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# Epstein–Barr virus LMP1 protein promotes proliferation and inhibits differentiation of epithelial cells via activation of YAP and TAZ

Deo R. Singh[a], Scott E. Nelson[a], Abigail S. Pawelski[a], Alisha S. Kansra[a], Stuart A. Fogarty[a], Jillian A. Bristol[a], Makoto Ohashi[a], Eric C. Johannsen[a,b], and Shannon C. Kenney[a,b,1]

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**Latent Epstein–Barr virus (EBV) infection promotes undifferentiated nasopharyngeal carcinomas (NPCs) in humans, but the mechanism(s) for this effect has been difficult to study because EBV cannot transform normal epithelial cells in vitro and the EBV genome is often lost when NPC cells are grown in culture. Here we show that the latent EBV protein, LMP1 (Latent membrane protein 1), induces cellular proliferation and inhibits spontaneous differentiation of telomerase-immortalized normal oral keratinocytes (NOKs) in growth factor-deficient conditions by increasing the activity of the Hippo pathway effectors, YAP (Yes-associated protein) and TAZ (Transcriptional coactivator with PDZ-binding motif). We demonstrate that LMP1 enhances YAP and TAZ activity in NOKs both by decreasing Hippo pathway-mediated serine phosphorylation of YAP and TAZ and increasing Src kinase-mediated Y357 phosphorylation of YAP. Furthermore, knockdown of YAP and TAZ is sufficient to reduce proliferation and promote differentiation in EBV-infected NOKs. We find that YAP and TAZ are also required for LMP1-induced epithelial-to-mesenchymal transition. Importantly, we demonstrate that ibrutinib (an FDA-approved BTK inhibitor that blocks YAP and TAZ activity through an off-target effect) restores spontaneous differentiation and inhibits proliferation of EBV-infected NOKs at clinically relevant doses. These results suggest that LMP1-induced YAP and TAZ activity contributes to the development of NPC.**

EBV | LMP1 | NPC | YAP | TAZ

## Significance

Undifferentiated nasopharyngeal carcinomas (NPCs) are EBV-infected, but EBV infection is not sufficient to transform epithelial cells and NPCs routinely lose EBV when cultured. Here we demonstrate that the latent EBV protein, LMP1, is required and sufficient for the ability of EBV infection to induce cell proliferation and inhibit spontaneous differentiation in a telomerase-immortalized normal oral keratinocyte cell line (NOKs) when cells are grown in the absence of growth factors. Furthermore, we show that LMP1 activates the Hippo pathway effectors, YAP and TAZ, and that an FDA-approved drug that inhibits YAP and TAZ activity (ibrutinib) through off-target effects reverses this LMP1 effect at clinically relevant doses. These results suggest that activation of YAP and TAZ by EBV promotes NPC.

Epstein–Barr virus (EBV) is a gamma herpesvirus that causes infectious mononucleosis and contributes to the development of both B cell and epithelial cell tumors in humans, including undifferentiated nasopharyngeal carcinoma (NPC) and gastric carcinoma (1–4). Like all herpesviruses, EBV persists in the host for life and has both latent and lytic forms of infection. EBV-infected B cell and epithelial cell malignancies are composed almost entirely of latently infected cells, and the viral proteins and RNAs (including microRNAs) expressed during the latent forms of EBV infection are thought to be the main mediators of EBV-induced transformation (2–4). The major reservoir for persistent latent EBV infection in healthy humans is the memory B cell compartment, while lytic EBV infection occurs in B cell receptor-activated B cells and plasma cells (5–9). EBV infection in normal differentiated oropharyngeal epithelial cells is completely lytic, without concomitant latent viral infection (8, 9).

Undifferentiated NPCs are almost universally EBV-infected (1), and are composed of undifferentiated nasopharyngeal epithelial cells that have "type II" EBV latency. Cells containing type II EBV latency express only three latent viral proteins (EBNA1, LMP1, and LMP2A), as well as the virally encoded "BARTs" microRNAs (Bam-HI A rightward transcripts) and the small EBV-encoded nuclear RNAs (2). EBV is consistently found in the earliest (pre-malignant) stages of NPC (3), but the mechanism(s) by which EBV promotes NPC in humans remain incompletely defined. In contrast to the efficient transforming effect of EBV infection in B cells, EBV infection of normal oropharyngeal epithelial cells in vitro does not confer a selective growth advantage unless cells are grown in the absence of supplemental growth factors (4), and EBV-infected normal epithelial cells routinely lose the EBV genome unless viral infection is selected for by inserting an antibiotic resistance gene into the EBV genome. Cell lines derived from human NPC tumors also usually lose the EBV genome when cultured in vitro.

Thus, while EBV infection is likely essential for promoting the earliest stages of NPC tumor development, persistent EBV infection may not be required to sustain fully formed NPC tumors in vitro, presumably because mutations and/or epigenetic modifications of cellular oncogenes and tumor suppressor genes replace the functions of EBV transforming proteins/viral micro RNAs for growth in vitro. For example, LMP1 activates NF-κB signaling (1), and a recent study showed that cellular mutations which activate NF-κB signaling occur much

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The authors declare no competing interest.

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[1]To whom correspondence may be addressed. Email: skenney@wisc.edu.

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more frequently in NPC tumors that lose LMP1 expression compared to tumors that express LMP1 (5). Nevertheless, as NPC tumors in humans (and in xenograft models) do not lose the EBV genome, even though they sometimes lose expression of the LMP1 and LMP2A proteins, expression of other viral genes (in particular, the highly expressed viral microRNAs) may confer a growth and/or survival advantage to NPCs in vivo, for example, by inhibiting the host immune response to the tumor.

The EBV oncoprotein, LMP1, is a constitutively active mimic of CD40 that activates numerous different signaling pathways, including NF-κB, MAPK, PI3K, and Src in B cells (6–8). LMP1 is expressed in early NPC lesions and is thought to play a major role in promoting the development of NPC (6). However, in comparison with B cells, the effects of LMP1 in epithelial cells (particularly when LMP1 is expressed at biologically relevant levels) have been much less studied. Many of the previous reports examining LMP1 functions in epithelial cells were performed using overexpression studies in EBV-negative carcinoma cell lines that were already transformed and unable to differentiate [and in some cases HPV (human papilloma virus)-infected]. In such studies, LMP1 overexpression has been reported to increase proliferation, induce epithelial-to-mesenchymal transition (EMT), increase cellular migration and invasion, activate canonical and non-canonical NF-κB signaling, and enhance expression of the EGFR and EGFR signaling (reviewed in reference (6)). In addition, LMP1 overexpression (derived from B95.8 strain EBV) in SCC12F squamous carcinoma cells inhibits differentiation (9), and LMP1 over-expression in CNE carcinoma cells activates TAZ function (a Hippo pathway effector) (10). However, whether LMP1 induces the various phenotypes described above when expressed at biologically relevant levels in EBV-infected normal epithelial cells, and whether LMP1 also activates YAP (Yes-associated protein) function (the other Hippo pathway effector), is not known.

Here we have compared the effect of wild-type AG876 EBV strain infection, versus infection with an LMP1-deleted (ΔLMP1) AG876 EBV mutant, in a telomerase-immortalized normal oral keratinocyte cell line (NOKs). NOKs provide an excellent model system to study EBV in epithelial cells since they can be stably infected with EBV (in the presence of antibiotic selection) and retain the ability to differentiate (4, 11–13). Although we recently showed that EBV infection of NOKs inhibits spontaneous differentiation and promotes proliferation when cells are grown in monolayer culture in the absence of supplemental growth factors (14), suspended in methylcellulose (12), or "rafted" (12), the mechanism(s) for these effects and the specific viral protein(s) and/or viral RNAs required have not been previously identified. We show here that mutation of the EBV LMP1 gene greatly reduces the ability of EBV infection to enhance cellular proliferation and inhibit spontaneous differentiation in NOKs when growth factors are limiting. Furthermore, we demonstrate that expression of LMP1 (derived from B95.8 strain EBV) at biologically relevant levels is sufficient to induce proliferation and inhibit differentiation in NOKs. Most importantly, we have discovered that LMP1's ability to induce proliferation and block differentiation in NOKs is at least partially mediated through activation of the Hippo pathway effectors, YAP and TAZ, and show that an FDA-approved drug that inhibits YAP and TAZ activity (ibrutinib) reverses these LMP1 effects. Thus, YAP and/or TAZ inhibitors may be novel therapies for treating LMP1-dependent epithelial tumors.

