate the effects of rapamycin on YY1 regulation of BRLF1 transcription in epithelial cells and B cells by:

1. Examining the effects of YY1 knockdown on BRLF1 expression in epithelial cells and B cells during mTORC1 inhibition and EBV lytic replication.
2. Determining how YY1 localizes differently based on rapamycin treatment and EBV lytic induction in epithelial cells and B cells.

CHAPTER II

MATERIALS AND METHODS

Materials

EBV-positive AGS-BDneo epithelial cells and EBV-positive Raji B cells were obtained from L. Hutt-Fletcher and ATCC, respectively. F-12 Ham's media and Roswell Park Memorial Institute (RPMI) media were purchased from Thermo Fisher Scientific. Fetal bovine serum was purchased from Gibco. Rapamycin, tetradecanoyl phorbol acetate (TPA), and sodium butyrate were purchased from Sigma Aldrich. Rapamycin and TPA were suspended in DMSO while sodium butyrate was suspended in sterile 1xPBS. Paraformaldehyde, 96%, ACROS Organics was purchased from Thermo Fisher Scientific. A QIAGEN GeneSolution YY1 siRNA (1 nmol) Kit and AllStars Negative Control siRNA were purchased from QIAGEN. Lipofectamine 2000 and Lipofectamine RNAiMax transfection reagents were purchased from Invitrogen. For RNA extraction, TRIzol™ reagent was purchased from Invitrogen. Power SYBR Green RNA-to-C_T™ 1-Step Kit was purchased from Applied Biosystems. BRLF1 forward primer (5'-CACCATGAGGCCTAAAAAGGATCG-3') and BRLF1 reverse primer (5'-CTAAAATAAGCTGGTGTCAAAAAT-3'), and GAPDH forward primer (5'-CTCCTCCTGTTCGACAGTCAGC-3') and GAPDH reverse primer (5'-CCCAATACGACCAAATCCGTT-3') were purchased from Eurofins Scientific MWG Biotech Incorporation.

Immobilon®-P nitrocellulose membranes were purchased from Millipore. PermaFluor Mountant mounting glue was purchased from Thermo Fisher Scientific. Primary anti-mouse YY1 and anti-rabbit alpha tubulin antibodies were purchased from Santa Cruz Biotechnology. Anti-rabbit Lamin-B1 antibody was purchased from Cell Signaling Technology. Goat-anti-mouse and goat-anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibodies, and AlexaFluor 488 conjugated donkey-anti-mouse secondary antibody were purchased from Jackson Immunoresearch. Hoescht stain was purchased from Sigma-Aldrich.

Cell Culture and Inhibitor Treatment

AGS-BDneo cells were cultured in Thermo Fisher Scientific F-12 Ham's media containing 10% fetal bovine serum, fungicide, penicillin, streptomycin, and 500 μg/mL G418 in 75 cm^2 culture flasks until confluent. When confluent, cells were washed twice with sterile 1xPBS and non-enzymatic cell dissociation solution (Cellstripper®) was applied to cells to collect dissociated cells. The dissociated cells were then either re-suspended in Thermo Fisher Scientific F-12 Ham's media with additives, or used for experiments. Raji cells were cultured in Thermo Fisher Scientific RPMI media containing 10% fetal bovine serum, fungicide, penicillin, and streptomycin in 75 cm^2 flasks. Both cell lines were maintained at 37°C and 5% CO_2 with their media changed every two to three days. Cells were passaged when cell confluency was >80%.

Both AGS-BDneo cells and Raji cells were treated with 5 nM rapamycin at different time intervals. Control cells or cells designated for chemical induction were not treated with rapamycin. Cells designated for rapamycin treatment either remained

unwashed until cell harvesting or had their media removed, were washed with sterile 1xPBS, had complete media added back, and were then treated with 5 nM rapamycin. AGS-BDneo cells were chemically induced into lytic replication using 5 ng/mL TPA and 0.75 mM sodium butyrate. Raji cells were chemically induced using 20 ng/mL TPA and 3 mM sodium butyrate. Both AGS-BDneo cells and Raji cells were induced 24-48 hours prior to cell harvesting.

Protein Extraction, SDS-PAGE, and Western Blotting

Protein extracts were created by harvesting cells, washing them twice with 1xPBS, re-suspending them in ELB lysis buffer (0.25 M NaCl, 5 mM EDTA, 0.1% NP-40, 50 mM pH 7 HEPES, protease/phosphatase inhibitors), and freeze-thawing twice for 12 minutes. Cells were then centrifuged for 10 minutes at 4°C, and supernatants were collected and quantified to determine protein concentrations. Equal amounts of protein (20-40 μg) were loaded onto a 10% SDS-PAGE gel, and electrophoresed at 200 V for 30-40 minutes. Proteins were then transferred onto Immobilon®-P (Millipore) membranes at 100 milliamps overnight.

Western blots were probed with anti-YY1, anti-alpha tubulin, and anti-Lamin-B1 antibodies (Santa Cruz or Cell Signaling) in 1:250, 1:500, and 1:500 dilutions in 0.25% milk block, respectively. Secondary goat-anti-mouse or goat-anti-rabbit HRP antibodies (Jackson Immunoresearch) diluted at 1:5000 in 0.25% milk block were then used to completely immunostain blots. Fully immunostained Western blots were then visualized using chemiluminescent reagents (Advansta) and a Li-Cor blot developer with Image Studio Digits software. Protein levels were also quantified using the same software.

siRNA Transfection

AGS-BDneo cells cultured in 60 x 15 mm plates had their media with additives and G418 removed. Cells were washed with sterile 1xPBS, and had Thermo Fisher Scientific F-12 Ham's media without additives added back. YY1 knockdown was achieved using a QIAGEN GeneSolution siRNA (1 nmol) Kit, and an AllStars Negative Control siRNA was used as a transfection control. Transfection mixture reactions for AllStars Negative control siRNA and YY1 siRNAs (Lipofectamine 2000 or Lipofectamine RNAiMax and Thermo Fisher Scientific F-12 Ham's media without additives) were incubated at room temperature for 20 minutes. Designated control plates were given a mixture of Lipofectamine 2000 or Lipofectamine RNAiMax transfection reagent, Thermo Fisher Scientific F-12 Ham's media without additives, and 1 nmol of AllStars Negative Control siRNA per plate. Plates designated for rapamycin treatment, chemical induction, or both conditions were given a mixture of Lipofectamine 2000 or Lipofectamine RNAiMax transfection reagent, Thermo Fisher Scientific F-12 Ham's media without additives, and 1 nmol of each of the four YY1 siRNAs per plate. AGS-BDneo cells were transfected for 24 hours, after which media was removed, cells were washed with sterile 1xPBS, and cells either had their media replaced with Thermo Fisher Scientific F-12 Ham's media with additives or had their media replaced and were treated with 5 nM rapamycin. After 48 hours, designated cells were chemically induced. Cells were harvested after 72 hours for either SDS-PAGE and Western blotting, or qRT-PCR.

Like the siRNA transfection protocol followed for AGS-BDneo cells, transfection mixture reactions for AllStars Negative Control and YY1 siRNAs (Lipofectamine

RNAiMax and Thermo Fisher Scientific RMPI media without additives) were incubated at room temperature for 20 minutes. During the 20-minute incubation period, Raji cells cultured in 75 cm^2 flasks had their cells removed and were centrifuged for 5 minutes. After the first spin, media was removed and cells were re-suspended in sterile 1xPBS. Instead of re-suspending the cells in Thermo Fisher Scientific RMPI media with additives or sterile 1xPBS after the second spin, cells were re-suspended in cold, Thermo Fisher Scientific RMPI media without additives. In control electroporation cuvettes, 500 μL of cell re-suspension were added along with 100 μL of Thermo Fisher Scientific RMPI media without additives, and 1 μL (1 nmol) of the AllStars Negative Control siRNA per cuvette. In YY1 siRNA electroporation cuvettes, 500 μL of cell re-suspension were added along with 100 μL of Thermo Fisher Scientific RMPI media without additives, and 0.25 μL (1 nmol) of each of the 4 YY1 siRNAs per cuvette.

Each set of electroporation cuvettes were mixed and incubated on ice for 10 minutes. Each sample was electroporated at 950 μF, 250 V for 2 seconds to ensure cell permeability to siRNA transfection, and samples were incubated on ice for an additional 10 minutes. The electroporated samples were then transferred into designated 25 cm^2 flasks with Thermo Fisher Scientific RMPI media with additives, and incubated for a 3-day period. Designated flasks were treated with 5 nM rapamycin after 24 hours, and chemically induced after 48 hours. After 72 hours, cells were harvested for SDS-PAGE and Western blotting.

