Title: Epstein-Barr virus latent membrane protein 2A enhances MYC-driven cell cycle progression in a mouse model of B lymphoma

Running title: A novel link of EBV and MYC in the cell cycle

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Key Points

- Latent membrane protein 2A augments MYC oncogene in driving the cell cycle by increasing protein instability of a tumor suppressor p27kip1.
- Latent membrane protein 2A potentiates MYC expression to overcome a cell cycle checkpoint without disrupting p53 tumor suppressor pathway.

Abstract

Elevated expression of MYC is a shared property of many human cancers. Epstein-Barr virus (EBV) has been associated with lymphoid malignancies, yet collaborative roles between MYC and EBV in lymphomagenesis are unclear. EBV latent membrane protein 2A (LMP2A) functions as B cell receptor (BCR) mimic known to provide survival signals to infected B cells. Co-expression of human MYC and LMP2A in mice (LMP2A/λ-MYC) accelerates B lymphoma onset compared to mice expressing human MYC alone (λ-MYC mice). Here we show a novel role of LMP2A in potentiating MYC to promote G1-S transition and hyperproliferation by downregulating cyclin-dependent kinase inhibitor p27kip1 in a proteasome-dependent manner. Expressing a gain-of-function S10A mutant of p27kip1 has minor effect on tumor latency. However, pre-tumor B cells from λ-MYC mice expressing homozygous S10A mutant show a significant decrease in the percentage of S-phase cells. Interestingly, LMP2A is able to counteract the anti-proliferative effect of the S10A mutant to promote S-phase entry. Finally, we show that LMP2A expression correlates with higher levels of MYC expression and suppression of p27kip1 prior to lymphoma onset. Our study demonstrates a novel function of EBV LMP2A in maximizing MYC expression resulting in hyperproliferation and cellular transformation into cancer cells in vivo.
Introduction

The cell cycle is a tightly regulated process governed by cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors. Cell cycle checkpoints are important cellular mechanisms preventing uncontrolled proliferation caused by oncogenic stimuli. Retinoblastoma (Rb) and p53 pathways are critical pathways preventing premature cell cycle progression by inducing cell cycle arrest or apoptosis. Though pathways and mutations leading to malignancies are different depending on cancer types, many routes converge to increase MYC expression through chromosomal translocation, amplification of c-MYC transcription, and protein stabilization. MYC is a basic helix-loop-helix (bHLH) transcription factor regulating many target genes in most, if not all, cell types. MYC heterodimerizes with a binding partner, Max, and binds to CACGTG-containing DNA sequences. Recent studies suggest that the main function of MYC is to upregulate transcription of its target genes, which in turn, indirectly lead to repression of certain genes, including those encoding CDK inhibitors. MYC is important for B lymphocyte activation and proliferation. Constitutive expression of MYC in murine B cells leads to increases in the percentage of cells in S and G2/M phases of the cell cycle. MYC activation increases activities of cyclin-CDK complexes, resulting in the hyperphosphorylation of Rb and release of E2F transcription factors to upregulate S-phase genes. MYC promotes the expression of D-type cyclins, E2F, and contributes to the transcription repression of genes encoding CDK inhibitors p27\(^{Kip1}\), p21\(^{Cip1}\), and p15\(^{Ink4b}\), leading to the progression into S-phase. MYC also induces apoptosis via the upregulation of p19\(^{ARF}\) and induction of the p53 pathway. Mice
lacking p53, Cdkn2a, and Cdkn1b show rapid onset of MYC-induced lymphomagenesis, suggesting importance of cell cycle regulators in preventing excessive proliferation caused by MYC.

Latent infection of Epstein-Barr virus (EBV), a member of gammaherpesviruses, is associated with cellular hyperproliferation observed in posttransplant lymphoproliferative disorders (PTLD), as well as malignancies including Hodgkin’s disease, and non-Hodgkin’s lymphoma. EBV is linked with a role in suppressing apoptosis induced by MYC in infected B cells and thus aids MYC in cell growth and proliferation. Latent membrane protein 2A (LMP2A) is encoded by EBV and found in most latency programs. LMP2A contains an ITAM (immunoreceptor tyrosine activation motif) similar to that of the host BCR and provides BCR-like survival signals to B cells. Furthermore, a recent study suggest that LMP2A can contribute to proliferation of B cells during an early phase of EBV-induced B cell proliferation, suggesting a pro-proliferative role of LMP2A in the cellular transformation process.

In a mouse model of EBV latent infection, mice expressing LMP2A and human MYC transgene (LMP2A/λ-MYC) demonstrate accelerated lymphomagenesis compared to mice expressing human MYC alone (λ-MYC mice), which is similar to another study showing that constitutive activation of the BCR leads to a rapid onset of MYC-induced lymphoma. The p19ARF-p53 pathway is an important mechanism controlling aberrant proliferation induced by pathological expression of MYC. Therefore, mutations and inactivation of p53 pathway are frequently found in MYC-induced tumorigenesis.
However, p53 pathway inactivation is absent in LMP2A/λ-MYC tumors suggesting that LMP2A utilizes a different mechanism to promote MYC-driven lymphomagenesis.