## Results

**LMP1 Expression Is Essential for the Ability of EBV to Induce Proliferation and Inhibit Differentiation under Growth Factor Restricted Conditions in NOKs.** To examine the role of LMP1 in the context of the intact viral genome in EBV-infected NOKs, we used CRISPR/Cas9 to insert an out-of-frame mutation at amino acid 13 of the LMP1 protein in the EBV AG876 virus strain as described in the *SI Appendix, Materials and Methods* section. Cord blood B cells or NOKs were infected with either wild-type EBV ("WT-EBV") or ΔLMP1 EBV and stable EBV-infected clones were obtained using G418 selection in NOKs or by selecting for B cell outgrowth on a feeder layer in cord blood B cells. In contrast to WT-EBV infected cells, B cells infected with ΔLMP1 EBV did not express LMP1 but expressed similar levels of two other EBV latency proteins (EBNA1 and EBNA2) (*SI Appendix*, Fig. S1).

We next asked if LMP1 expression is required for the ability of EBV infection to increase proliferation of NOKs grown in the absence of supplemental growth factors. Equal numbers of uninfected, WT-EBV infected or ΔLMP1-infected cells were plated at sub-confluent conditions in KSFM (keratinocyte serum-free media) media without supplemental EGF or bovine pituitary extract. Nine days later, cellular proliferation for each condition was measured using a luminescence-based "cell titer glo" assay. As shown in Fig. 1*A*, WT-EBV infected NOKs proliferate much more efficiently than uninfected cells under growth factor-restricted conditions, while the ΔLMP1-infected cells grow similarly as the uninfected NOKs. Thus, LMP1 expression is required for the ability of WT-EBV to induce proliferation of NOKs grown in growth factor-restricted conditions.

As uninfected NOKs spontaneously differentiate in monolayer culture when grown at subconfluent conditions without growth factors, and we previously showed that WT-EBV infection inhibits this differentiation (14), we determined if LMP1 expression is also required for this EBV effect. Immunoblot analyses were performed to compare expression levels of cell cycle markers and markers of epithelial cell differentiation when uninfected, WT-EBV infected, or ΔLMP1-infected NOKs were grown in growth-factor restricted conditions for 24 h. As shown in Fig. 1 *B* and *C* and *SI Appendix*, Fig. S2, uninfected NOKs spontaneously express differentiation-dependent cellular proteins such as involucrin, KLF4, BLIMP1, TGM1, IRF6, and ZNF750 and lose expression of the proliferation marker, PCNA (Proliferating cell nuclear antigen), when grown in the absence of EGF and BPE, while WT-EBV infected cells express a much lower level of differentiation markers and higher levels of PCNA. Expression of p21 (a marker for cell cycle inhibition) is also increased in the uninfected NOKs in comparison to the WT-EBV infected NOKs (*SI Appendix*, Fig. S2). In contrast to the WT-EBV infected cells, NOKs infected with the ΔLMP1 virus express higher levels of differentiation markers (similar to the uninfected NOKs) and lose PCNA expression while increasing p21 expression.

To determine whether LMP1 expression alone is sufficient to block spontaneous differentiation of NOKs, NOKs were stably infected with a control lentiviral vector, or a lentivirus expressing LMP1, and activation of proliferation and differentiation markers was examined when cells were cultured without supplemental EGF and BPE. As shown in Fig. 1 *D* and *E*, LMP1 expression alone is sufficient to promote proliferation, and inhibit spontaneous differentiation, in EBV-negative NOKs when growth factors are limiting. LMP1 expression in the LMP1 lentivirus-infected NOKs was confirmed to be similar to the level of LMP1 found in WT-EBV infected NOKs (Fig. 1*F*). These results indicate that LMP1 expression is both required, and sufficient, for the ability of WT-EBV to inhibit spontaneous differentiation and promote proliferation of NOKs when growth factors are restricted.

**LMP1 Expression Is also Required for WT-EBV Infection to Induce an EMT Phenotype and Activate Canonical and Non-canonical NF-κB Signaling in NOKs.** We also examined whether

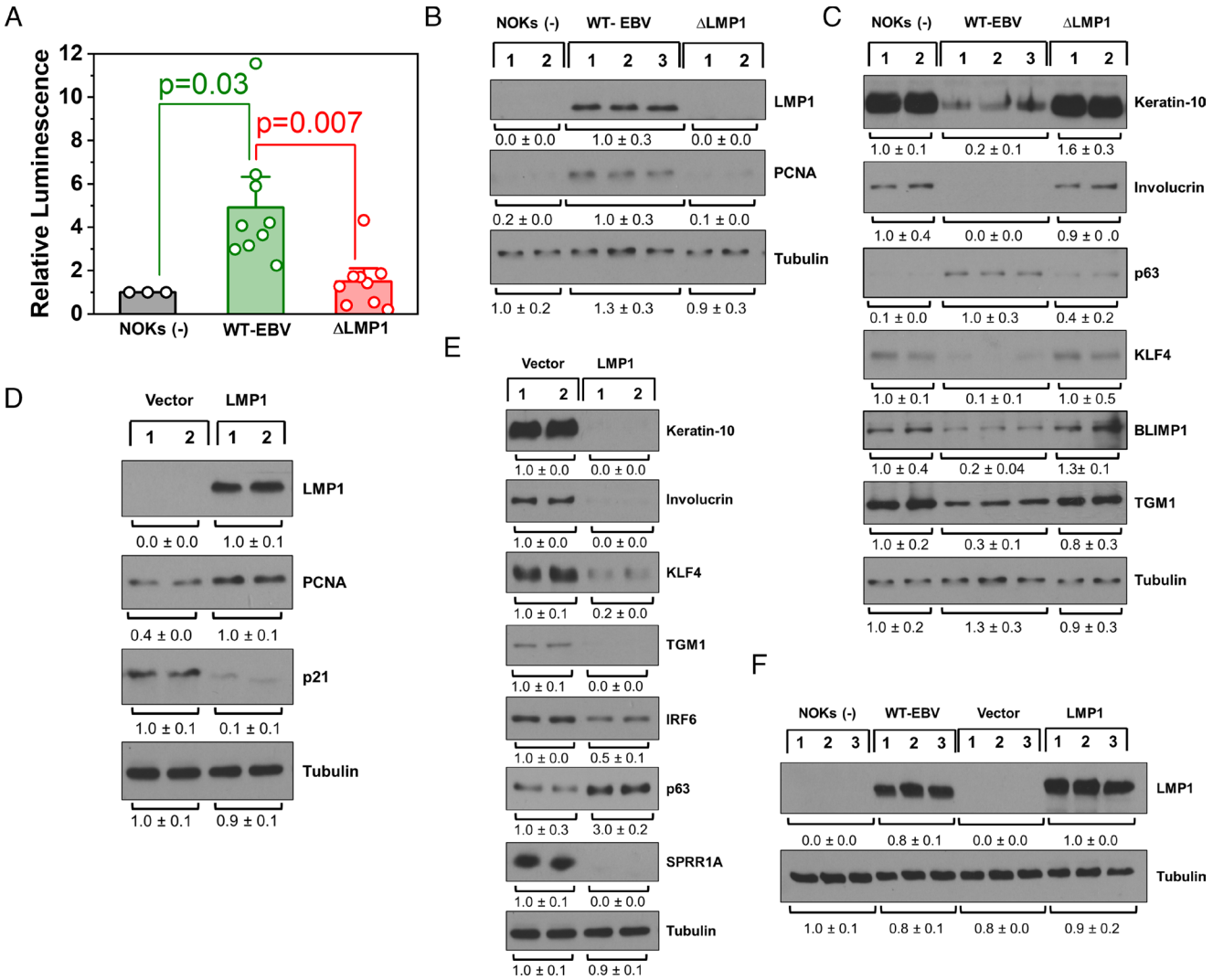


**Fig. 1.** LMP1 is required and sufficient to induce proliferation and inhibit spontaneous differentiation of NOKs. (*A*) Proliferation assays (using cell titer glo) were performed in uninfected, wild-type EBV (WT-EBV) infected or LMP1 deleted EBV (ΔLMP1) infected NOKs plated at subconfluent conditions in the absence of growth factors for 9 d. The results in uninfected NOKs are set as 1. (*B* and *C*) Uninfected, WT-EBV infected or ΔLMP1 infected NOKs were plated at subconfluent conditions in the absence of any supplemental growth factors and immunoblot analysis was performed 24 h later to examine expression of LMP1, PCNA or various differentiation marker proteins (Keratin-10, Involucrin, KLF4, BLIMP1, TGM1), Δp63 (expressed in undifferentiated cells) or tubulin as indicated. The same cellular extracts were used in the Fig. 1 *B* and *C* blots. (*D* and *E*) NOKs infected with control (Vector) or LMP1-expressing (LMP1) lentiviruses were plated at subconfluent conditions in the absence of supplemental growth factors. 24 h later western blots were performed to examine expression of LMP1, or various cell cycle (PCNA, p21) or differentiation (Keratin-10, Involucrin, KLF4, TGM1, IRF6, SPRR1A) markers, Δp63 or tubulin as indicated. The same cellular extracts were used in the Fig. 1 *D* and *E* blots. (*F*) Immunoblot analysis showing LMP1 expression in uninfected, WT-EBV infected NOKs versus NOKs infected with control or LMP1 expressing lentivirus vectors. The western blots were quantitated using ImageJ software, normalized to Tubulin for each condition, and presented as mean ± SD. The NOKs (−) or Vector control results were set as 1 for differentiation marker blots and p21 blots and the WT-EBV or LMP1 results were set as 1 for the PCNA, Δp63 and LMP1 blots.