Antibody Staining for Immunocytochemical Analysis

AGS-BDneo cells were cultured in 35 x 10 mm plates with Thermo Fisher Scientific F-12 Ham's media with additives and coverslips prior to antibody staining. Raji cells were cultured in 25 cm^2 flasks with Thermo Fisher Scientific RMPI media with additives. Designated AGS-BDneo cells and Raji cells were treated with 5 nM rapamycin for 24 hours. After 24 hours, designated cells were chemically induced and incubated for an additional 24 hours. For AGS-BDneo cells, coverslips were transferred to a 24-well plate with each well containing sterile 1xPBS after a total of 48 hours. Coverslips were washed with sterile 1xPBS, and then fixed with 0.4% paraformaldehyde (1xPBS, 0.4 g paraformaldehyde incubated at 65°C for 1 hour) for 15 minutes at room temperature. Fixative was removed, and coverslips were incubated at room temperature with incubation mix (1xPBS, 0.3% BSA, 5% donkey serum, 0.1% Triton-X) for 10 minutes. Cells were then incubated with incubation mix plus primary anti-YY1 antibody (1:200 dilution) for 1 hour at 37°C, washed 4 times with sterile 1xPBS, and incubated with incubation mix and AlexaFluor 488 conjugated donkey-anti-mouse secondary antibody (1:400 dilution; Jackson Immunoresearch) for 40 minutes at 37°C with aluminum foil covering the 24-well plate. Cells were washed 4 times with sterile 1xPBS with the 4^{th} wash containing 1 nmol of Hoechst Stain (1:2000 dilution; Sigma Aldrich). Coverslips were then mounted onto slides with PermaFluor Mountant mounting glue (Thermo Fisher Scientific).

The same procedure was performed for Raji cells, except cells were washed and stained in 1.5-mL microfuge tubes. Raji cells were transferred from their flasks to 15-mL

Falcon tubes, centrifuged for 5 minutes, and transferred to 1.5 mL microfuge tubes in which washing and staining was performed. To ensure that cells were not completely washed away during washing and staining steps, the amount of time cells were washed was reduced. In between the removal and addition of reagents, cells were centrifuged for 3 minutes and supernatant was decanted. After cells were stained with 1 nmol of Hoechst stain (Sigma-Aldrich), the stain was removed, cells were re-suspended in PermaFluor Mountant mounting glue (Thermo Fisher Scientific), and cells were mounted onto slides.

Both AGS-BDneo and Raji cell slides were examined using a KEYENCE BZ-X700 Series All-in-One Fluorescence Microscope. Images were viewed using BZ-X Viewer software. A haze reduction feature was used on Raji cell images to reduce the blurriness of the images due to the natural clumping of the cells, and the bright fluorescence of YY1. YY1 fluorescence levels in the cytoplasm and nuclei of AGS-BDneo cells was measured using BZ-X Analyzer software at a 10-s exposure, 40x objective, and x1.6 zoom. The same software was used to measure YY1 fluorescence levels in the cytoplasm and nuclei of Raji cells except cells were viewed at a 2-s exposure, 40x objective, and x1.6 zoom.

Nuclear and Cytoplasmic Extractions

AGS-BDneo cells and Raji cells were cultured in 100 x 20 mm plates and 25 cm^2 flasks, respectively. Cells either received no treatment, were treated with 5 nM rapamycin, chemically induced, or were both treated with rapamycin and chemically induced. After 72 hours, cells were harvested. Cells were washed with sterile 1xPBS and cell pellets were re-suspended in CE buffer (10 mM HEPES 7.6, 60 mM KCl, 1 mM

EDTA, 1 mM DTT, 0.7% NP-40, protease/phosphatase inhibitors). Cells were incubated on ice for 5 minutes before they were centrifuged at 4°C and had their supernatant transferred to new 1.5-mL microfuge tubes (cytoplasmic extracts). Nuclei pellets were washed twice with CE buffer without 0.7% NP-40. Nuclei pellets were then re-suspended in ELB lysis buffer (0.25 M NaCl, 5 mM EDTA, 0.1% NP-40, 50 mM pH 7 HEPES, protease/phosphatase inhibitors), freeze-thawed twice for 12 minutes, and were sonicated for 10 seconds at 2 watts (RMS) before being centrifuged for 10 minutes at 4°C. The resulting supernatants were then transferred to new 1.5-mL microfuge tubes (nuclear extracts). Protein concentrations were quantified prior to SDS-PAGE and Western blotting.

Quantitative Real-Time PCR (qRT-PCR)

AGS-BDneo were cultured and treated similarly to the protocol outlined for siRNA transfection. Total RNA was extracted using the TRIzol™ reagent purchased from Invitrogen, and following the manufacturer's instructions. RNA concentrations were quantified prior to qRT-PCR using a Nano-Drop® ND-1000 Spectrophotometer and its accompanying software. qRT-PCR reactions (20 μL) were performed using the Power SYBR Green RNA-to-C_T™ 1-Step Kit (Applied Biosystems), 10 ng of total RNA, and 20 pmol of each BRLF1 primer as shown in the manufacturer's instructions. RNA samples were done in triplicate as well as the positive control (GAPDH) and negative control (RNase-free water). Reactions were performed and analyzed with an Applied Biosystems Step One Plus RT-PCR machine and its accompanying software.

Data Analysis

Western blots immunostained with anti-YY1 antibody for YY1 knockdown had YY1 protein quantification values normalized using alpha tubulin as a loading control. Image Studio Digits software was used to quantify protein levels. Fold change in BRLF1 expression via qRT-PCR was quantified using mean ΔC_T values. Mean ΔC_T values were normalized relative to the control. Western blots immunostained with anti-YY1 antibody for YY1 localization had YY1 protein quantification values normalized using alpha tubulin as a cytoplasmic loading control, and Lamin-B1 as a nuclear loading control. Protein levels were quantified using Image Studio Digits software. Normalized values were held to their respective conditions. BZ-X Analyzer software was used to quantify YY1 fluorescence and determine YY1 localization via immunocytochemical analysis. For Aim 2, YY1 localization data was also represented as ratios of the normalized values held to their respective conditions. Ratios with values above 1.0 were considered to represent YY1 localization predominating in the nucleus; ratios with values below 1.0 were considered to represent YY1 localization predominating in the cytoplasm. Standard error was calculated by dividing the standard deviation of all the normalized values by the square of the number of conditions. Statistical significance was calculated using a student two-tailed T-test, and only $p<0.05$ were considered significant.

CHAPTER III

RESULTS

Previous studies in our lab have demonstrated that rapamycin treatment of epithelial cells and B cells caused cell-specific alterations in YY1 regulation of BZLF1 expression. It was also demonstrated that YY1 primarily localized in the nucleus of B cells rather than in the cytoplasm during mTORC1 inhibition. In epithelial cells, YY1 localization was non-specific when compared to YY1 localization in B cells. These findings suggest that YY1 does play a role in regulating EBV gene expression during mTORC1 inhibition. In this study, I wanted to see the how mTORC1 inhibition, via rapamycin, affected YY1 regulation of BRLF1 gene expression in both epithelial cells and B cells.

YY1 Knockdown Occurs More Readily in Epithelial Cells Than in B Cells

The aim of this study was to successfully knockdown YY1 in epithelial cells and B cells. In previous studies from our lab, YY1 knockdown was achievable in epithelial cells, with expression decreased more than 60%. The level of YY1 knockdown in B cells, however, was more difficult to achieve. AGS-BDneo epithelial cells and Raji B cells were used to perform YY1 knockdown via siRNA transfection to see if YY1 knockdown could be achieved in both cell types.

RNAi was propagated via siRNA transfection in both epithelial cells and B cells to determine the extent of YY1 knockdown. siRNA transfection was performed using QIAGEN GeneSolution YY1 siRNAs and an AllStars Negative Control siRNA to compare levels of YY1 knockdown relative to a control. For epithelial cells, Lipofectamine RNAiMax transfection reagent was used to achieve YY1 knockdown. After 3 days, YY1 knockdown in epithelial cells was assayed via SDS-PAGE and Western blotting. It was confirmed that YY1 knockdown was achieved in epithelial cells (Figure 5A). When compared to the control, YY1 expression decreased by 65%. The same Western blot was probed with alpha tubulin to confirm that each sample was equally loaded prior to Western blot analysis.

Sufficient YY1 knockdown was not achieved in B cells. There were four different protocols created to knockdown YY1, and none of them yielded a YY1 knockdown comparable to the knockdown that occurred in epithelial cells. The first protocol involved using Lipofectamine 2000 and electroporation as previously stated. Cells were also incubated for 4 days to see if they required a longer incubation period to be transfected. After 4 days, YY1 knockdown was assayed via SDS-PAGE and Western blotting, and Western blot analysis was performed similarly to that of epithelial cells. There was only a 10.5% decrease in YY1 expression in B cells (Figure 5B).

The second protocol involved using Lipofectamine 2000 and electroporation, but this time cells were electroporated for 2 seconds, 5 seconds, and 8 seconds. This was performed to see how long cells could be electroporated to ensure siRNA transfection and viability. Like the first protocol, YY1 knockdown was assayed via SDS-PAGE and

Western blotting. While YY1 expression decreased by 10% when cells were electroporated for 2 seconds, YY1 knockdown was still insufficient compared to YY1 knockdown in epithelial cells. YY1 expression did not decrease in cells that were electroporated for 5 seconds or 8 seconds (Figure 5C).

