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Positive regulation of *HIF-1A* expression by EBV oncoprotein LMP1 in nasopharyngeal carcinoma cells

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ABSTRACT

Latent membrane protein 1 (LMP1) is a pivotal viral oncoprotein that contributes to the carcinogenesis of Epstein–Barr virus (EBV)-associated malignancies, including nasopharyngeal carcinoma (NPC). We investigated the regulation of hypoxia-inducible factor 1-α (HIF-1α) by LMP1. In NPC cells, we found that LMP1 significantly enhanced the HIF-1α mRNA level, and not only the protein amount as described previously. Mechanistically, the stability of the HIF-1α transcript was remarkably prolonged by LMP1 via reduced expressions of RNA-destabilizing proteins tristetraprolin (TTP) and pumilio RNA-binding family member 2 (PUM2) through C-terminal activation region 1 (CTAR1) and CTAR3 interaction with the ERK1/2 and STAT3 signaling pathways, respectively, in parallel with hindrance of PUM2 binding to the HIF-1α mRNA 3′-untranslated region (3′-UTR). On the other hand, *HIF-1A* promoter activity was also obviously facilitated by the LMP1 CTAR1-recruited ERK1/2/NF-κB pathway. Intriguingly, in this scenario, augmented HIF-1α further exhibited positive auto-regulation of its own gene transcription. Our results showed the first time that LMP1 directly up-regulates *HIF-1A* transcription and post-transcription in NPC cells, in addition to providing evidence of an increase in the HIF-1α mRNA level caused by a tumor-associated virus under normoxic conditions.

Introduction

Evidence has revealed that a reciprocal interaction between tumor-associated virus infection and host cells partially explains the intricate mechanism of tumorigenesis. For instance, although Epstein–Barr virus (EBV) infection is an etiological factor of nasopharyngeal carcinoma (NPC), a highly prevalent epithelial malignant disease in Taiwan, early events of genetic abnormalities in epithelial cells may pave the way for establishment of latent virus infection in precancerous lesions and malignant cells [1–4]. During latent EBV infection, a limited set of viral latent proteins, small RNAs and microRNAs is expressed in cancer cells, among which latent membrane protein 1 (LMP1) plays a pivotal role in viral carcinogenesis. Through self-aggregation, lipid-raft partitioning and tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) and TRAF-associated death domain protein (TRADD) binding to C-terminal activation regions (CTARs), LMP1 engages multiple signaling pathways, such as canonical and non-canonical NF-κB, extracellular regulated kinase (ERK)-MPAK, c-Jun N-terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3K)/Akt and Janus kinase (JAK)-3/STAT pathways. These signaling pathways modulate numerous biological pathways, including cell proliferation, apoptosis, invasion, metastasis, lymph-angiogenesis, epithelial–mesenchymal transition (EMT), and also genetic and epigenetic alterations [5,6]. Intriguingly, via exosomes delivery, a non-cell-autonomous action of LMP1 on neighboring cell growth has been observed, and the tumor microenvironment has been described [7].

Angiogenesis is one of the critical steps in tumor development [8]. In NPC cells, the underlying mechanism of LMP1-induced angiogenesis is up-regulation of the α subunit of hypoxia-inducible factor 1 (HIF-1α) via Siah1 E3 ubiquitin ligase-mediated degradation of prolyl hydroxylases (PHDs) 1 and 3 [9,10], although co-expression of LMP1 and HIF-1α in NPC biopsies is still controversial

Abbreviations: LMP1, latent membrane protein 1; EBV, Epstein–Barr virus; NPC, nasopharyngeal carcinoma; HIF-1α, hypoxia-inducible factor-1α; TTP, tristetraprolin; PUM2, pumilio RNA-binding family member 2; TRAF, tumor necrosis factor receptor (TNFR)-associated factors; TRADD, TRAF-associated death domain protein; ERK, extracellular regulated kinase; JNK, c-Jun N-terminal kinase; PI3K, phosphatidylinositol 3-kinase; JAK, Janus kinase; EMT, epithelial–mesenchymal transition; PHD, prolyl hydroxylase; pVHL, von Hippel–Lindau protein; RBP, RNA-binding protein; VEGF, vascular endothelial growth factor; EPO, erythropoietin.

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[11,12]. HIF-1 is a heterodimeric transcription factor composed of a constitutively expressed β subunit (also called aryl hydrocarbon receptor nuclear translocator, ARNT) and an oxygen-sensitive α subunit. It has been proven that under normoxic conditions, HIF-1α undergoes rapid degradation via the ubiquitin-proteasome pathway through von Hippel–Lindau protein (pVHL) binding followed by PHD-mediated hydroxylation of the oxygen-dependent degradation domain (ODDD), whereas accumulation due to reduced PHD activity and pVHL binding ability has been observed in response to hypoxia. Subsequently, stabilized HIF-1α associates with HIF-1β in the nucleus, followed by targeting of the hypoxia-responsive element (HRE) of the downstream gene promoters to activate transcription [13–18].

Several studies have indicated that various translational or post-translational modifications, such as phosphorylation, SUMOylation, S-nitrosylation and acetylation, are involved in the synthesis or degradation of HIF-1α, either dependent or independent of pVHL and oxygen [19]. In addition to complicated control at the protein level of HIF-1α, notably, transcription and post-transcriptional regulation of *HIF-1A* through transcriptional factors binding to the promoter, RNA-binding proteins (RBPs) binding to the mRNA 5′-untranslated region (5′-UTR) or 3′-UTR, or microRNAs also contribute to turnover of *HIF-1A* expression [20–23]. Of interest, identification of the HRE within the *HIF-1A* promoter and epigenetic evidence further indicated an auto-transactivation ability of HIF-1α [24–26].