loss of LMP1 expression affects two widely reported phenotypes of overexpressed LMP1 (increased EMT and increased NF-κB signaling) in EBV-infected NOKs. Immunoblot analyses were performed in uninfected NOKs or NOKs infected with wildtype or ΔLMP1 EBV to examine expression levels of EMT markers and NF-κB signaling markers. As shown in Fig. 2*A* and *SI Appendix*, Fig. S3, NOKs infected with WT-EBV, as previously reported (13, 15), have increased markers of EMT (including decreased E-cadherin expression, and increased fibronectin and vimentin expression) in comparison to the uninfected cells. In contrast, NOKs infected with ΔLMP1 EBV express more E-cadherin, and less fibronectin, N-cadherin, and vimentin, compared to WT-EBV infected cells. In addition, expression of LMP1 alone was sufficient to induce the EMT phenotype in NOKs (Fig. 2*B*). Thus, LMP1 expression at biologically relevant levels is sufficient, and required, for induction of the EMT phenotype in EBV-infected NOKs.

Likewise, we found that EBV infection induces activation of canonical and non-canonical NF-κB signaling in NOKs in an LMP1-dependent manner. LMP1 overexpression in epithelial cells has been previously shown to activate both of canonical and non-canonical NF-κB signaling (16) and NPC cells (which have high levels of NF-κB) require both canonical and non-canonical NF-κB signaling for survival in vitro (17, 18). As shown in Fig. 2*C* and *SI Appendix*, Fig. S4, NOKs infected with WT-EBV have more p100 to p52 cleavage (a marker of noncanonical NF-κB signaling), and more phosphorylated p65 protein (a marker of canonical NF-κB signaling) in comparison with the uninfected NOKs, confirming that EBV infection induces both canonical and non-canonical NF-κB signaling in NOKs. In contrast, NOKs infected with ΔLMP1 EBV have less p100 to p52 cleavage, and less phosphorylated p65, in comparison to the WT-EBV infected cells. Expression of LMP1 alone in NOKs is also sufficient to

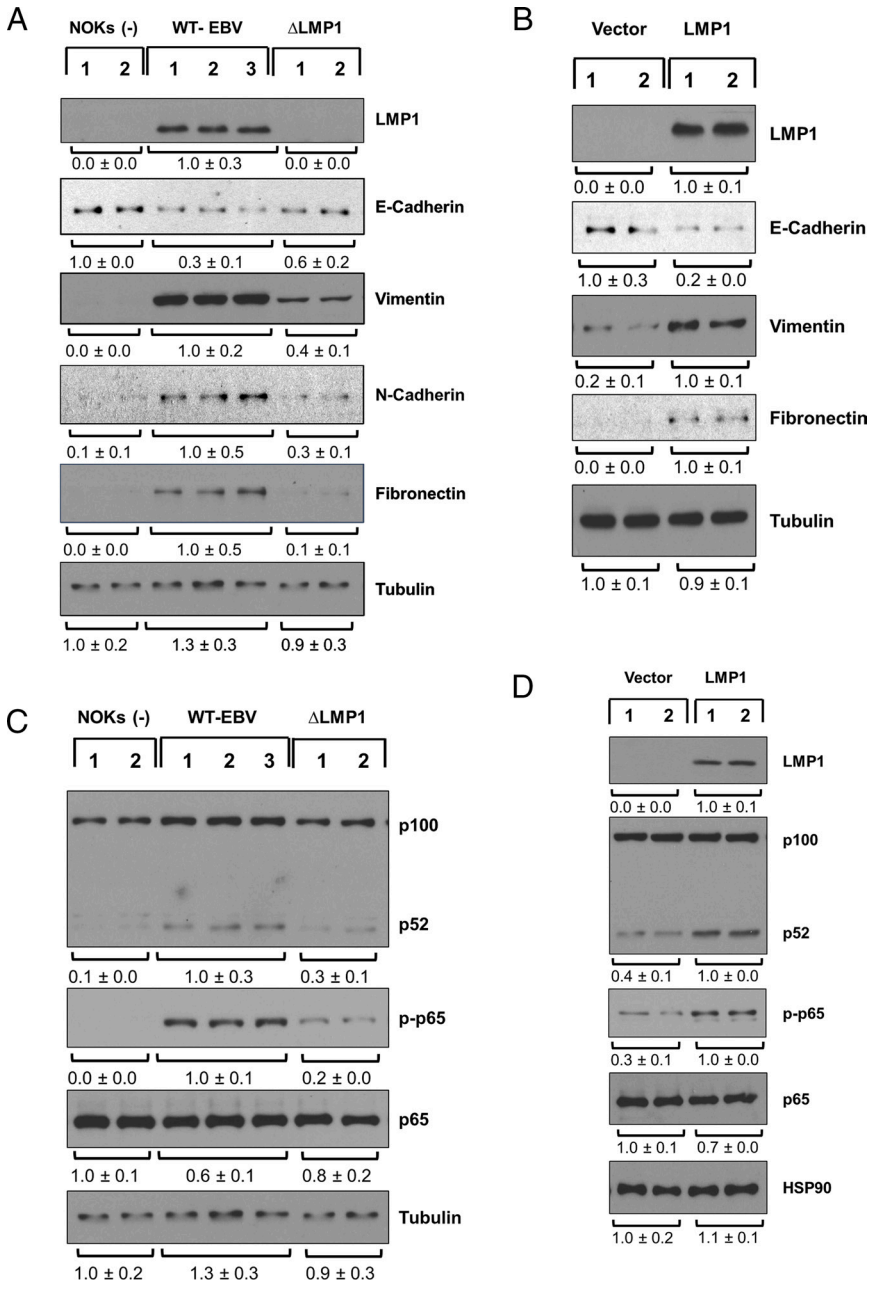


**Fig. 2.** LMP1 is required and sufficient to induce EMT phenotype and NF-κB signaling in NOKs. (*A*) Uninfected NOKs, WT-EBV infected NOKs or ΔLMP1 infected NOKs were plated at subconfluent conditions in the absence of any supplemental growth factors and immunoblot analyses were performed 24 h later to examine expression of LMP1, various EMT markers (decreased E-cadherin, and increased vimentin, N-cadherin and fibronectin)) as indicated. (*B*) NOKs infected with control or LMP1 expressing lentiviruses were plated as described above and 24 h later western blots were performed to examine expression of LMP1, or various EMT markers as indicated. (*C*) Uninfected NOKs, WT-EBV infected or ΔLMP1 infected NOKs were plated at subconfluent conditions in the absence of any supplemental growth factors and immunoblot analyses were performed 24 h later to examine expression of markers for increased canonical NF-κB signaling (phospho-p65) or non-canonical NF-κB signaling (p52) as indicated. (*D*) NOKs infected with control or LMP1 expressing lentiviruses were plated as described above and 24 h later western blots were performed to examine expression of LMP1, or NF-κB signaling markers as indicated.Tubulin or HSP 90 served as a loading control. The same cellular extracts were used in the Fig. 2 *A* and *C* blots (and also used in the Fig. 1 *B* and *C* blots). The extract used in Fig. 2*B* is the same as that used in Fig. 1 *D* and *E*. The western blots were quantitated using ImageJ software and normalized to loading control for each condition. The NOKs (−) or Vector control result was set as 1 for the Tubulin, HSP90, E-cadherin and p65 blots and the WT-EBV or LMP1 result was set to 1 for other EMT markers, p-p65, p52 and LMP1 blots. The values for p-p65 were obtained by normalizing p-p65 to total p65. The results are presented as mean ± SD.

induce both canonical and non-canonical NF-κB signaling in uninfected NOKs (Fig. 2*D*).