The third protocol involved using Lipofectamine RNAiMax as the transfection reagent in combination with electroporation. Like the results from the first two protocols, adequate YY1 knockdown was not achieved in B cells (Figure 5D). Finally, the fourth protocol involved using Lipofectamine RNAiMax and electroporation, but this time cells were cultured in a 6-well plate and electroporated at a higher voltage (310 V). Cells were also either washed with sterile 1xPBS after 5 or 24 hours, or not washed at all. Cells with designated wash times after 5 or 24 hours were initially plated in plain RPMI media and had their media replaced with RPMI media with additives after being washed with sterile 1xPBS. The other cells that were not washed with sterile 1xPBS were cultured and maintained in RPMI media with additives. After 3 days, YY1 knockdown was assessed similarly to that of the previous protocols. Like the outcomes of the previous protocols, YY1 knockdown was not achieved in B cells (Figure 5E).

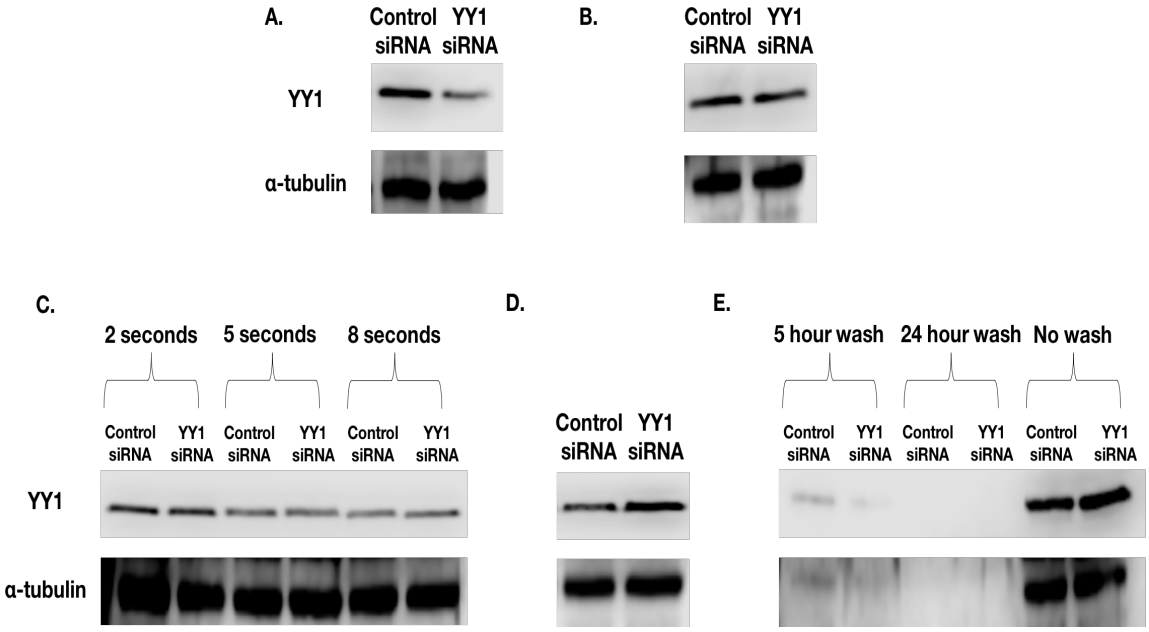


Figure 5. YY1 Knockdown Occurs More Readily in Epithelial Cells Than in B Cells. (A) AGS-BDneo epithelial cells and (B-E) Raji B cells were cultured in their respective media, and transfected with YY1 siRNAs and an AllStars Negative Control siRNA. Protein lysates were prepared for both cell types, and were subjected to SDS-PAGE and Western blotting using antibodies against YY1 and alpha tubulin. (A) YY1 knockdown in AGS-BDneo epithelial cells. (B) YY1 knockdown in Raji B cells using Lipofectamine 2000 transfection reagent. (C) YY1 knockdown in Raji B cells with 2-second, 5-second, and 8-second electroporation time intervals. (D) YY1 knockdown in Raji B cells using Lipofectamine RNAiMax transfection reagent. (E) YY1 knockdown in Raji B cells with 5-hour and 24-hour washes, and no wash with sterile 1xPBS. The results are represented as separate experiments.

YY1 Positively Regulates BRLF1 Expression in Epithelial Cells During mTORC1 Inhibition and Lytic Replication

Our lab has demonstrated that YY1 knockdown is possible in epithelial cells, and that loss of YY1 during mTORC1 inhibition downregulates BZLF1 expression. While it was determined that YY1 knockdown upregulated BZLF1 expression in B cells treated with rapamycin, the level of YY1 knockdown was insufficient compared to YY1 knockdown in epithelial cells. The effects of YY1 knockdown and rapamycin on BRLF1

expression remain unknown in epithelial cells and B cells. I wanted to determine whether YY1 was acting as an activator or repressor of BRLF1 transcription, and if rapamycin could alter YY1 transcriptional regulation of BRLF1 in epithelial cells and B cells. Due to insufficient YY1 knockdown occurring in Raji B cells, only AGS-BDneo epithelial cells were used to assess the effects of YY1 knockdown and rapamycin on BRLF1 expression.

AGS-BDneo epithelial cells either received no treatment or were treated with 5 nM rapamycin, chemically induced with TPA and sodium butyrate, or a combination of the two. RNA was extracted from the cells and used for quantitative real-time PCR (qRT-PCR). RNase-free water was used a negative control and GAPDH was used as a positive control to ensure sufficient qRT-PCR reactions occurred. When compared to the control, there was an increase in BRLF1 transcript levels when YY1 knockdown occurred (Figure 6). The increase in BRLF1 transcript levels yielded a $p<0.05$, which was statistically significant. There was a slight increase in BRLF1 transcript levels when YY1 knockdown occurred in chemically induced cells, but it was not statistically significant (Figure 6). When comparing BRLF1 transcript levels in chemically induced control siRNA-transfected cells to chemically induced control siRNA-transfected cells treated with rapamycin, there was a six-fold increase in BRLF1 transcript levels (Figure 6). The six-fold increase in BRLF1 transcript levels yielded a statistically significant $p<0.05$. When YY1 knockdown occurred in chemically induced cells treated with rapamycin, there was a decrease in BRLF1 transcript levels compared to control siRNA-transfected cells with

the same combination treatment (Figure 6). The decrease in BRLF1 transcript levels between those cells yielded a statistically significant $p<0.05$.

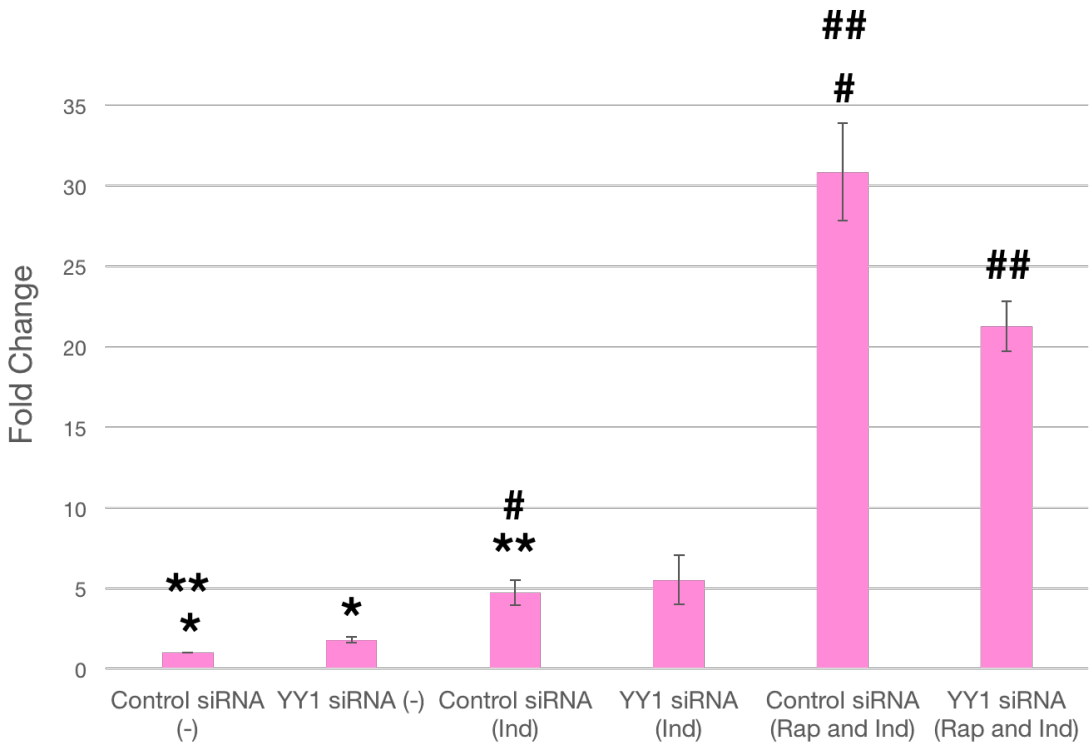


Figure 6. YY1 Positively Regulates BRLF1 Expression in Epithelial Cells During mTORC1 Inhibition and Lytic Replication.
AGS-BDneo epithelial cells were cultured in their respective media and were either treated with 5 nM rapamycin, chemically induced with TPA and sodium butyrate, or a combination of the two. After 3 days, RNA extraction was performed and qRT-PCR was used to assess fold change in BRLF1 transcript levels. The results are the means ± SEM of three separate experiments. The data were normalized relative to the control. * = $p<0.05$ relative to YY1 knockdown cells (with no treatment); ** = $p<0.05$ relative to chemically induced cells with no YY1 knockdown; # = $p<0.05$ relative to chemically induced cells treated with rapamycin and no YY1 knockdown; ## = $p<0.05$ relative to YY1 knockdown cells chemically induced and treated with rapamycin.