In this study, we investigate a role of LMP2A in altering the cell cycle regulation independent of the p53 pathway. We demonstrate that LMP2A cooperates with MYC in disrupting the G₁ checkpoint through the downregulation of a CDK inhibitor p27kip1 leading to premature S-phase entry. We also show that LMP2A expression leads to high MYC levels, indicating a novel relationship between MYC and LMP2A. Our study sheds new light into an intricate collaboration of EBV and host oncoproteins in malignant transformation.

**Materials and Methods**

**Mice**

Tg6 line of EμLMP2A transgenic mice²⁰,²¹ and λ-MYC mice²⁸ has been described and are in the C57BL/6 background. Mice expressing S10A mutant of p27kip1 (Cdkn1b<sup>tm2Jro</sup>) mice) were constructed previously²⁹ and obtained from The Jackson Laboratory and are in the 129 background. Tumor mice were sacrificed when lymph node tumors could be observed externally or mice were moribund. Animals were maintained at Northwestern University’s Center for Comparative Medicine, in accordance with University animal welfare guidelines.

**Tumor and spleen cell isolation**

Pre-tumor spleens were isolated from 21-25 days old mice. Magnetic activated cell sorting using CD19 positive selection (Miltenyi Biotec) were used to purify splenic B
cells. Tumor-bearing lymph nodes were dissociated and treated with 155 mM ammonium chloride to lyse red blood cells. Approximately 90% of tumor cells in tumor-bearing lymph nodes were of B-cell lineage and thus did not required further cell sorting.

Flow cytometry and cell cycle analysis

Five million purified pre-tumor B cells were fixed in ice-cold 70% ethanol. Cells were stained with Ki-67-FITC antibody and DNA was stained with PI/RNase staining buffer according to the manufacturer’s instructions (BD Biosciences). For the viability assay, primary tumor cells were stained with 7-AAD (Invitrogen) and anti-CD19-APC antibody (eBioscience) following Nutlin-3 (CalBiochem) treatments. All samples were analyzed using the FACS-CantoII flow cytometer (BD Biosciences) and FlowJo v 9.2 software (Tree Star, Inc.).

Histology

Tumor-bearing lymph nodes were fixed in 10% buffered formalin phosphate (Fisher Scientific) followed by paraffin embedding. Pre-tumor spleens were frozen in tissue embedding medium (Tissue-Tek). Tumors were sectioned and stained with hematoxylin and eosin (tumor lymph nodes), or with anti-B220 antibody (pre-tumor spleens) at the Mouse Histology and Phenotyping Laboratory at Northwestern University. Stained tissue sections were imaged using EVOS XL Core digital inverted microscope (Advanced Microscopy Group) using 20X magnification lense.

Real-time RT-PCR

Total RNA was isolated from purified pre-tumor B cells using RNeasy RNA extraction kit with DNase I digestion from QIAGEN. To generate cDNA, High-Capacity cDNA Reverse Transcription kit from Applied Biosystems was used. Real-time PCR was
carried out on Applied Biosystems Step One Plus machine using primers specific for the indicated genes. Fast SYBR green master mix from Applied Biosystems was used in all reactions. The ΔΔCt method was used to normalize gene expression to Gapdh.

**Immunoblots**

Pre-tumor B cells or tumor cells were lysed in lysis buffer with protease and phosphatase inhibitor cocktails (Roche, Mannheim, Germany). Lysates were electrophoretically separated by SDS/PAGE (Bio-Rad). Protein was transferred to a nitrocellulose membrane (Whatmann), and incubated with primary antibodies. Membranes were probed with IRDye secondary antibodies (Li-Cor Biosciences). The protein bands were visualized with Odyssey Fc Western Blotting Imager and analyzed with Image Studio version 2.0 (Li-Cor Biosciences).

**Statistical analysis**

Two-tailed Student-\( t \) test, one-way and two-way ANOVA, and survival analysis were analyzed using GraphPad (GraphPad Software, Inc.). \( P < 0.05 \) is considered statistical significant.