**LMP1 Activates the Hippo Pathway Effectors, YAP and TAZ, in NOKs by Decreasing Hippo-Mediated Serine Phosphorylation of YAP and TAZ and Increasing Src Kinase Activity.** YAP and its paralog TAZ (WWTR1) are the transcriptional effector genes of the tumor suppressor Hippo signaling pathway and are commonly activated in epithelial cancers (19–23). The Hippo signaling pathway inhibits YAP and TAZ via activation of the LATS1/2 kinases, which phosphorylate YAP and TAZ at multiple sites. This phosphorylation inhibits YAP/TAZ activity by causing YAP/TAZ to be sequestered in the cytoplasm through interaction with 14-3-3 proteins and promoting degradation of YAP and TAZ by the proteasome (19–29). In contrast, dephosphorylation of YAP and TAZ at these serine residues allows the proteins to

enter the nucleus and activate target genes via interaction with DNA-bound co-factors, particularly the four TEAD (TEA domain transcription factor) family members (19–29).

As we recently showed that WT (Akata strain) EBV infection decreases Hippo-mediated serine 127 YAP phosphorylation and serine 89 TAZ phosphorylation in NOKs (30), and over-expression of LMP1 in CNE carcinoma cells was previously reported to increase nuclear translocation and activity of TAZ (10), we examined the effect of WT-EBV versus ΔLMP1 EBV infection on YAP and TAZ in NOKs grown under conditions that allow Hippo signaling as described in the *SI Appendix, Materials and Methods* section. As shown in Fig. 3*A* and *SI Appendix*, Fig. S5, WT-EBV infected NOKs have reduced S127 YAP and S89 TAZ phosphorylation in comparison with the uninfected NOKs, while the levels of total YAP and TAZ are not significantly different. Furthermore, in comparison with the WT-EBV infected NOKs, ΔLMP1 EBV-infected NOKs have higher levels of both S127 YAP and S89 TAZ phosphorylation, suggesting that the ability of WT-EBV infection to inhibit Hippo-mediated phosphorylation of YAP and TAZ is at least partially mediated by LMP1. To examine whether LMP1 expression in uninfected NOKs is sufficient to inhibit S127 YAP and S89 TAZ phosphorylation, we also compared the levels of total YAP and TAZ

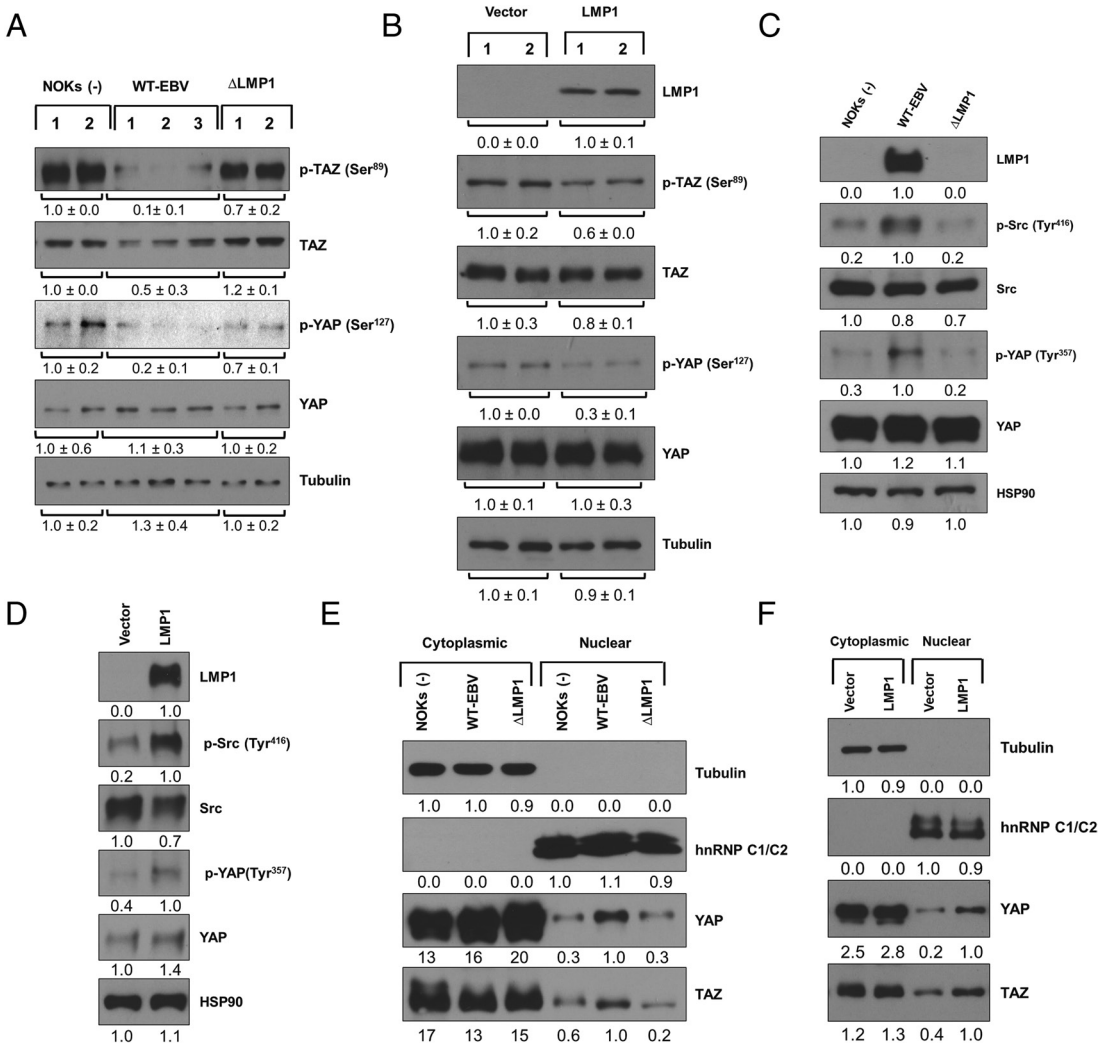


**Fig. 3.** LMP1 reduces hippo-mediated inhibitory serine phosphorylation of YAP and TAZ and promotes Src-mediated tyrosine phosphorylation of YAP. (*A*) Immunoblot analysis was performed on extracts from uninfected, WT-EBV infected or ΔLMP1-infected NOKs to examine expression of LMP1, total versus S89-phosphorylated TAZ, or total versus S127-phosphorylated YAP, as indicated. The extract used in Fig. 3*A* is the same as that used in Fig. 1 *B* and *C*. Tubulin serves as a loading control. (*B*) Immunoblot analysis was performed on extracts from NOKs infected with a control lentivirus vector versus a LMP1 expressing lentivirus vector to examine expression of LMP1, total versus S89-phosphorylated TAZ, or total versus S127-phosphorylated YAP, as indicated. The extract used in Fig. 3*B* is the same as that used in Fig. 2*B*. Tubulin serves as a loading control. (*C*) Immunoblot analysis was performed on extracts from uninfected, WT-EBV infected or ΔLMP1-infected NOKs to examine expression of LMP1, total versus activated (tyrosine 416 phosphorylated) Src, or total versus activated (tyrosine 357 phosphorylated) YAP as indicated. HSP90 serves as a loading control. (*D*) Immunoblot analysis was performed on extracts from NOKs infected with a control lentivirus vector versus a LMP1 expressing lentivirus to examine expression of LMP1, total versus activated (tyrosine 416 phosphorylated) Src, or total versus activated (tyrosine 357 phosphorylated) YAP as indicated. HSP90 serves as a loading control. (*E*) Uninfected, WT-EBV infected or ΔLMP1-infected NOKs were separated into nuclear and cytoplasmic fractions as described in the *SI Appendix, Materials and Methods* section and immunoblots performed to compare the amounts of nuclear versus cytoplasmic YAP and TAZ. hnRNP C1/C2 served as a control for a known nuclear protein and tubulin as a control for a known cytoplasmic protein. (*F*) NOKs infected with a control lentivirus vector versus a LMP1 expressing lentivirus were separated into nuclear and cytoplasmic fractions and immunoblots performed to compare the amounts of nuclear versus cytoplasmic YAP and TAZ. The western blots were quantitated using ImageJ software and normalized to loading control for each condition. The NOKs (−) Vector control result was set as 1 for the Tubulin, HSP90, YAP, TAZ, Src, p-YAP (Ser$^{127}$), and p-TAZ (Ser$^{89}$) blots and the WT-EBV or LMP1 result was set to 1 for the LMP1, p-YAP (Tyr$^{357}$) and p-Src (Tyr$^{416}$) blots. The values for p-proteins have been obtained by normalizing them to their respective total protein amounts. The results are presented as mean ± SD. in Fig. 3*A* and Fig. 3*B*. In Fig. 3 *E* and *F*, for the tubulin blots, the result of the NOKs (−) or Vector control in cytoplasmic extracts was set as 1. For the hnRNP blots, the nuclear NOKs (−) or Vector control result was set as 1. For the YAP and TAZ blots, the nuclear WT-EBV or LMP1 result was set as 1.

versus S127 phosphorylated YAP and S89 phosphorylated TAZ in EBV-negative NOKs infected with the lentivirus control vector or the LMP1-expressing lentivirus vector. As shown in Fig. 3*B*, LMP1 expression alone is sufficient to inhibit Hippo-mediated phosphorylation of YAP and TAZ serine residues in NOKs.