YY1 Localization Determination via SDS-PAGE and Western Blotting

Post-translational modifications imposed on YY1 can alter its function and localization within the cell [32, 38]. Our lab has previously demonstrated that YY1 localization varies between epithelial cells and B cells during mTORC1 inhibition and lytic replication. In epithelial cells, YY1 did not specifically localize in a high concentration in neither the nucleus nor cytoplasm while YY1 localized in a higher concentration in the nucleus of B cells. I wanted to examine whether YY1 localization was more predominant in the nucleus or cytoplasm of epithelial cells and B cells treated with rapamycin and undergoing lytic replication. I also wanted to see if creating nuclear and cytoplasmic extracts and assaying YY1 protein concentrations via SDS-PAGE and Western blotting was a more sensitive method for determining YY1 localization.

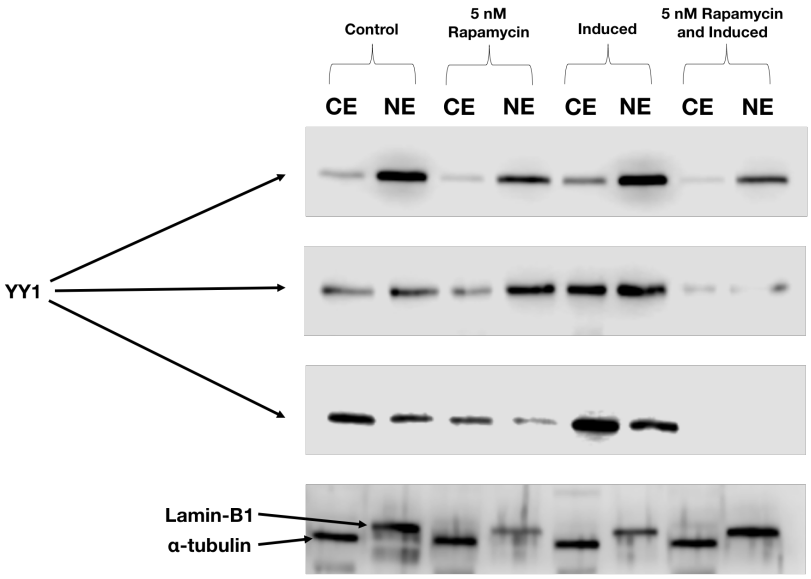
Nuclear and cytoplasmic extracts were created from AGS-BDneo epithelial cells and Raji B cells that either received no treatment or were treated with 5 nM rapamycin, chemically induced with TPA and sodium butyrate, or a combination of the two. YY1 localization was assayed via SDS-PAGE and Western blotting using anti-YY1 antibody, and anti-alpha tubulin and anti-Lamin-B1 antibodies were used to as cytoplasmic and nuclear loading controls, respectively. In epithelial cells, YY1 primarily localized in the nucleus of cells that received no treatment, were treated with rapamycin, or were chemically induced (Figure 7A-7C). YY1 nuclear localization drastically increased in cells that were chemically induced, but it was not statistically significant (Figure 7A-7C). Cells chemically induced and treated with rapamycin primarily had YY1 localizing in the cytoplasm; however, there were instances in which YY1 would not appear under those

conditions (Figure 7A). The increase in cytoplasmic YY1 localization in chemically induced cells and sudden decrease in cytoplasmic YY1 localization in chemically induced cells treated with rapamycin was statistically significant ($p<0.05$) relative to the control (Figure 7B).

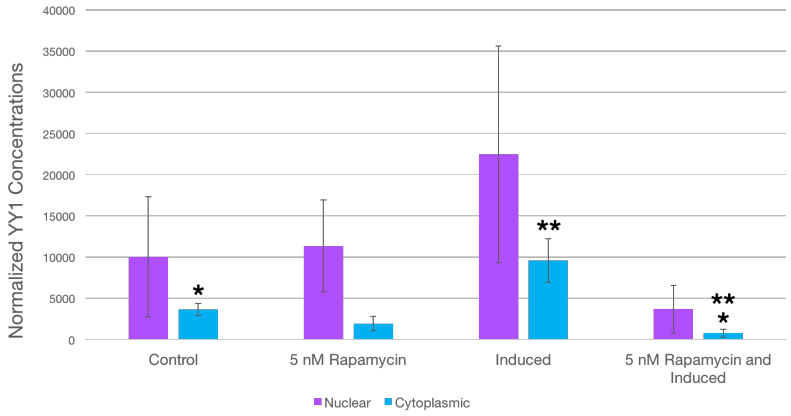
In B cells, YY1 localization was equally distributed in cells that received no treatment (Figure 7D-7F). When cells were treated with rapamycin, there was an increase in nuclear YY1 localization relative to the control (Figure 7E and 7F). The decrease in YY1 localization in cells treated with rapamycin relative to chemically induced cells treated with rapamycin was statistically significant ($p<0.05$). Cytoplasmic YY1 localization predominantly occurred in chemically induced cells and chemically induced cells treated with rapamycin (Figure 7E and 7F). The subsequent decrease in nuclear YY1 localization in chemically induced cells treated with rapamycin relative to the control was significant ($p<0.05$).

AGS-BDneo

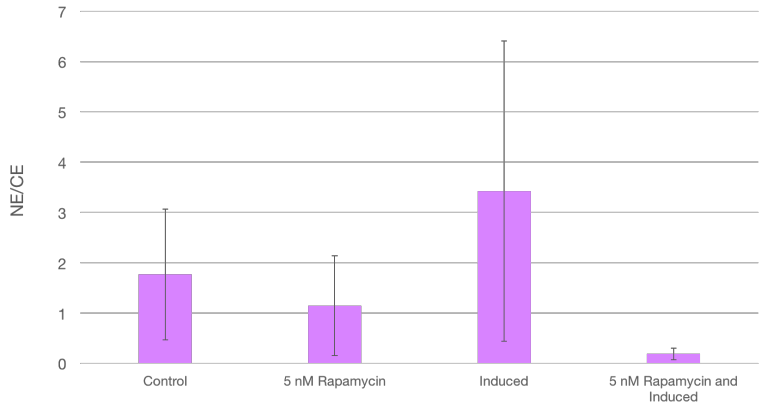
A.



B.



C.