**Results**

**LMP2A couples with MYC to promote G₁-S transition**

Since previous studies suggested that LMP2A promotes cell cycle progression in the context of deregulated MYC, \(^{24,25} \) we chose to investigate mechanisms by which LMP2A disrupts the cell cycle regulation. Since p53 pathway inactivation is frequent in MYC-driven lymphomagenesis, the status of p53 pathway may interfere with analysis of the cell cycle when comparing tumor cells from LMP2A/λ-MYC and λ-MYC mice. Thus, we
chose to examine cell cycle profiles of pre-tumor B cells since the p53 pathway is intact at this stage in both LMP2A/λ-MYC and λ-MYC mice. Purified B cells from 3-week-old pre-tumor LMP2A/λ-MYC mice demonstrated high proliferative characteristics with a significant increase in the percentage of Ki-67-positive cells (Figure 1A and B) and percentage of S-phase cells (Figure 1C and D) compared to those isolated from λ-MYC mice of the same age. The hyperproliferative phenotype of LMP2A/λ-MYC pre-tumor B cells was also evident histologically. While wild-type (WT) C57BL/6 as well as LMP2A and λ-MYC transgenic mice formed B cell follicles in secondary lymphoid organs such as the spleen, LMP2A/λ-MYC mice showed disorganized B cell follicles (Fig. 1E) and an increase in B220+ population. WT, LMP2A, and λ-MYC mice had comparable percentages of immature and mature B cells. LMP2A/λ-MYC B220+ cells were IgM-IgD−, likely as a result of LMP2A replacing the requirement for the BCR 20,30,31 (Fig. S1 and S2 with other detailed analyses). These data indicate that LMP2A promotes S-phase entry and hyperproliferation of B lymphocytes prior to malignant transformation.

LMP2A expression disrupts a cell cycle checkpoint during G1

We further delineated changes in cell cycle regulators of the G1 checkpoint in LMP2A-negative and –positive cells by Western blot analyses. Interestingly, of all CDK inhibitors important for preventing premature progression from G1 to S phase, p27kip1 was the only CDK inhibitor significantly downregulated in pre-tumor LMP2A/λ-MYC B cells compared to λ-MYC B cells (Figure 2A and B). There was also a moderate increase in phosphorylated Rb and cyclin A (a marker for S phase) in LMP2A/λ-MYC B cells, suggesting that low levels of p27kip1 results in the disruption of the Rb pathway in G1.
checkpoint and leads to pre-mature S-phase entry. However, quantitative real-time PCR (qRT-PCR) revealed that Cdkn1b mRNA levels were similar between LMP2A/λ-MYC and λ-MYC pre-tumor B cells (Figure 2C). These data agreed with previous studies demonstrating that MYC can promote Cdk1b transcriptional repression. 32,33 Taken together, LMP2A does not contribute to Cdkn1b transcriptional repression mediated by MYC, but rather downregulates p27<sub>kip1</sub> at the protein level to promote cell cycle progression.

**Instability of p27<sub>kip1</sub> in LMP2A/λ-MYC and λ-MYC pre-tumor B cells**

Since p27<sub>kip1</sub> is largely controlled by posttranslational modification and proteolysis, 34-36 we further explored the possibility that LMP2A could increase the instability of p27<sub>kip1</sub> to promote cell cycle progression. Pre-tumor B cells of LMP2A/λ-MYC and λ-MYC littermates were treated with the protein synthesis inhibitor cycloheximide (CHX) for 1, 2, and 3 hours. LMP2A/λ-MYC B cells showed a slightly shorter half-live compared to λ-MYC B cells after CHX treatment. However, the reduction of p27<sub>kip1</sub> was significantly diminished when LMP2A/λ-MYC B cells were co-treated with both CHX and MG-132, a proteasome inhibitor (Figure 3A and 3B). Furthermore, when treated with MG-132 alone, LMP2A/λ-MYC B cells had twice the level of p27<sub>kip1</sub> compared to vehicle treated cells. In contrast, MG-132 had minimal effect on p27<sub>kip1</sub> expression in λ-MYC B cells (Fig. 3C). These data indicate that LMP2A promotes p27<sub>kip1</sub> degradation through a proteasome-dependent process.
Expression of S10A mutant form of p27\textsuperscript{kip1} (p27\textsuperscript{S10A}) in MYC-driven lymphomagenesis