In addition to the Hippo pathway, numerous other factors regulate YAP and/or TAZ activity (19–29). Src family kinase (SFK) proteins indirectly increase YAP and TAZ activity by inhibiting Hippo pathway-mediated phosphorylation of YAP and TAZ via effects on other cellular proteins (31), and also more directly activate YAP and TAZ activity by directly phosphorylating the LATS1 protein and inactivating it (32), and directly phosphorylating YAP tyrosine residue 357, thereby increasing its transcriptional activity and nuclear translocation (33). As LMP1 was previously shown to activate Src kinase in EBV-infected B cells (34), and Src-mediated phosphorylation of the focal adhesion kinase protein was reported in EBV-infected epithelial cells (35), we examined whether WT-EBV infection and/or ΔLMP1 infection increases Src activity, and/or Y357 YAP phosphorylation, in NOKs. As shown in Fig. 3*C* and *SI Appendix*, Fig. S6*A*, Src activity was increased in WT-EBV infected, but not ΔLMP1-infected NOKs, as indicated by the increased level of Y416 phosphorylated Src (a marker for activated Src) in WT-EBV infected cells in comparison with the uninfected or ΔLMP1 infected NOKs. In contrast, the levels of total Src protein were similar in WT-EBV infected, ΔLMP1-infected, and uninfected NOKs.

We next determined whether there is also more Y357 phosphorylated YAP in WT-EBV infected cells compared to uninfected or ΔLMP1 infected NOKs, since SFKs directly phosphorylate YAP residue Y357 (33). We found that WT EBV infected NOKs have a higher level of YAP Y357 phosphorylation in comparison with the uninfected cells, and LMP1 expression contributes to this effect (Fig. 3*C* and *SI Appendix*, Fig. S6*A*). Furthermore, expression of LMP1 alone in EBV-negative NOKs was sufficient to enhance YAP Y357 phosphorylation (Fig. 3*D* and *SI Appendix*, Fig. S6*B*). These results indicate that LMP1 increases YAP and TAZ activity not only by inhibiting Hippo pathway-mediated S127 YAP and S89 TAZ phosphorylation, but also by increasing Src activity (or the activity of related SFK members) and inducing YAP Y357 phosphorylation.

To determine whether LMP1 promotes the ability of YAP and TAZ to enter the nucleus, we separated the nuclear and cytoplasmic fractions of NOKs and performed immunoblot analyses to ask if LMP1-expressing NOKs have a higher level of nuclear YAP and/or TAZ in comparison with NOKs without LMP1 expression. These experiments revealed that WT-EBV infected NOKs have more YAP and TAZ in the nucleus compared to uninfected NOKs, and that this effect requires LMP1 (Fig. 3*E* and *SI Appendix*, Fig. S7*A*). Furthermore, expression of LMP1 alone was found to be sufficient to increase nuclear YAP and TAZ levels in EBV-negative NOKs (Fig. 3*F* and *SI Appendix*, Fig. S7*B*).

**YAP and TAZ Activation Are Required for the Ability of LMP1 to Induce Proliferation, Inhibit Differentiation, and Induce EMT in NOKs.** As YAP and TAZ induce keratinocyte proliferation and inhibit keratinocyte differentiation (36–39), we next examined whether the ability of LMP1 to induce YAP and/or TAZ activity is required for its ability to activate proliferation and/or inhibit differentiation in NOKs. We knocked down YAP and/or TAZ expression using two different sets of siRNAs (small interfering RNAs) for each gene and performed immunoblot analyses to quantitate expression of various different markers of cell cycle progression or differentiation in uninfected or WT-EBV infected NOKs grown in growth factor-restricted conditions. As shown in Fig. 4*A* and *SI Appendix*, Fig. S8, knockdown of either YAP or TAZ expression alone increased expression of multiple differentiation markers in WT-EBV infected cells, and decreased expression of the proliferation marker PCNA. Furthermore, in most cases, simultaneous knockdown of YAP and TAZ together resulted in a greater increase in differentiation marker expression, and greater loss of PCNA expression, compared to loss of YAP or TAZ alone, although TAZ knock-down alone had a greater effect than YAP knockdown alone on KLF4 expression (Fig. 4*A* and *SI Appendix*, Fig. S8). Similarly, knockdown of YAP and TAZ together also inhibited the ability of LMP1 expression by itself to induce proliferation and inhibit differentiation in uninfected NOKs (Fig. 4*B* and *SI Appendix*, Fig. S9). As YAP and TAZ have been previously shown to induce EMT (10, 25, 27), we also examined whether YAP and/or TAZ contribute to LMP1's ability to induce the EMT phenotype. As shown in Fig. 4 *A* and *B* and *SI Appendix*, Fig. S10 *A* and *B*, knockdown of YAP and TAZ greatly reduced the expression of the EMT markers, vimentin and fibronectin, in WT-EBV infected or LMP1-expressing NOKs cells. Thus, YAP and TAZ activation by LMP1 is required for induction of the EMT phenotype. Finally, although the interactions between the Hippo signaling pathway and NF-κB pathways are extremely complex, and are both context and cell-type dependent (40, 41), we also determined if knockdown of YAP and/or TAZ activity affects the ability of EBV to activate canonical and/or non-canonical NF-κB signaling in NOKs. As shown in *SI Appendix*, Fig. S11, the amount of both phospho-p65 and p52 was decreased in EBV-infected NOKs cells when YAP and TAZ were knocked down, suggesting that YAP and TAZ contribute to both canonical and non-canonical NF-κB activation in EBV-infected NOKs. Conversely, we found that knock-down of p100/52 decreased the amount of total TAZ (*SI Appendix*, Fig. S12) although further studies are required to determine whether this also results in a decrease in nuclear (active) TAZ. As we did not find that knockdown of either p65 and/or p100/52 components of NF-κB altered the ability of WT-EBV infection to inhibit differentiation or induce EMT in NOKs (*SI Appendix*, Fig. S13), NF-κB signaling does not appear to contribute directly to these EBV-mediated phenotypes.

**Ibrutinib and Dasatinib Both Decrease EBV's Ability to Enhance YAP and TAZ Activity in NOKs.** Given our findings that activation of YAP and TAZ is essential for the ability of EBV infection to enhance proliferation, inhibit differentiation and induce the EMT phenotype in NOKs, we next examined whether treating EBV-infected NOKs with the FDA-approved drugs, Ibrutinib or dasatinib, reduces EBV-induced activation of YAP and/or TAZ, and/or reverses any of the EBV-mediated phenotypes in NOKs. Ibrutinib was chosen for study since this first-generation BTK inhibitor (which is used to treat B cell malignancies such as CLL) was recently shown to prevent YAP nuclear localization via an off-target effect (42), and has also been reported to inhibit Src kinase activity via an off-target effect (43). Dasatinib (which is used to treat a number of different malignancies) was chosen for study because it inhibits Src kinase activity through its on-target effect (44), and EBV infection promotes Src-kinase mediated YAP Y357 phosphorylation in NOKs. As shown in Fig. 5*A*, treatment of WT-EBV infected NOKs with ibrutinib greatly reduced expression of the proliferation marker PCNA, while increasing expression of cell cycle inhibitor p21 and keratinocyte differentiation markers keratin-10, KLF4, and IRF6, even though LMP1 expression was increased. Ibrutinib also reversed the ability of EBV infection to induce the EMT phenotype in NOKs (Fig. 5*A*) and increased Hippo pathway-mediated YAP S127 and TAZ S89 phosphorylation