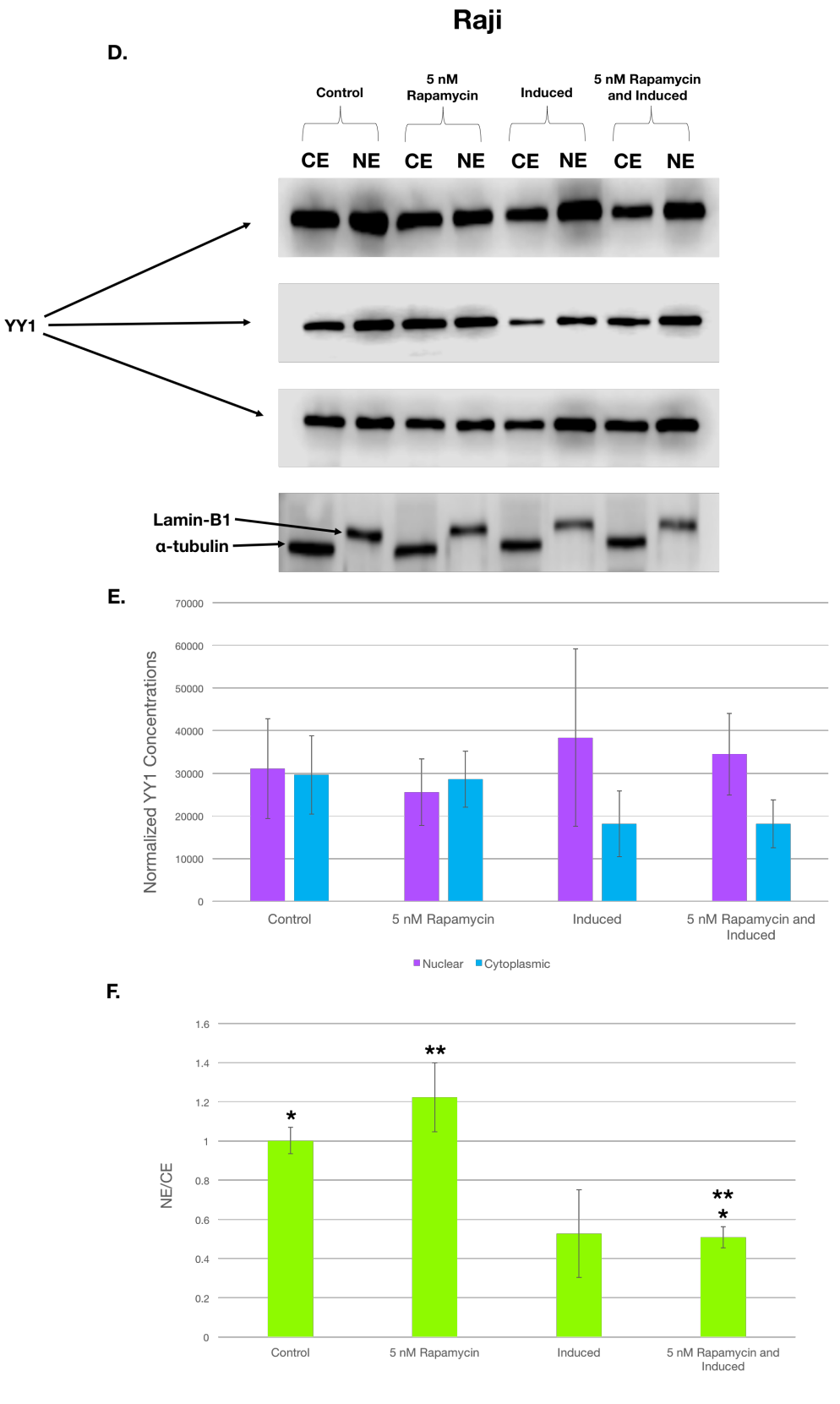

Raji
D.
Control
5 nM Rapamycin
Induced
5 nM Rapamycin and Induced
CE NE CE NE CE NE CE NE
YY1
Lamin-B1
α-tubulin
E.
Normalized YY1 Concentrations
70000
60000
50000
40000
30000
20000
10000
0
Control
5 nM Rapamycin
Induced
5 nM Rapamycin and Induced
Nuclear
Cytoplasmic
F.
NE/CE
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
**
*
Control
5 nM Rapamycin
Induced
5 nM Rapamycin and Induced

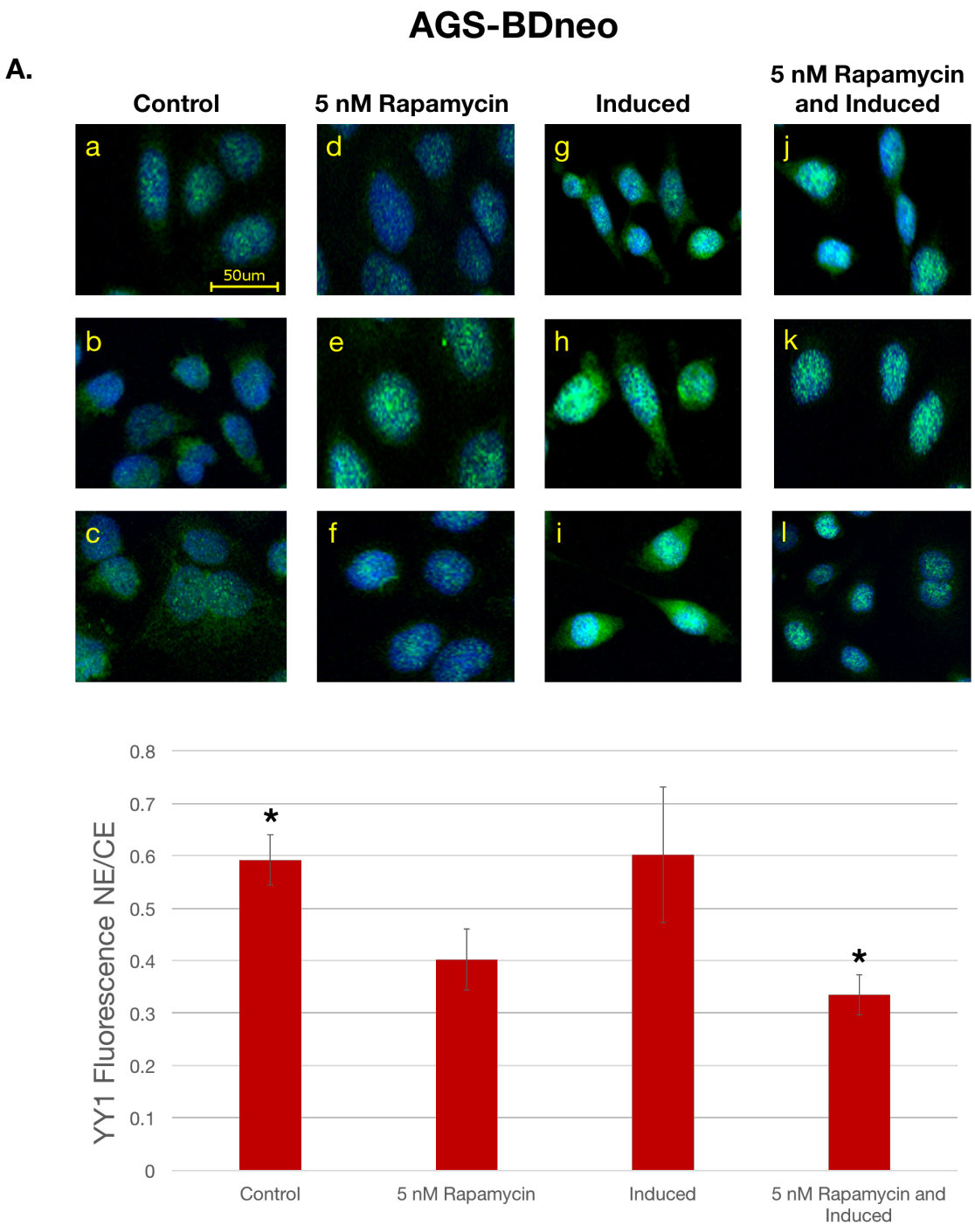
Figure 7. YY1 Localization Determination via SDS-PAGE and Western Blotting. (A-C) AGS-BDneo epithelial cells and (D-F) Raji B cells were cultured in their respective media and were either treated with 5 nM rapamycin, chemically induced with TPA and sodium butyrate, or a combination of the two. After 3 days, nuclear and cytoplasmic extracts were created and YY1 localization was assayed via SDS-PAGE and Western blotting. YY1 localization data for both cell types are represented as normalized YY1 concentrations (B and E) and ratios normalized to their respective loading control (C and F). The results are the means ± SEM of three separate experiments. (B) * = $p<0.05$ relative to chemically induced cells treated with rapamycin; ** = $p<0.05$ relative to chemically induced cells treated with rapamycin. (F) * = $p<0.05$ relative to chemically induced cells treated with rapamycin; ** = $p<0.05$ relative to chemically induced cells treated with rapamycin.

YY1 Localization Determination via Immunocytochemical Analysis

While our lab has investigated YY1 localization via SDS-PAGE and Western blotting, YY1 localization has yet to be investigated through immunocytochemical analysis. I wanted to see if YY1 localization in epithelial cells and B cells could be validated through another method of protein localization analysis. AGS-BDneo epithelial cells and Raji B cells either received no treatment or were treated with 5 nM rapamycin, chemically induced with TPA and sodium butyrate, or a combination of the two before they were fixed and stained onto slides. The nuclei of both cell types were stained with Hoescht stain (Sigma-Aldrich) to define the boundary between the nucleus and cytoplasm, and YY1 was tagged with an anti-YY1 antibody and AlexaFluor 488 conjugated donkey-anti-mouse secondary antibody (Jackson Immunoresearch) to make it fluoresce green in the cells. Prepared cell slides were examined using a KEYENCE BZ-X700 Series All-in-One Fluorescence Microscope, and YY1 fluorescence was quantified using BZ-X Analyzer software. For both epithelial cells and B cells, 120 cells were examined per condition for a total of 960 cells.

My data suggests that YY1 localization in epithelial cells is predominantly in the cytoplasm (Figure 8A). Cells that received no treatment or were chemically induced had more nuclear YY1 localization, but YY1 localization was still predominant in the cytoplasm (Figure 8A). There was a slight increase in nuclear YY1 localization in chemically induced cells relative to the control, but it was not statistically significant. YY1 localization increased in the cytoplasm in cells treated with rapamycin, and further increased in chemically induced cells treated with rapamycin (Figure 8A). Cytoplasmic YY1 localization in chemically induced cells treated with rapamycin relative to the control was statistically significant ($p<0.05$).

In B cells, YY1 localization primarily occurred in the nucleus. Nuclear YY1 localization was the highest in cells treated with rapamycin (Figure 8B). Nuclear YY1 localization slightly decreased and was comparably more cytoplasmic in chemically induced cells (Figure 8B). In chemically induced cells treated with rapamycin, YY1 localization became slightly more nuclear relative to chemically induced cells (Figure 8B). There was no statistical significance when examining YY1 localization in B cells treated rapamycin, chemically induced, or a combination of the two.


AGS-BDneo
A.
Control
5 nM Rapamycin
Induced
5 nM Rapamycin and Induced
a
b
c
d
e
f
g
h
i
j
k
l
50um
YY1 Fluorescence NE/CE
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
Control
5 nM Rapamycin
Induced
5 nM Rapamycin and Induced

B.