To examine the importance of p27\textsuperscript{kip1} in LMP2A-mediated lymphomagenesis, we crossed our transgenic mice to knock-in mice in which serine 10 of p27\textsuperscript{kip1} was replaced by alanine (p27\textsuperscript{S10A}). We chose the p27\textsuperscript{S10A} knock-in mutant mice since previous studies demonstrated a tumor-suppressing phenotype compared to WT p27\textsuperscript{kip1} in a mouse model of lung cancer,\textsuperscript{29} a lymphoproliferative disorder,\textsuperscript{37} and SV40-induced tumorigenesis.\textsuperscript{38} In addition, murine embryonic fibroblasts (MEFs) from p27\textsuperscript{S10A/S10A} mice have a nuclear localization phenotype\textsuperscript{29} and are more refractory to degradation mediated by SV40-induced transformation compared to MEFs from WT mice.\textsuperscript{38} Since the role of S10 phosphorylation of p27\textsuperscript{kip1} had not been tested in the context of deregulated MYC \textit{in vivo}, the S10A mutant could act as a gain-of-function form of p27\textsuperscript{kip1} and would allow us to test if the expression of this mutant would influence lymphoma development in our model. We hypothesized that expression of p27\textsuperscript{S10A} would prevent LMP2A-mediated p27\textsuperscript{kip1} suppression and would delay lymphomagenesis to be similar to that of λ-MYC mice. However, we found the median survival time of all LMP2A/λ-MYC mice was very similar (36-38 days). We noted a small subset of LMP2A/λ-MYC\textsuperscript{+/S10A} and LMP2A/λ-MYC\textsuperscript{S10A/S10A} mice were tumor-free at day 50 when all LMP2A/λ-MYC\textsuperscript{+/+} developed tumors. One LMP2A/λ-MYC\textsuperscript{S10A/S10A} mouse lived tumor-free beyond median survival time of λ-MYC\textsuperscript{+/+} (122 days). All λ-MYC mice regardless of p27\textsuperscript{kip1} genotypes had similar time-to-lymphoma onset. This observation suggests that p27\textsuperscript{S10A} has a minimal effect on the latency time of lymphoma development in our model (Fig. 4A).
P53-mediated apoptosis is an important tumor suppressive mechanism counteracting MYC oncogenic function. To examine whether p27<sup>S10A</sup> expression would change the status of p53 pathway, primary tumor cells were treated with the MDM2 inhibitor Nutlin-3, which leads to the release of p53 from its negative regulator. All LMP2A/λ-MYC tumor cells were sensitive to Nutlin-3 treatments, resulting in a sharp decrease in viability compared to control at 3 hours post-treatment, suggesting that these cells have an intact p53 pathway after activation of p53 upon Nutlin-3 treatment. All λ-MYC tumor cells were resistant to Nutlin-3 treatments (Fig. 4B), indicating that p53 is mutated or the pathway is inactivated similar to previous reports. 24,27 Previous studies reported that, tumors with defect in p53-19<sup>ARF</sup>-MDM2 pathway show aberrant accumulation or stabilization of p19<sup>ARF</sup> and p53 due to the disruption of p53 negative feedback loop. 24,27,39-41 Excessive accumulation of p53 and p19<sup>ARF</sup> proteins in λ-MYC tumor cells was evident but was absent in all LMP2A/λ-MYC tumors, regardless of genotypes of p27<sup>kip1</sup> (Fig. 4C). These data indicate that LMP2A effectively promotes lymphoma development without perturbing the p53 pathway and tumor promotion by LMP2A is unaffected by p27<sup>S10A</sup> expression. Despite the strong differences in tumor onset time and p53 status, all tumors from LMP2A/λ-MYC and λ-MYC mice were histologically similar with the “starry sky” appearance resembling Burkitt lymphoma (Fig. 4D and Fig. S3). Taken together, these data indicate that p27<sup>S10A</sup> expression does not prevent p53 pathway inactivation in λ-MYC tumors and that the p53 pathway is still functionally intact in all LMP2A/λ-MYC tumors examined.
LMP2A antagonizes p27<sup>S10A</sup> function

To investigate if the p27<sup>S10A</sup> mutant could alter the cell cycle profile at earlier steps of lymphomogenesis, spleen mass was analyzed in mice at 3 weeks of age. P27<sup>S10A</sup> expression did not significantly change the relative spleen mass or the ratios of B:T cells, although there was a small decrease in relative spleen weight of mice with homozygous p27<sup>S10A</sup> compared to those with WT p27<sup>kip1</sup> (Fig. S4). We found all LMP2A/λ-MYC pre-tumor B cells were >90% Ki-67 positive (Fig. 5A). Interestingly, there was a dose-dependent decrease in the percentage of Ki-67 positive B cells with the expression of heterozygous and homozygous p27<sup>S10A</sup> in λ-MYC pre-tumor B cells (Fig. 5A). Furthermore, cell cycle analyses showed a significant dose-dependent increase in G<sub>1</sub>-cells and a significant decrease in S-phase cells in λ-MYC pre-tumor B cells with the expression of one or 2 alleles of p27<sup>S10A</sup>. These data indicate that p27<sup>S10A</sup> impedes S-phase entry in early events of lymphoma development. However, the effect of p27<sup>S10A</sup> in blocking G<sub>1</sub>-S transition was lost in all LMP2A/λ-MYC pre-tumor B cells (Fig. 5B), indicating that LMP2A can counteract the anti-proliferative function of p27<sup>S10A</sup> to promote MYC-driven cell cycle progression.