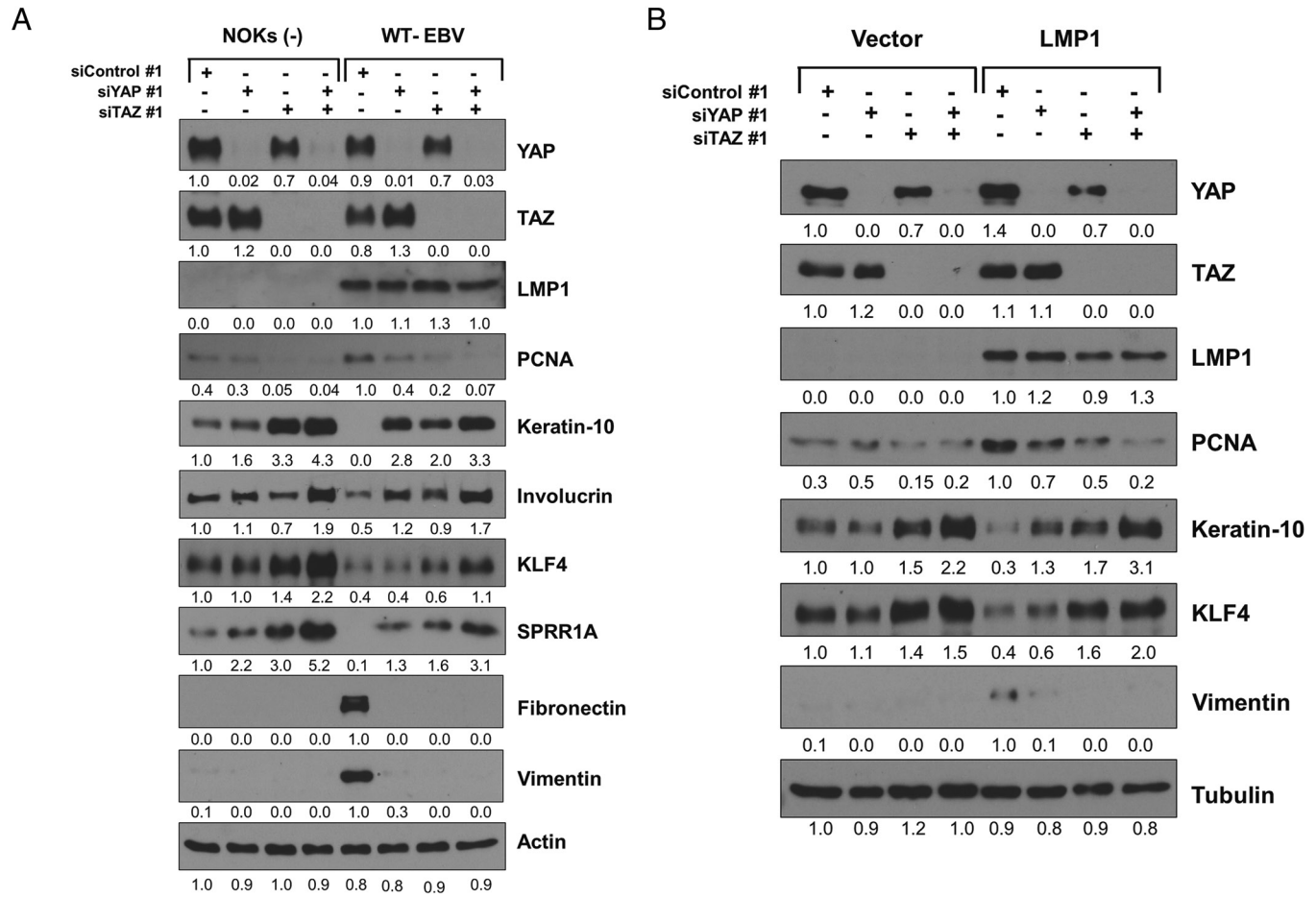


**Fig. 4.** YAP and TAZ are required for EBV-induced proliferation, inhibition of differentiation and EMT in NOKs. Uninfected or WT-EBV infected NOKs (*A*) or NOKs infected with control lentivirus or LMP1-expressing lentivirus (*B*) were treated with or without control siRNAs or siRNAs targeting YAP or TAZ (alone or in combination as indicated). Two days later, western blot analysis was performed to examine the effects of YAP and/or TAZ knockdown on YAP and TAZ expression, LMP1 expression, PCNA, and expression of various different keratinocyte differentiation markers or markers for EMT. The immunoblots were quantitated using ImageJ software and normalized to the loading control for each condition. The NOKs (−) Vector siControl result was set as 1 for the Tubulin, YAP, TAZ, Keratin-10, KLF4, SPRR1A, and Involucrin blots and the WT-EBV and LMP1 siControl result was set as 1 for the LMP1, PCNA, Fibronectin, and Vimentin blots.

(Fig. 5*B*), confirming the previous report showing that ibrutinib inhibits YAP activity in melanoma cells (42). Of note, NOKs do not express the BTK protein (*SI Appendix*, Fig. S14). A dose titration experiment confirmed that ibrutinib decreases PCNA expression and increases differentiation in WT-EBV infected NOKs even at very low doses that can be achieved safely in patients (45) (Fig. 5*C*). A dose titration experiment also confirmed that ibrutinib increases Hippo pathway-mediated YAP S127 and TAZ S89 phosphorylation at low doses (Fig. 5*D*). In addition, Ibrutinib decreased the amount YAP Y357 phosphorylation and Src activity (Fig. 5*E*) in WT-EBV infected NOKs, suggesting that the ability of ibrutinib to inhibit YAP/TAZ activity is at least partially mediated via off-target inhibition of Src (or related SFK members) activity. The Src kinase inhibitor dasatinib also reduced PCNA expression, increased differentiation marker expression, and reduced markers of EMT phenotype in WT-EBV infected NOKs (*SI Appendix*, Fig. S16*A*). As expected, dasatinib decreased the amount of Src-phosphorylated YAP Y357, as well as total Src kinase activity in WT-EBV infected NOKs (Fig. 5*E*). A dose titration experiment confirmed that these effects occur at drug levels that can be obtained in patients (46) (*SI Appendix*, Fig. S16 *B* and *C*). Furthermore, dasatinib increases Hippo-pathway mediated serine phosphorylation of YAP and TAZ in NOKs (*SI Appendix*, Figs. S15 and S16*D*).

To confirm that ibrutinib blocks the ability of EBV infection to enhance proliferation of NOKs grown in the absence of growth factors, uninfected or WT-EBV infected NOKs were plated at subconfluent conditions (in the absence of growth factors) in the presence or absence of Ibrutinib (0.05 μM) and the number of viable cells in each condition relative to the starting number of cells was determined by trypan blue staining 7 d later (Fig. 5*F*). As expected, EBV-infected NOKs proliferated more than uninfected NOKs under these growth conditions. Most importantly, however, a clinically relevant dose of ibrutinib reduced proliferation of the EBV-infected NOKs (Fig. 5*F* and *SI Appendix*, Fig. S17), while having little effect on the proliferation of uninfected NOKs (Fig. 5*F*). These results suggest that ibrutinib (and possibly dasatinib) could be promising agents to inhibit growth of EBV-induced epithelial tumors that continue to express LMP1 and require YAP and/or TAZ for proliferation and/or their EMT phenotype.

## Discussion

The undifferentiated form of NPC is almost universally associated with EBV infection, but the specific mechanism(s) by which EBV promotes the development of NPC are still being defined. EBV infection occurs as a very early event in NPC and the viral genome is already present in pre-invasive lesions such as carcinoma in situ (3). The EBV LMP1 membrane protein, which mimics CD40 signaling and is expressed during certain forms of latent viral infection, is thought to contribute to early NPC development by