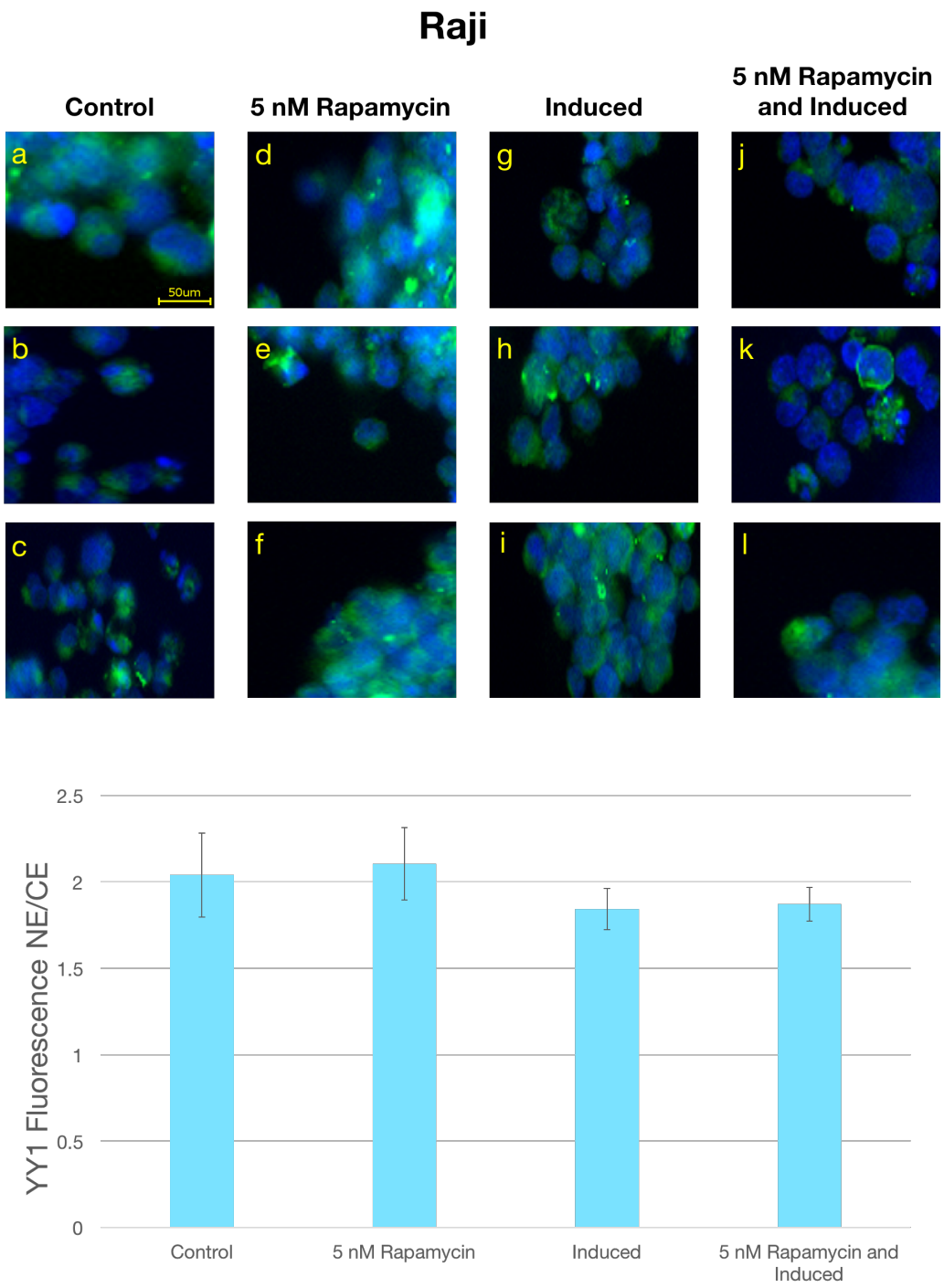

Raji
Control
5 nM Rapamycin
Induced
5 nM Rapamycin and Induced
a
b
c
d
e
f
g
h
i
j
k
l
50um
YY1 Fluorescence NE/CE
2.5
2
1.5
1
0.5
0
Control
5 nM Rapamycin
Induced
5 nM Rapamycin and Induced

Figure 8. YY1 Localization Determination via Immunocytochemical Analysis. (A) AGS-BDneo epithelial cells and (B) Raji B cells were cultured in their respective media and were either treated with 5 nM rapamycin, chemically induced with TPA and sodium butyrate, or a combination of the two. After 3 days, cells were fixed and stained onto slides that were visualized using a KEYENCE BZ-X700 Series All-in-One Fluorescence Microscope. YY1 fluorescence was quantified using BZ-X Analyzer software. The results are the means ± SEM of three separate experiments. The data were calculated as ratios and normalized relative to the control. (A) * = $p<0.05$ relative to chemically induced cells treated with rapamycin.

CHAPTER IV

DISCUSSION

While YY1 knockdown in AGS-BDneo epithelial was easily achieved (Figure 5A), YY1 knockdown in Raji B cells proved to be difficult (Figure 5B-5E). In epithelial cells, YY1 knockdown decreased YY1 expression by 65% relative to the control. That suggests that siRNA transfection was successful and that AGS-BDneo epithelial cells are more capable of endocytosing siRNA molecules complexed to lipids. YY1 knockdown in B cells was not achieved to the extent of YY1 knockdown in epithelial cells.

Immune cells are notoriously difficult to transfect. Primary lymphocytes are known to be highly resistant to non-viral transfection methods involving cationic lipids and polymer reagents [29]. The use of conventional transfection methods for immune cells, such as B cells, can result in high rates of cell death and low transfection efficiency [21]. Immune cells are also susceptible to induced apoptosis when they are introduced to foreign nucleic acids. The introduction and accumulation of foreign DNA in the cytoplasm of monocytes, dendritic cells, and macrophages has been shown to trigger apoptotic cell death [35]. It was suggested that apoptotic cell death occurred due to the functioning of the cells in response to viral infection. B cells share similar characteristics with the cells mentioned in the study, which suggests that B cells may also undergo apoptosis when introduced to foreign siRNA molecules [35]. Increased B cell death would contribute to decreased siRNA transfection efficiency.

Insufficient YY1 knockdown in B cells may also be contributed to YY1's role in B cell physiology. YY1 is a known regulator of germinal centers—sites within lymphoid organs that aid in B cell maturation and differentiation [16, 21]. YY1 binds to germinal center-specific genes and regulates gene expression programs involved in B cell development and function [16]; therefore, YY1 knockdown may not be feasible in B cells due YY1's critical role in maintaining and regulating B cell physiology. Loss of YY1 could also impact B cell viability.

Lipofectamine 2000 transfection reagent and electroporation were initially used to enhance siRNA transfection of B cells. When YY1 expression only decreased by 10.5%, it was thought that the duration of cell electroporation might have been the cause of insufficient knockdown (Figure 5B). Electroporation is a commonly used method to aid in siRNA transfection of resistant cells such as B cells, so I tested different time intervals to establish how long cells should be electroporated to ensure efficient siRNA transfection and cell viability. It was confirmed that 2 seconds was the longest time interval to electroporate B cells and achieve YY1 knockdown (Figure 5C). YY1 expression decreased by 10% relative to the control. When cells were electroporated for more than 2 seconds, YY1 knockdown was unsuccessful possibly due to decreased cell viability.

For the third trial of YY1 knockdown in B cells, the transfection reagent was changed from Lipofectamine 2000 to Lipofectamine RNAiMax. It was presumed that the transfection reagent used for the first two trials might have contributed to poor siRNA transfection efficiency. When B cells were transfected using Lipofectamine RNAiMax in

combination with electroporation, YY1 knockdown did not occur (Figure 5D). The final trial for YY1 knockdown in B cells involved plating the cells in a 6-well plate with different media, and washing them with sterile 1xPBS at different time intervals during their incubation period. Cells that were cultured for 24 hours in plain RPMI media prior to being washed did not yield enough protein to assess if YY1 knockdown occurred or not (Figure 5E). Cell density and viability may have decreased when cells were both cultured in plain RPMI media and washed with sterile 1xPBS before having their media replaced with enriched RPMI media. Cells that were washed after 5 hours with sterile 1xPBS initially indicated that YY1 knockdown occurred; however, when protein extract loading was assessed using alpha tubulin as a loading control, the faintness and smear of the bands indicated that there was not enough protein to determine whether YY1 knockdown occurred or not (Figure 5E).