To delineate the molecular mechanisms responsible for the failure of p27<sup>S10A</sup> in blocking G<sub>1</sub>-S transition in LMP2A/λ-MYC pre-tumor B cells but successfully preventing S-phase entry in λ-MYC pre-tumor B cells, pre-tumor B cells were subjected to Western blot analyses. We found an opposite trend of increasing p27<sup>kip1</sup> and decreasing MYC levels in representative λ-MYC<sup>+/S10A</sup> and λ-MYC<sup>S10A/S10A</sup> compared to λ-MYC<sup>+/+</sup>. In addition, we found a decrease in cyclin A in λ-MYC<sup>S10A/S10A</sup> pre-tumor B cells, corresponding to a
lower percentage of B cells in S-phase shown in Fig. 5B. LMP2A/λ-MYC<sup>S10A/S10A</sup> pre-tumor B cells did not show an increase in p27<sup>kip1</sup> level compared to LMP2A/λ-MYC<sup>+/+</sup> cells. Furthermore, MYC was expressed at high levels in all LMP2A/λ-MYC pre-tumor B cells, regardless of p27<sup>S10A</sup> expression (Fig. 6A-B).

Since p27<sup>S10A/S10A</sup> showed a nuclear translocation phenotype, the low level of total p27<sup>kip1</sup> in LMP2A/λ-MYC pre-tumor B cells suggests another degradation process plays a role in LMP2A-mediated p27<sup>kip1</sup> degradation. Previous studies showed that Cks1 and Skp2, both are constituents of SCF<sup>Skp2</sup> complex regulating nuclear p27<sup>kip1</sup> degradation, were increased in pre-cancerous B cells from Eμ–Myc mice compared to WT B cells. We found similar Skp2 levels in both LMP2A/λ-MYC and λ-MYC pre-tumor B cells, while there was an increase in Cks1 protein level in LMP2A/λ-MYC B cells. There was also an elevated Cks1 mRNA in LMP2A/λ-MYC compared to λ-MYC B cells but the difference was not statistically significant (Fig. S5). These data suggest that high MYC levels in LMP2A/λ-MYC B cells promotes further p27<sup>kip1</sup> degradation, at least in part, by upregulating Cks1 in the SCF<sup>Skp2</sup> complex, which is active in late G<sub>1</sub> and S phase. Overall, these results indicate that LMP2A expression can antagonize the function of p27<sup>S10A</sup> by maintaining MYC at high level, leading to continued downregulation of p27<sup>kip1</sup> levels. In the absence of LMP2A, the gain-of-function p27<sup>S10A</sup> is able to increase total p27<sup>kip1</sup> level, which in turn, inversely correlates with MYC expression.
Discussion

This study reports the collaborative efforts of MYC and EBV LMP2A in disrupting the cell cycle prior to progression to malignancy. The complex relationship of a tumor virus and a human oncogene is exemplified in our model that demonstrates EBV provides an optimal intracellular environment conducive to malignant transformation. Our studies confirm previous studies on how two important tumor suppressor pathways counteract MYC and reveal how EBV changes the equation to favor the pro-proliferative function of MYC.

Low levels of p27kip1 are observed in several human cancers and murine models of cancers indicate that p27kip1 is a dose-dependent tumor suppressor. Mice hemizygous for p27kip1 (p27+/−) display an intermediate phenotype in body weight and cell proliferation between p27−/− and p27+/+ mice. The loss of one or 2 copies of p27kip1 demonstrates a dose-dependent decrease in tumor latency in γ-irradiation and carcinogen-induced tumor models. Our studies are compatible with these studies as expression of one or 2 copies of the gain-of-function S10A mutant in λ-MYC pre-tumor B cells results in a dose-dependent decrease in the percentage of cells in S-phase and that are Ki-67 positive.

Germ line deletion or mutation of the p27kip1 gene, CDKN1B, in human cancer is rare, implicating other mechanisms play a major role in regulating p27kip1 in cancer. MYC promotes transcriptional repression of the p27kip1 gene, but p27kip1 is largely controlled at the protein level. MYC promotes p27kip1 protein degradation by
upregulating transcription of negative regulators of \( p27^{\text{Kip1}} \), \(^{42,51-53}\) including \( Cks1 \) and \( Skp2 \). Furthermore, phosphorylation of \( p27^{\text{kip1}} \) at different sites controls degradation and subcellular localization of \( p27^{\text{kip1}} \) at different time points in the cell cycle. There are at least 2 pathways regulating \( p27^{\text{kip1}} \) degradation. In \( G_0 \) and early \( G_1 \), \( p27^{\text{kip1}} \) is phosphorylated at serine 10 and is exported from the nucleus. Cytoplasmic \( p27^{\text{kip1}} \) is targeted for proteasomal degradation mediated by the Kip1 ubiquitination-promoting complex (KPC). \(^{54}\) In late \( G_1 \) and \( S \) phase, \( p27^{\text{kip1}} \) is phosphorylated at threonine 187 and recognized by the SCF\(^{\text{Skp2}} \) complex, which ubiquitinates \( p27^{\text{kip1}} \) and targets it for the degradation by the proteasome in the nucleus. \(^{55,56}\) Mice lacking \( Csk1 \) or \( Skp2 \) showed an increase in \( p27^{\text{kip1}} \) level and delayed MYC-induced tumorigenesis. \(^{42,52}\) We found that \( p27^{S10A} \) fails to increase total \( p27^{\text{kip1}} \) levels in LMP2A/\( \lambda \)-MYC\(^{+/S10A} \) and LMP2A/\( \lambda \)-MYC\(^{S10A/S10A} \) pre-tumor B cells, indicating that LMP2A still efficiently promotes nuclear degradation of \( p27^{\text{kip1}} \) mediated by SCF\(^{\text{Skp2}} \) complex in late \( G_1 \) and \( S\)-phase and may do so via \( Cks1 \) upregulation. Another possibility is that high MYC levels in pre-tumor B cells from LMP2A/\( \lambda \)-MYC mice makes the degradation of \( p27^{\text{kip1}} \) very rapid (Fig. 6). However, both cytoplasmic and nuclear degradation pathways may be required to act in concert. Future studies will further examine the role of SCF\(^{\text{Skp2}} \) complex in LMP2A-mediated lymphoma development.