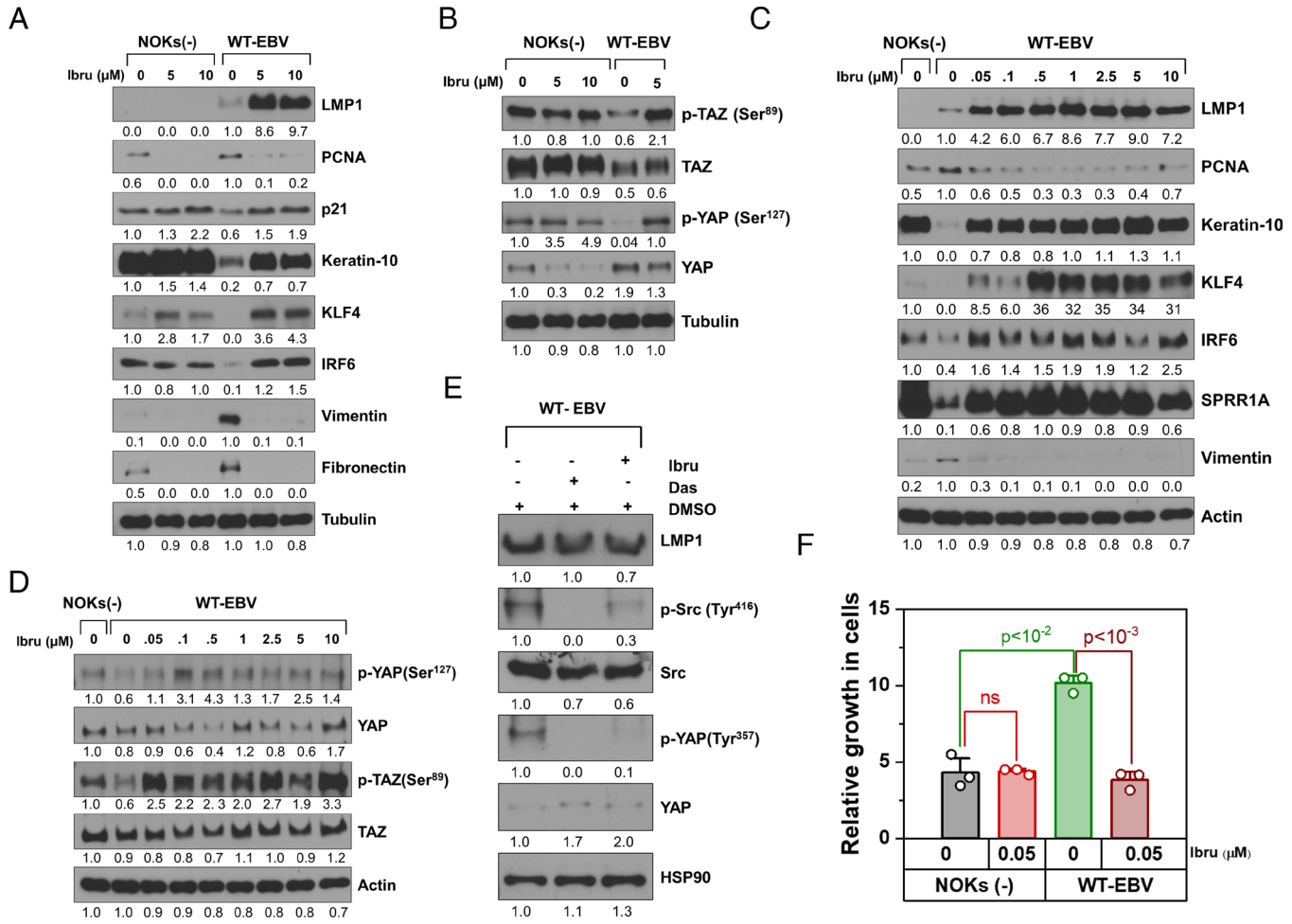


**Fig. 5.** Ibrutinib inhibits the ability of EBV infection to induce proliferation and inhibit differentiation in NOKs. Uninfected or WT EBV infected NOKs (grown at sub-confluent conditions in the absence of supplemental growth factors) were treated with or without ibrutinib (Ibru) at the doses indicated. Immunoblot was performed 2 d later to examine expression of LMP1, PCNA, p21, KLF4, vimentin, fibronectin or tubulin (*A*), total YAP and TAZ versus Hippo-mediated YAP and TAZ inhibitory serine phosphorylation (*B*), or LMP1, PCNA, EMT marker vimentin and a variety of epithelial cell differentiation markers in an ibrutinib dose titration study (*C*), Comparison of total YAP and TAZ versus Hippo-mediated serine phosphorylation of YAP and TAZ in cells treated with different doses of ibrutinib (*D*). Comparison of total Src versus activated (tyrosine phosphorylated) Src, or total YAP versus activated (tyrosine phosphorylated) YAP in WT-EBV infected NOKs treated with 1μM ibrutinib (Ibru) or 1 μM dasatinib (Das) for 1 h. (*E*). The same extracts were used in the Fig. 5 *A* and *B* blots, and the Fig. 5 *C* and *D* blots were derived from the same cellular extracts. The immunoblots were quantitated using ImageJ software and normalized to the loading control for each condition. In Fig. 5 *A*–*D*, the untreated NOKs (-) result was set as 1 for the loading control, differentiation markers, p21, YAP, TAZ, p-YAP (Ser$^{127}$), p-TAZ (Ser$^{89}$) blots and the WT-EBV untreated result was set as 1 for the LMP1, PCNA, Fibronectin, Vimentin, Src, p-YAP (Tyr$^{357}$), and p-Src (Tyr$^{416}$) blots. The p-YAP (Ser$^{127}$), p-YAP (Tyr$^{357}$), p-Src (Tyr$^{416}$), and p-TAZ (Ser$^{89}$) were normalized to the total YAP, Src, and TAZ respectively. In Fig. 5*E*, the untreated WT-EBV result was set as 1 for the LMP1, p-Src (Tyr$^{416}$), Src, p-YAP (Tyr$^{357}$), YAP, and HSP90 blots. (*F*) Uninfected NOKs or WT-EBV infected NOKs were grown in sub-confluent conditions (in the absence of growth factors) in the presence or absence of 0.05 μM Ibrutinib (Ibru) (or DMSO) and the number of cells (relative to the starting number) at day 7 for each condition was determined by trypan blue staining.

enhancing NF-κB signaling, as well as inducing an EMT phenotype (6). Although fully formed NPC tumors often lose LMP1 expression (17), LMP1 is uniformly expressed in early pre-invasive NPC lesions (3). Thus, LMP1 functions may be most essential for the initiation of NPC and become less important as additional cellular mutations are acquired. Here we show that LMP1 expression is required for the ability of EBV to induce cellular proliferation and inhibit spontaneous differentiation in telomerase-immortalized NOKs when growth factors are limiting. Furthermore, we demonstrate that LMP1 activates the Hippo pathway effectors, YAP and TAZ, and show that activation of YAP and TAZ are both required for EBV to enhance cellular proliferation, inhibit spontaneous differentiation, and induce EMT in NOKs. These results reveal that LMP1-induced activation of YAP and TAZ activity is a likely major mechanism by which EBV promotes early NPC.

Induction of YAP and/or TAZ activity may be universally important for the ability of human DNA tumor viruses to promote epithelial cell tumors. The HPV, which plays a key role in causing both human cervical carcinomas and human head and neck tumors, activates YAP function in virally infected epithelial cells via multiple different mechanisms. For example, the E7 oncoprotein increases the nuclear localization and activity of YAP by promoting degradation of PTPN14 (Protein tyrosine phosphatase non-receptor type 14), a negative regulator of YAP activity, and this effect is required for the ability of high-risk HPV E7 proteins to induce proliferation and inhibit differentiation of HPV-infected basal epithelial cells (47–49). In addition, the HPV E6 oncoprotein increases YAP nuclear localization via its interaction with cellular PDZ domain protein (50). Furthermore, activation of endogenous YAP1 activity is sufficient to cause rapid cervical carcinomas in mice even in the absence of E6 or E7 expression (51). The Kaposi Sarcoma virus herpesvirus, KSHV, encodes a viral GPCR (G protein-coupled receptor) that activates YAP and TAZ activity by blocking LATS (the cellular kinases in the Hippo pathway that phosphorylate YAP and TAZ to inhibit their functions) (52). In addition, simian virus 40 and Merkel cell

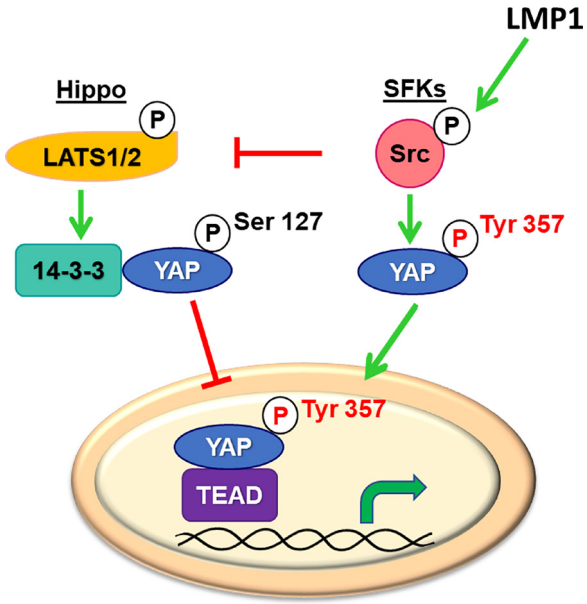


**Fig. 6.** LMP1 activates YAP and TAZ activity in NOKs by multiple mechanisms. LMP1 inhibits Hippo-mediated serine phosphorylation of YAP and TAZ, promoting the ability of YAP and TAZ to enter the nucleus and turn on YAP/TAZ/TEAD-dependent target genes. LMP1 activation of Src results in inhibition of LATS kinase activity as well as an activating tyrosine phosphorylation of YAP that increases its nuclear localization and transcriptional activity.

polyomavirus small T oncoproteins activate YAP by inhibiting NF2, resulting in reduced YAP function (53).

Although one previous paper reported that overexpressed LMP1 activates TAZ activity in CNE carcinoma cells by interacting with the actin inhibitory protein, gelsolin, and thereby decreasing activity of the LATS Hippo kinases (10), this report shows that the EBV LMP1 protein activates YAP activity, and also the first to show that it activates TAZ and YAP activity in the context of EBV-infected telomerase-immortalized NOKs that can differentiate. Since the previous paper was performed using LMP1 overexpression in the CNE carcinoma cell line [which was initially thought to be derived from a human NPC tumor but more recently was shown to be contaminated with HPV-infected HeLa cells (54)], there is also a worry that HPV-encoded E6 or E7 proteins contributed to the results in the previous paper.