Although the effects of YY1 knockdown and rapamycin on BRLF1 transcription in B cells could not be determined via qRT-PCR due to inadequate YY1 knockdown, those effects were still determined in epithelial cells. The increase in BRLF1 transcript levels in YY1 knockdown epithelial cells that received no treatment or were chemically induced relative to the control supports the role of YY1 in maintaining EBV latency (Figure 6). The absence of YY1 in epithelial cells permits BRLF1 transcription to occur due to decreased YY1 repression of BRLF1. When epithelial cells were treated with rapamycin and chemically induced, there was a drastic increase in BRLF1 transcript levels in cells transfected with control siRNA and a decrease in BRLF1 transcript levels in YY1 knockdown cells with the same treatment combination (Figure 6). Based on those

results, it is suggested that YY1 is a positive regulator of BRLF1 transcription when mTORC1 is inhibited via rapamycin. When mTORC1 is inhibited, its inhibition negates YY1 repression of BRLF1 and helps to promote BRLF1 expression. That suggests that inhibited mTORC1 acts as a positive regulator of BRLF1 expression in the presence of YY1, and that active mTORC1 acts on YY1 to modify its role in transcription. When YY1 is absent during mTORC1 inhibition, inhibited mTORC1 has nothing to repress. Inhibited mTORC1 may then act as a negative regulator of BRLF1 during EBV lytic replication by exerting its repressive activity on BRLF1 when it would normally repress YY1 repressor activity.

YY1 localization within epithelial cells and B cells varied during mTORC1 inhibition and lytic replication. When YY1 localization was investigated in epithelial cells via SDS-PAGE and Western blotting, localization primarily occurred in the nucleus when cells received no treatment, were treated with rapamycin, or were chemically induced (Figure 7A-7C). YY1 localization primarily occurred in the cytoplasm of cells that were chemically induced and treated with rapamycin (Figure 7C). That suggests that active mTORC1 may phosphorylate YY1 and cause it to translocate into the nucleus where it can positively regulate BRLF1 transcription. When mTORC1 is inhibited via rapamycin, YY1 remains in the cytoplasm because it is unable to be phosphorylated by mTORC1. Some YY1 may get recruited to the nucleus during mTORC1 inhibition to aid in maintaining EBV latency, but YY1 will mainly reside in the cytoplasm. Cytoplasmic YY1 localization will also occur when mTORC1 is inhibited and when EBV switches from latent to lytic. YY1 acting as a positive regulator of BRLF1 transcription during

mTORC1 inhibition is further supported by data collected when BRLF1 transcript levels were investigated using qRT-PCR (Figure 6).

The variance in YY1 localization in chemically induced cells and chemically induced cells treated with rapamycin was due to YY1 being cleaved or completely disappearing (Figure 7A). Normalized YY1 localization data is provided to show the degree of nuclear and cytoplasmic YY1 localization (Figure 7B). When YY1 protein bands were quantified (Figure 7A), it showed YY1 localization predominating in the nucleus regardless of cell treatment (Figure 7B); however, when representing the data as ratios, YY1 localization was shown to predominant in the cytoplasm during mTORC1 inhibition and chemical induction (Figure 7C). It is unknown why YY1 was cleaved predominantly in the cytoplasm and sometimes degraded when cells were chemically induced and treated with rapamycin. It is possible that EBV can promote YY1-targeted degradation under certain conditions.

When assaying YY1 localization in B cells via SDS-PAGE and Western blotting, YY1 was predominantly localizing in the nucleus of cells that were treated with rapamycin (Figure 7D-7F). Those results suggest that during mTORC1 inhibition, YY1 could be recruited to the nucleus by EBV to promote latency by repressing BRLF1 expression. When cells were chemically induced, or chemically induced and treated with rapamycin, YY1 localization increased in the cytoplasm relative to the control. That suggests that YY1 remains in the cytoplasm due to inhibited mTORC1 not being able to phosphorylate it and cause it to translocate to the nucleus. YY1 residing in the cytoplasm during EBV lytic replication also allows the virus to switch from latent to lytic, and

promote viral replication. Normalized YY1 localization data shows an unequal distribution of YY1 in the nucleus and cytoplasm of B cells (Figure 7E); however, when YY1 localization data was represented as ratios, YY1 localization was shown to be equally distributed in the nucleus and cytoplasm of cells that received no treatment (Figure 7F). YY1 was sometimes cleaved in B cell cytoplasmic extracts; however, it was inconsistent relative to YY1 cleavage in epithelial cells (Figure 7A).

Immunocytochemical analysis of YY1 localization in epithelial cells differs from data collected on determining YY1 localization via SDS-PAGE and Western blotting. When quantifying YY1 fluorescence in epithelial cells, YY1 localization primarily occurred in the cytoplasm of cells regardless of treatment or no treatment (Figure 8A). Cells that received no treatment or were chemically induced had more nuclear YY1 localization relative to cells treated with rapamycin or chemically induced and treated with rapamycin; however, YY1 localization was still predominantly cytoplasmic (Figure 8A). Increased nuclear YY1 localization in chemically induced epithelial cells further supports YY1's role as a positive regulator of BRLF1 activity. When cells were treated with rapamycin or were chemically induced and treated with rapamycin, YY1 localization occurred more in the cytoplasm than in the nucleus (Figure 8A). During mTORC1 inhibition via rapamycin, increased YY1 localization in the cytoplasm supports data suggesting that inhibited mTORC1 is not able to phosphorylate YY1—thus allowing it to remain in the cytoplasm even during EBV lytic replication.

While immunocytochemical analysis data on the effects of no treatment or rapamycin treatment on YY1 localization in B cells supports data collected via SDS-

PAGE and Western blotting, the effects of chemical induction on YY1 localization varies (Figures 7F and 8B). When examining YY1 localization via SDS-PAGE and Western blotting, YY1 localization in chemically induced B cells primarily occurred in the cytoplasm, and became increasingly cytoplasmic when chemically induced cells were also treated with rapamycin (Figure 7F); however, immunocytochemical analysis data suggests that YY1 localization increases in the nucleus when cells are chemically induced and treated with rapamycin (Figure 8B). Increased nuclear YY1 localization in chemically induced B cells treated with rapamycin suggests that other nuclear localization signals are causing YY1 to localize more in the nucleus, or EBV is possibly recruiting some YY1 to the nucleus to modulate the volume of viral transcripts being produced during lytic replication.

The mTOR pathway is a critical host cell-signaling pathway that viruses can manipulate to promote viral replication. Increases in mTOR activity have been shown to increase viral replication and cell growth in cells infected with adenovirus or HMCV [9]. When cells were treated with mTOR inhibitors like RAD001, decreased mTOR activity attenuated viral replication and reduced incidence of viral infection [9]. Rapamycin, another well-known mTORC1 inhibitor, has also been shown to alter viral replication of viruses such as EBV. When treated with rapamycin, EBV lytic replication increases in epithelial cells and decreases in B cells [3]. Rapamycin alters EBV lytic replication in both epithelial cells and B cells by exerting its repressive effects on both mTORC1 and YY1. YY1, a crucial transcriptional regulator of gene expression, negatively regulates EBV immediate-early genes BZLF1 and BRLF1 that are critical for the initiation and

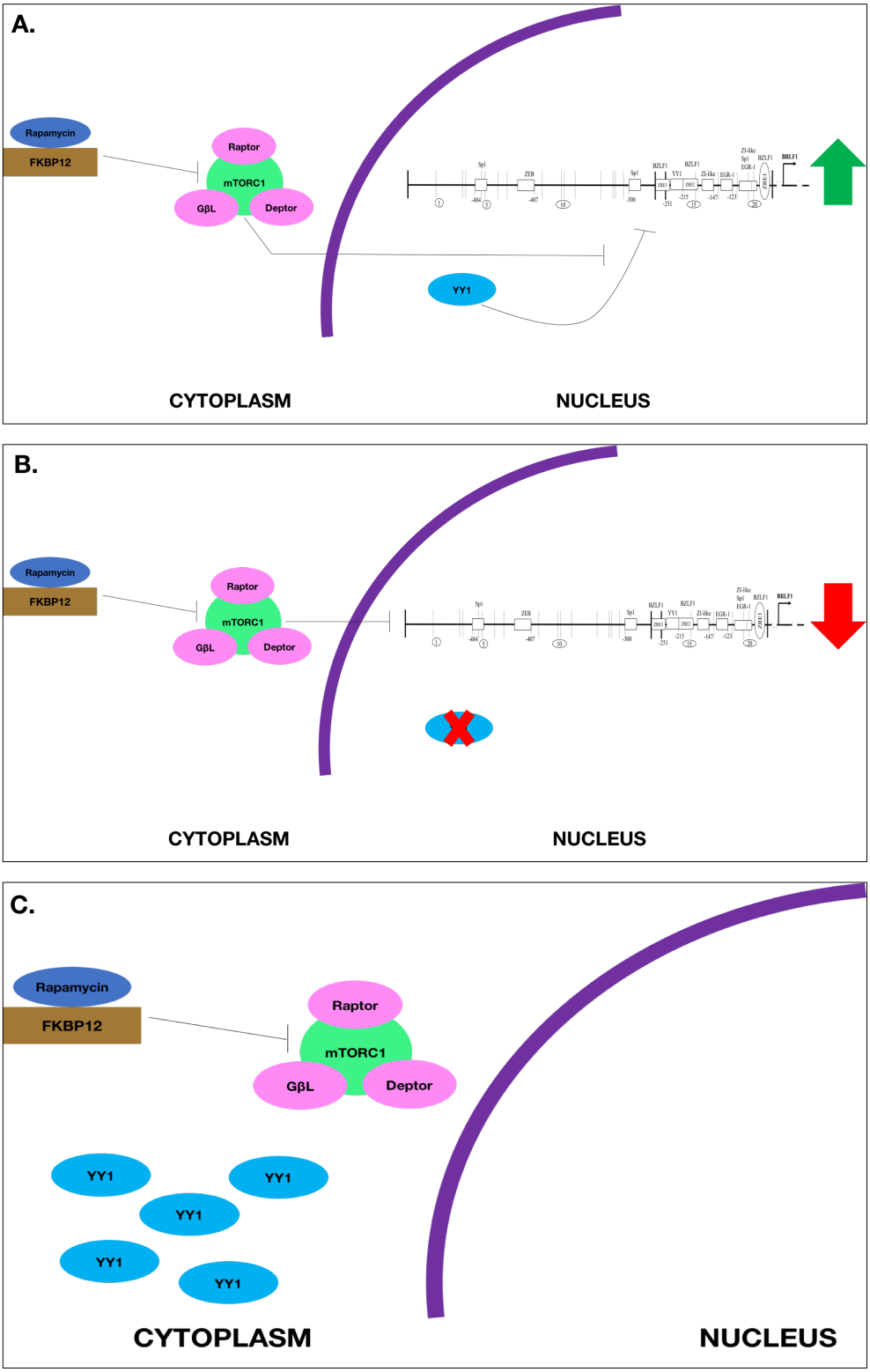
progression of EBV lytic replication [3]. While it has been established how rapamycin alters YY1 regulation of BZLF1 in epithelial cells and B cells, its effects on YY1 regulation of BRLF1 had yet to be determined.