It is important to note that regulatory processes of \( p27^{\text{kip1}} \) in cancers can be different in different cell types and oncogenic stimuli. Immortalized MEFs from \( p27^{S10A/S10A} \) mice are more resistant to \textit{in vitro} transformation by K-Ras but show similar transformation ability induced by MYC compare to WT MEFs. \(^{50}\) These data suggest that K-Ras and MYC
suppress p27\textsuperscript{kip1} through different mechanisms during cellular transformation and could explain the difference in tumor suppressing function of p27\textsuperscript{S10A} in our MYC model and K-Ras-induced tumorigenesis in previous studies.\textsuperscript{29,38} Furthermore, the localization of p27\textsuperscript{kip1} in quiescent or activated B cells is different from that of T cells and MEFs. P27\textsuperscript{kip1} is mainly localized in the cytoplasm of resting B cells and is translocated to the nucleus upon BCR activation. Defects in the suppression of serine 10 phosphorylation on p27\textsuperscript{kip1} inhibit the nuclear translocation process and thus contribute to autoimmunity and a lymphoproliferative disorder and expression of p27\textsuperscript{S10A} corrects this anomaly.\textsuperscript{37} Although homozygous p27\textsuperscript{S10A} expression significantly reduced percentage of S-phase pre-tumor B cells from λ-MYC mice (Fig. 6B), it had only a small effect in tumor latency (Fig 4A). Therefore, p27\textsuperscript{S10A} may be a mild gain-of-function mutant in MYC-driven lymphoma model.

Our study is the first to show an underlying mechanism of EBV LMP2A in promoting cell cycle progression via p27\textsuperscript{kip1} downregulation. A recent study showed that LMP2A is important for cell proliferation and efficient long-term growth following the transformation of primary B cells.\textsuperscript{23} LMP2A may be dominant in promoting MYC-induced p27\textsuperscript{kip1} downregulation in latency program I where EBNA1 and LMP2A appear to be uniquely expressed.\textsuperscript{57,58} Furthermore, \textit{in vitro} BCR stimulation in primary normal B cells results in the downregulation of p27\textsuperscript{kip1}, G\textsubscript{1}-S transition and proliferation of B cells.\textsuperscript{37} Given that LMP2A is a BCR mimic, pathways leading to p27\textsuperscript{kip1} degradation in this study may be similar to a stimulated BCR, which involves the Erk and/or Akt pathway.\textsuperscript{37} Inhibition of Akt induces apoptosis in B cells from LMP2A transgenic mice.\textsuperscript{59} Furthermore, the
inhibition of Lyn kinase or mammalian target of rapamycin (mTOR) pathway leads to decreases in spleen and tumor sizes in LMP2A/λ-MYC mice compared to λ-MYC mice, suggesting LMP2A is able to mimic the BCR signal through the Akt/mTOR pathway to promote lymphomagenesis.

The dramatic downregulation of p27\textsuperscript{kip1} in pre-tumor B cells and the accelerated lymphomagenesis in LMP2A/λ-MYC mice correspond with a previous study showing that E\textsubscript{μ}–Myc mice lacking p27\textsuperscript{kip1} have rapid tumor onset compared to those with WT p27\textsuperscript{kip1}, suggesting the importance of p27\textsuperscript{kip1} in suppressing MYC-driven lymphomagenesis. However, p53 status in tumors of E\textsubscript{μ}–Myc; p27/\textsuperscript{-} was not assessed. The relationship of how MYC represses p27\textsuperscript{kip1} is better documented than how p27\textsuperscript{kip1} can negatively regulate MYC. It is possible that p27\textsuperscript{kip1} is a part of negative feedback network controlling MYC level. Since LMP2A expression associates with the continued p27\textsuperscript{kip1} repression, even when homozygous p27\textsuperscript{S10A} is present, the importance of p27\textsuperscript{kip1} in LMP2A-mediated lymphomagenesis is still unclear and future study will address this issue.