Our results here, in agreement with the previous paper, confirm that LMP1 expression inhibits Hippo pathway-mediated phosphorylation of TAZ, and are the first to show that LMP1 also inhibits Hippo pathway-mediated phosphorylation of YAP. We have also discovered that in addition to inhibiting the Hippo pathway, LMP1 activates YAP and TAZ function via its ability to activate Src kinase activity in NOKs. Src kinase, and related SFKs, phosphorylate the YAP Y357 residue, promoting YAP nuclear localization and transcriptional function even when the Hippo pathway is active (33). This model is summarized in Fig. 6. Importantly, since the previous report confirmed that human NPC tumors have enhanced nuclear TAZ, particularly when these tumors express LMP1 (10), the ability of LMP1 to activate TAZ activity is not only relevant to the effects of EBV infection in normal epithelial cells, but also to the role of LMP1 in promoting NPC tumors in humans.

Our findings here are also the first to show that LMP1 activation of YAP/TAZ activity is required for the ability of EBV infection in telomerase-immortalized NOKs to promote epithelial cell proliferation, inhibit differentiation, and induce EMT. Although YAP and TAZ both interact with TEAD family members and share many common transcriptional targets, a subset of target genes is specific to YAP or TAZ, and the phenotypes of YAP and TAZ knock-out cells are not always similar (55). While most previous studies examining the effects of HPV oncoproteins on the Hippo pathway have concentrated upon examining the effects on YAP activation, our results here suggest that TAZ activation may be at least as important as YAP activation for EBV's ability to promote the development of NPC tumors. Similar to our results here, recent studies in HPV-infected, or HPV E7-expressing keratinocytes have suggested that YAP target genes in normal keratinocytes are primarily involved in inhibiting differentiation and promoting proliferation, rather than for activating "classic" YAP target genes such as CYR61 and CTGF (49). The fact that LMP1 induces a YAP- and TAZ- dependent EMT phenotype in NOKs, which does not appear to be the case in E7-expressing keratinocytes, suggests that induction of the EMT phenotype in normal epithelial cells may require both YAP and TAZ activation. Consistent with this, LMP1-induced TAZ activation was previously shown to induce an EMT phenotype in CNE cells (10), which presumably already have activated YAP due to their HPV infection (54).

Our finding that the activation of both YAP and TAZ contributes to LMP1-induced proliferation, and inhibition of differentiation, in NOKs also mirrors previous findings showing that expression of constitutively active YAP and/or TAZ increases proliferation and inhibits differentiation in normal mouse and human keratinocytes, and that TEAD family members are also required for these effects (36–39). TEAD family members, which are DNA-binding transcription factors that directly interact with YAP and TAZ to mediate their transcriptional effects, inhibit proliferation, and induce spontaneous differentiation, in telomerase-immortalized human keratinocytes at least partly by limiting activity of the KLF4 transcription factor (37). Since we previously showed that KLF4 [a master regulator of epithelial cell differentiation (56)] cooperates with PRDM1 (BLIMP1) to activate LMP1 expression in EBV-infected NOKs (57), the ability of the YAP/TAZ/TEAD axis to inhibit KLF4 activity may serve as an autoinhibitory mechanism to regulate LMP1 expression in EBV-infected basal epithelial cells.

The finding that two different human DNA tumor viruses, HPV and EBV, that cause epithelial cell tumors both enhance YAP and/or TAZ activity [albeit through different mechanism(s)] suggests that active forms of YAP and TAZ contribute to the life cycles of these viruses independent of any effects on EBV- and HPV-induced tumor development. In the case of HPV, the ability of E7 to promote YAP activity in undifferentiated basal epithelial cells (the normal site of HPV latent infection) has been proposed to prolong the life span and proliferation of this latent viral reservoir (helping to maintain the pool of latently HPV-infected cells), while not inhibiting epithelial cell differentiation to the extent that differentiation-dependent HPV lytic infection cannot occur in at least a portion of the infected epithelial cells (49).

In the case of EBV infection, the virus may not normally infect, or persist, in undifferentiated basal epithelial cells and instead preferentially infects differentiated epithelial cells where it directly enters the lytic form of viral infection (58). Since we have recently shown that YAP and TAZ collaboratively activate lytic EBV infection in epithelial cells (30), and found that LMP1 expression induced by KLF4 and BLIMP1 expression during early differentiation of EBV-infected NOKs promotes efficient lytic EBV reactivation (57), we hypothesize that LMP1's ability to activate YAP and TAZ activity in non-transformed epithelial cells normally serves to enhance lytic EBV reactivation, while extending the lifespan of lytically infected cells by at least partially attenuating differentiation. However, if EBV infects a basal epithelial cell, where it can enter viral latency due to the lack of KLF4 and

BLIMP1 expression, LMP1 expression associated with latent EBV infection could instead promote early NPC lesions by activating YAP and TAZ. For EBV infection of basal epithelial cells to lead to NPC development, the amount of nuclear YAP and TAZ expression likely needs to be high enough to induce the tumor-promoting effects of YAP and TAZ, but not so high as to induce the lytic form of EBV replication.

Finally, our finding that two FDA-approved drugs, ibrutinib and dasatinib, can inhibit the ability of EBV infection to promote proliferation, inhibit differentiation, and induce EMT in NOKs, and the demonstration that these effects are at least partially mediated through reduction of YAP and TAZ activity, suggests that these agents might be useful for the treatment of YAP- and/or TAZ-dependent NPC tumors. Whether these agents can inhibit the growth of authentic NPC tumors, especially when LMP1 expression is turned off, at non-toxic doses in vivo is an important area for future study.

## Materials and Methods

**Cell Lines and Cell Culture.** NOKs (a gift from the Karl Munger lab at Tufts University) is a telomerase-immortalized NOK cell line derived as previously described (59). NOKs were grown in KSFM supplemented with 12.5 mg bovine pituitary extract, and 0.1 μg epidermal growth factor per 500 mL of media (KSFM, Lifetech). EBV-infected NOKs cells (WT or mutant viruses) were maintained with 50 μg/mL G418 antibiotic selection in addition to the media/growth supplements used to grow NOKs.

**Creation of AG876 LMP1 Mutant Virus.** The LMP1 mutant of AG876 strain EBV was generated using CRISPR/Cas9. The details of generation of LMP1 mutant are described in *SI Appendix*.

**Creation of NOKs Cell Lines Stably Infected with WT or LMP1 EBV.** NOKs were co-cultured with TPA (Phorbol 12-myristate 13-acetate)/Sodium Butyrate treated Burkitt Lymphoma cells stably infected with WT AG876 EBV or LMP1 mutant. After washing off the BL cells, NOKs were selected for stable WT or LMP1 mutant EBV infection by treating cells with G418 (50 μg/mL).

**Creation of NOKs Lines Stably Expressing LMP1.** The B95.8 LMP1 gene was inserted into the lentivirus vector pCDH-MSCV-MCS-EF1a-GFP-Puro to obtain lentivector pCDH-MSCV-MCS-EF1a-GFP-Puro-LMP1. Control lentivector or LMP1 expressing lentivector were then packaged in 293T cells (using KSFM media) as previously described (60), filtered through a 0.8 μm filter and applied to NOKs for 24 h before selecting for lentivirus infected cells using puromycin selection (1 μg/mL).

**Immunoblot Analysis.** Immunoblots were performed as previously described (61).

**Analysis of YAP and TAZ Nuclear versus Cytoplasmic Location.** Nuclear and cytoplasmic separation was performed using the modified "REAP" method (62) as described in the *SI Appendix, Materials and Methods* section.

**NOKs proliferation assays.** Proliferation assays were performed using the Cell Titer-Glo assay (Promega, Madison, WI) or Trypan Blue staining as described in the *SI Appendix, Materials and Methods* section.

**siRNA Treatment of Cells.** Uninfected NOKs or EBV-infected NOKs were transfected with two different siRNA controls, or two different siRNAs directed against YAP, TAZ, p65 or p100/p52 using RNAiMAX following the manufacturer's instructions. Cells were harvested after 2 d post-transfection for immunoblot analysis.

**Data, Materials, and Software Availability.** All study data are included in the article and/or *SI Appendix*.

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Author affiliations: [a]Department of Oncology, University of Wisconsin School of Medicine and Public Health, WIMR II, Madison, WI 53705; and [b]Department of Medicine, University of Wisconsin School of Medicine and Public Health, WIMR II, Madison, WI 53705

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