The data from this thesis research project suggests that rapamycin alters YY1 regulation of BRLF1 transcription in epithelial cells and B cells in a cell-type specific manner. The effects of rapamycin on YY1 regulation of BRLF1 transcription are like the effects of rapamycin on YY1 regulation of BZLF1 transcription. During mTORC1 inhibition and lytic replication in epithelial cells, YY1 acts as an activator of BRLF1 transcription (Figure 9A). The increase in BRLF1 transcript levels and nuclear YY1 localization in chemically induced cells and cells treated with rapamycin supports YY1's role as a positive regulator. In the absence of YY1, inhibited mTORC1 itself is a negative regulator of BRLF1 transcription (Figure 9B). It was also concluded that cytoplasmic YY1 localization in epithelial cells may be attributed to inhibited mTORC1 not being able to phosphorylate and cause the translocation of YY1 to the nucleus (Figure 9C). In B cells, YY1 acts as a repressor of BRLF1 transcription. YY1 promotes EBV latency in B cells by repressing BZLF1 and BRLF1 activity [6, 30, 43]. Although qRT-PCR was not performed on B cells due to insufficient YY1 knockdown, increased nuclear YY1 localization during mTORC1 inhibition and increased cytoplasmic YY1 localization during mTORC1 inhibition and lytic replication suggests that YY1 still acts as a repressor in B cells (Figure 9D and 9E).

Further research needs to be conducted to assess if YY1 truly acts as a repressor of BRLF1 transcription during mTORC1 inhibition in B cells. Different methods for

siRNA transfection of B cells should be explored to ensure sufficient YY1 knockdown. When sufficient YY1 knockdown is achieved, the effects of rapamycin and YY1 knockdown on B cells could be assayed using qRT-PCR. YY1 localization should also be explored in conjunction with phases of the cell cycle. YY1 localization has been known to be responsive to different phases of the cell cycle, and cell signaling pathways involved with the cell cycle [32]. A major cell signaling pathway involved in cell cycle progression is the mTOR pathway [27]. It is already known that mTORC1 can interact with YY1 to mediate cellular processes like mitochondrial metabolism, but it could possibly alter YY1 localization during different phases of the cell cycle [11, 32]. Epithelial cell and B cell viability during decreased YY1 expression should also be investigated to further evaluate how critical YY1's role is in the physiology of both cell types. YY1's versatile role in cellular and viral transcription, and its presence in various types of cancer, makes it a viable target for the development of YY1-targeted therapies that could be used in combination with rapamycin to attenuate EBV infection and reduce the incidence of developing EBV-associated diseases.

AGS-BDneo


A.
Rapamycin
FKBP12
Raptor
mTORC1
GβL
Deptor
YY1
CYTOPLASM
NUCLEUS
B.
Rapamycin
FKBP12
Raptor
mTORC1
GβL
Deptor
CYTOPLASM
NUCLEUS
C.
Rapamycin
FKBP12
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mTORC1
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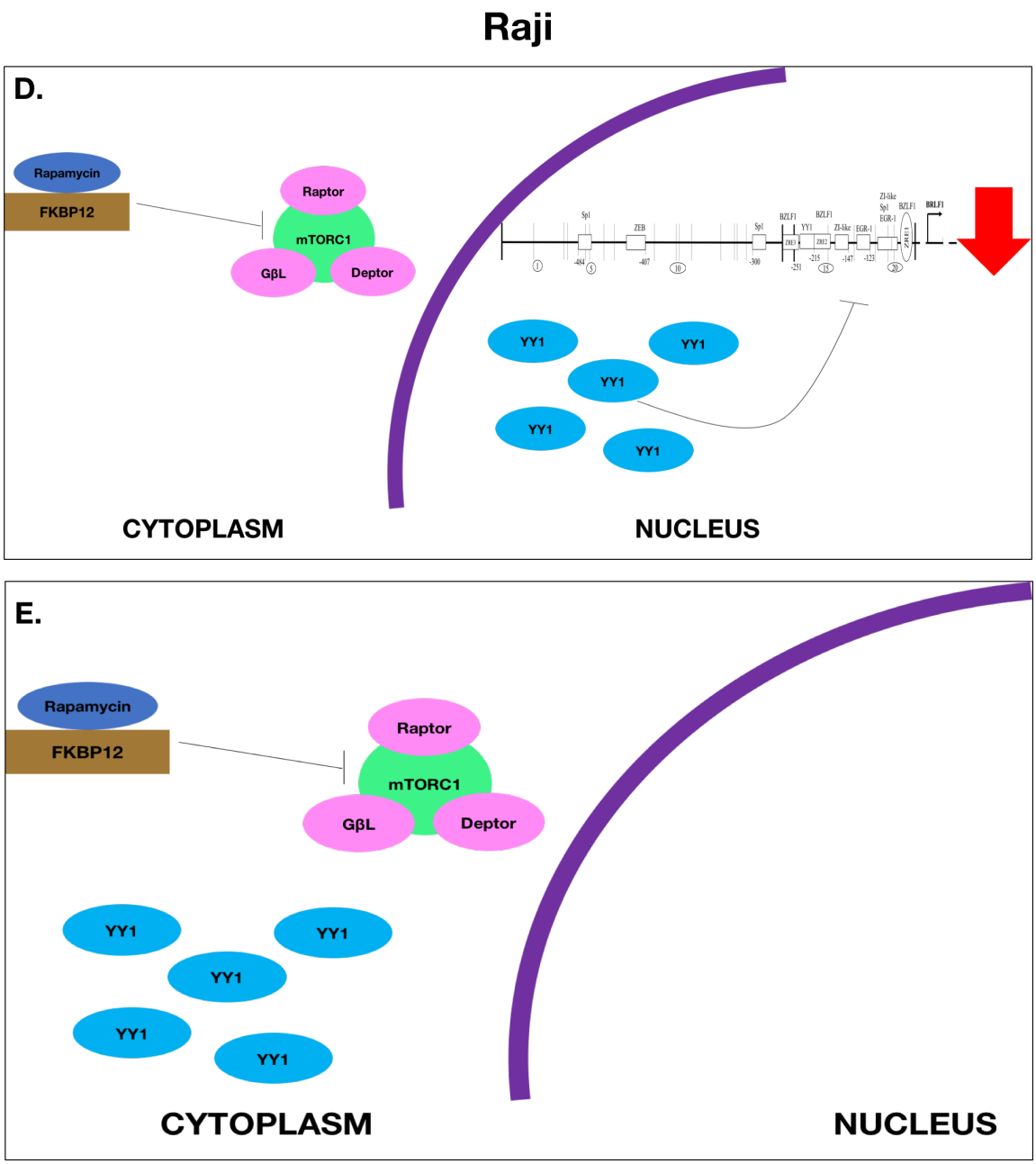

Figure 9. Cell Type-Specific YY1 Regulation of BRLF1 Transcription During mTORC1 Inhibition. AGS-BDneo epithelial cells (A) Inhibited mTORC1 negating YY1 repression of BRLF1, causing an upregulation in BRLF1 transcription. (B) Inhibited mTORC1 in the absence of YY1 negatively regulating BRLF1 transcription, causing a downregulation in BRLF1 transcription. (C) Increased cytoplasmic YY1 localization during mTORC1 inhibition due to inhibited mTORC1 not being able to phosphorylate and translocate YY1 to the nucleus. Raji B cells (D) Increased nuclear YY1 localization

during mTORC1 inhibition negatively regulating BRLF1, causing a downregulation in BRLF1 transcription. (E) Increased cytoplasmic YY1 localization during mTORC1 inhibition due to inhibited mTORC1 not being able to phosphorylate and translocate YY1 to the nucleus.

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