In λ-MYC B cells, our data suggest that the downregulation of p27\textsuperscript{kip1} and p53 pathway inactivation occurs at different time points – p27\textsuperscript{kip1} downregulation precedes p53 inactivation. We propose that MYC downregulates p27\textsuperscript{kip1} both at the transcription and protein level, leading to cell cycle progression. The remaining pool of p27\textsuperscript{kip1} then functions as part of a MYC repression network to control MYC. MYC can also induce apoptosis through the induction of p53 pathway. Both p53 and p27\textsuperscript{kip1} act in concert or
independently to prevent uncontrolled proliferation. However, selection pressure promotes the survival of only cells that contain a disruption in one or more components of p53 pathway. P27kip1 may act dominantly at an early stage of lymphomagenesis and the downregulation of p27kip1 may be less important following p53 pathway inactivation. In the presence of LMP2A, such as in EBV-infected B cells, p27kip1 is downregulated and MYC may be able to drive proliferation to a greater extent, thus outpacing apoptosis. Therefore, B cells expressing both deregulated MYC and LMP2A expand more readily without the need to mutate or inactivate the p53 pathway (Fig. 7).

In summary, we demonstrated for the first time the cooperative role of LMP2A and MYC in disrupting the cell cycle through promoting p27kip1 degradation at the early stage of lymphomagenesis. LMP2A perturbs MYC negative feedback loop, partially mediated by p27kip1, to accentuate MYC expression and accelerate lymphomagenesis without altering the p53 pathway. Our data shed a new light on how EBV infection aids MYC in counteracting with anti-proliferative cellular mechanisms and our results also emphasize critical roles of EBV infection in creating intracellular state favorable for malignant transformation.
Acknowledgement

The authors thank members of the Longnecker laboratory for help in completion of this study. This work is supported by the Northwestern University Mouse Histology and Phenotyping Laboratory and a Cancer Center Support Grant (NCI CA060553). R.L. is Dan and Bertha Spear Research Professor and is supported by the National Cancer Institute (CA073507 and CA133063). K.F. is supported by Training Program in Viral Replication T32AI060523-09. J.C. is a fellow of the American Heart Association.

Authorship contribution

K.F. and R.L. designed research, analyzed data, and wrote manuscript. K.F. and J.C. performed research.

Conflict-of-interests disclosure: The authors declare no competing financial interest.
References


**Figure legends**

**Figure 1.** Pre-tumor B cells from LMP2A/λ-MYC mice demonstrate highly proliferative phenotypes. **A)** Histograms of Ki-67-positive purified B cells from 3-week-old representative mice. The number in each histogram indicates the percentage of Ki-67 positive cells in purified B cells. **B)** Combined Ki-67 data from 3 experiments shown as mean ± SD. The differences in the percentage of Ki-67 positive cells were analyzed by 1-way ANOVA. ***P < 0.001. “ns” = not significant. **C)** Representative histograms of propidium iodine staining for DNA content in purified pre-tumor B cells. **D)** Pie charts show mean ± SD of cells in different phases of the cell cycle as analyzed in C). WT: n = 3, LMP2A: n = 3, LMP2A/λ-MYC: n = 4, λ-MYC: n = 4. **E)** Immunohistochemistry of B220+ cells in representative 3-week-old mouse spleens (X20). “No 1° Ab” = negative control with secondary antibody only.

**Figure 2.** P27<sup>Kip1</sup> is downregulated at the protein level, but not at the mRNA level, in pre-tumor B cells from LMP2A/λ-MYC mice compared to those in λ-MYC mice. **A)** Representative Western blots of cell cycle regulators in purified B cells from 3-week-old mice of indicated genotypes (C57BL/6 background). Each lane represents a protein sample from one mouse. **B)** P27<sup>Kip1</sup> protein expression in B cells from 3-week-old mice shown as a ratio to WT expression from 5 independent experiments. **C)** Quantitative real-time PCR analyses of Cdkn1b expression from two independent experiments using a wild-type (WT) mouse as a control for each experiment. All differences in mRNA and
protein expression were analyzed by one-way ANOVA. **P < 0.01. ***P < 0.001. “ns” not significant. Data represent mean ± SD.

**Figure 3. LMP2A facilitates p27kip1 degradation in a proteasome-dependent manner.** A) Representative Western blot analyses of p27kip1 expression after treatments with 25μg/ml cycloheximide (CHX) with or without 40μM MG-132 at indicated time points. The levels of p27kip1 were normalized to calnexin (a loading control) and the fold reductions of proteins were calculated using cells at 0 hour of each genotype as a control. B) Combined data from 3 experiments represent mean ± SD of p27kip1 level. Differences in p27kip1 protein expression were analyzed by two-way ANOVA. * P < 0.05. **P < 0.01. ***P < 0.001. C) Representative Western blot analyses of pre-tumor B cell treatment with vehicle control (DMSO), 40μM, or 60μM MG-132 for 2 hours. Gapdh and calnexin were used as loading controls. Left and right parts of images shown were from the same membrane and middle irrelevant lanes were removed. Experiments were repeated at least 3 times.

**Figure 4. Expression of S10A mutant confers minor effect on time-to-tumor onset of LMP2A/λ-MYC mice and has no effect on p53 pathway**

A) Kaplan-Meier curves indicating the percent survival of indicated genotypes. B) Viability of primary tumor cells (7-AAD−, CD19+) at 3 hours after 5μM or 10μM Nutlin-3 treatments from 3 independent experiments. Percentage of viability in vehicle control in each genotype was set at 100% C) Western blot analyses of representative primary tumor cells showed aberrant stabilization of p53 and/or p19ARF in tumors from λ-MYC
mice. **D)** Hematoxylin and eosin (H&E) staining of tumor-bearing lymph nodes demonstrating “starry sky” appearance in all tumor genotypes (X20).

**Figure 5. Homozygous p27\textsuperscript{S10A} expression significantly decreases S-phase pre-tumor B cells from λ-MYC mice but has no effect in LMP2A/λ-MYC pre-tumor B cells**

**A)** Percentage of Ki-67 positive pre-tumor B cells of each genotype. Data were combined from 5 experiments and represent mean ± SD. **B)** Pie charts and bar graphs demonstrating the cell cycle profile of pre-tumor B cells. Data were analyzed with one-way ANOVA and represent mean ± SD and. * = \( P < 0.05 \) and ** = \( P < 0.01 \). Inset showed pie charts representing mean percentages of pre-tumor B cells in each phase of the cell cycle.

**Figure 6. LMP2A expression associates with high MYC level, which inversely correlates with low p27\textsuperscript{kip1} levels**

**A)** Western blot analyses of cell cycle regulators in purified B cells from 3-week-old WT, LMP2A/λ-MYC\textsuperscript{+/+}, LMP2A/λ-MYC\textsuperscript{+/S10A}, LMP2A/λ-MYC\textsuperscript{S10A/S10A}, λ-MYC\textsuperscript{+/+}, λ-MYC\textsuperscript{+/S10A}, and λ-MYC\textsuperscript{S10A/S10A} mice. Cyclin A was used as an S-phase marker. Normalized levels of p27\textsuperscript{kip1} and MYC are shown as percentages to Gapdh (loading control). **B)** Bar graphs demonstrating MYC and p27\textsuperscript{kip1} protein expression in B cells from 3-week-old mice from 2 independent experiments. Each bar represents combined data from \( n = 4 \) with mean ± SD.
Figure 7. Proposed relationship of MYC, LMP2A, and p27\textsuperscript{kip1} in lymphoma development

(Top) In λ-MYC mouse model, overexpression of MYC results in repression of Cdkn1b transcription as well as increases p27\textsuperscript{kip1} degradation, leading to an increase in proliferation. The remaining pool of p27\textsuperscript{kip1} partially impedes MYC-induced cell cycle progression and downregulates MYC. Abundant expression of MYC also induces p53 pathway activation. Both p27\textsuperscript{kip1} and p53 are able to prevent uncontrolled proliferation for certain length of time. At later step, selection pressure forces deregulated MYC cells to overcome the apoptosis mechanism mediated by p53 and a lymphoma develops.

(Bottom) In the presence of LMP2A, p27\textsuperscript{kip1} turnover is more rapid and weakens the MYC repression network, resulting in high level of MYC. While p53 pathway is induced as a result of overexpression of MYC, cell cycle progression and proliferation outpaces the rate of apoptosis. Severely low level of p27\textsuperscript{kip1} may relieve the selection pressure and a lymphoma develops without the need to inactivate p53 pathway.
A. Isotype control  Ki-67 FITC

B. % Ki-67 positive cells

C. DNA content

D. G0/G1  S  G2/M

E. WT LMP2A LMP2A/\(\lambda\)-MYC \(\lambda\)-MYC
**A.** LMP2A/λ-MYC pre-tumor B cells

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λ-MYC pre-tumor B cells

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**B.**

**C.**

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A. % Viability

B. % Tumor-free survival

C. 

D.
A.

% Ki-67+ CD19+ cells

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G₀/G₁  S  G₂/M

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Fish_Figure 5
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B.

- **p27kip1**
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    - +/S10A
    - S10A/S10A
  - λ-MYC:
    - +/+
    - +/S10A
    - S10A/S10A

- **MYC**
  - LMP2A/λ-MYC:
    - +/+
    - +/S10A
    - S10A/S10A
  - λ-MYC:
    - +/+
    - +/S10A
    - S10A/S10A
Cdkn1b

LMP2A

MYC

P53 mutation and pathway inactivation

Time

Lymphomagenesis

p27kip1

Time

Lymphomagenesis

p27kip1

Cdkn1b
Epstein-Barr virus latent membrane protein 2A enhances MYC-driven cell cycle progression in a mouse model of B lymphoma

Kamonwan Fish, Jia Chen and Richard Longnecker

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