Compelling results suggest that a number of viral oncoproteins induce HIF-1α in human cancers, including EBV-associated NPC [9,10,27]. However, this is not only at the protein level; in this study, we uncovered further evidence of facilitation of *HIF-A* expression in the transcription and post-transcriptional processes by EBV oncoprotein LMP1, as well as positive autoregulation of HIF-1α in NPC cells.

Materials and methods

Cell lines and plasmids

EBV-negative human NPC cell lines HONE-1 and NPC-TW01, as well as derivative EBV-positive NPC cells HA and NA, were gifts from Dr. Ching-Hwa Tsai (National Taiwan University, Taiwan), and were cultured as described previously [28,29]. HONE-1 and NPC-TW01 were authenticated by genetic profiling using short tandem repeat (STR) analysis. Plasmid pcDNA3/LMP1, pSG5Flag-LMP1 and pSG5Flag-LMP1Δ232-351, pCMV-Flag-p50 and pCMV-Flag-p65, a dominant negative STAT3(Y705F) mutant, CMV.hTTP.HA.BGH3′/BS+, Flag-tagged-PUM2, pHA-HIF-1α, wild-type puroLMP1-386, and various deletion mutants of C-terminal activation regions, puro-LMP1-350, puro-LMP1-Δ189-222, puro-LMP1-Flag-350Δ189-222, puro-LMP1-Flag-231 and puro-LMP1-Flag-188, were generously provided by Dr. Lai-Fa Sheu (Taichung Tzu Chi Hospital, Taiwan), Dr. Kenneth Izumi (National Institute of Health, USA), Dr. Ching-Jin Chang (Academia Sinica, Taiwan), Dr. Robert J. Arceci (Johns Hopkins University, USA), Dr. Perry Blackshear (National Institute of Environment of Health Sciences, USA), Dr. Chi-Ying Huang (National Yang-Ming University, Taiwan), Dr. Kou-Juey Wu (China Medical University, Taiwan) and Dr. Jen-Yang Chen (National Health Research Institutes, Taiwan) [30], respectively. pCMV-IκBα and a dominant negative IκBα (S32A/S36A) mutant, pCMV-IκBαM, were purchased from Clontech (Mountain View, CA, USA). asLMP1/pcDNA3.1(−) plasmid was generated for antisense RNA expression. Briefly, an isolated full-length LMP1 fragment in an antisense orientation from asLMP1/yTA plasmid, which was obtained by PCR subcloning with primers (Supplementary Table S1) using pcDNA3/LMP1 plasmid as a template and T&A cloning vector (Yeastern Biotech, Taipei, Taiwan), was inserted into *Eco*RI/*Bam*HI restriction sites of pcDNA3.1(−) vector (Invitrogen, Carlsbad, CA, USA). Luciferase reporter driven by vascular endothelial growth factor (VEGF) promoter (pGL3-1.5kbVEGFprom), a 0.8-kb fragment containing the *HIF-1A* 5′-UTR (287-bp) and promoter (+1 to −541) region (pH800), and a NF-κB-responsive luciferase reporter (NF-κB-Luc) were gifts from Dr. Min-Liang Kuo (Kaohsiung Medical University, Taiwan), Dr. Carine Michiels (University of Namur, Belgium) [23] and Dr. Wen-Ling Shih (National Pingtung University of Science & Technology, Taiwan). pGL3XHRE, a luciferase reporter driven by a heterologous SV40 promoter containing three repeats of erythropoietin (EPO) HRE downstream of the luciferase gene, was also provided by Dr. Carine Michiels. To introduce specific mutation on the *HIF-1A* promoter, site-directed mutagenesis was performed using pH800 plasmid as a template together with paired mutagenic oligonucleotides (Supplementary Table S1) flanking the HRE (-282/-269 bp) (GenBank accession no. AF050115), carried out using a QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. The mutation sequence of construct pH800mt was verified by DNA sequencing. A full-length and progressive deletion of the HIF-1α mRNA 3′-UTR from 5′ to 3′ (GenBank accession no. NM_001530) downstream of the luciferase coding sequence (pLuc-HIF-1α-3′UTR (FL), F1 (87-1175), F2 (154-1175), F3 (229-1175), F4 (291-1175), F5 (325-1175), F6 (553-1175), F7 (704-1175) and F8 (1033-1175)) was kindly provided by Dr. Nadia Cherradi (Institut National de la Santé et de la Recherche Médicale, France) [22].

Transfection, reverse transcription PCR (RT-PCR) and Western blot analysis

Sixty to seventy percent confluent cells were transfected with plasmid mixture using jetPrime reagent (Polyplus-transfection, Illkirch, France) according to the manufacturer's instructions. After transfection, cells were harvested for RNA and protein extraction. For RT-PCR, the HIF-1α, LMP1 or GAPDH transcript was amplified with primers (Supplementary Table S1) or as described previously [28]. For Western blot analysis, mouse anti-LMP1 antibody and anti-ERK1/2 antibody were purchased from Dako (Glostrup, Denmark) and Invitrogen, respectively. Rabbit anti-HIF-1α antibody and anti-HuB antibody were purchased from Novus Biologicals (Littleton, CO, USA). Mouse anti-NF-κB p65 antibody, rabbit anti-NF-κB p50 antibody and anti-phospho-ERK1/2 antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-TTP antibody, rabbit anti-cyclin A antibody and anti-IκBα antibody were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Rabbit anti-PUM2 antibody and mouse anti-HuR antibody were purchased from OriGene Technologies (Rockville, MD, USA). Rabbit anti-YB1 antibody and anti-QKI-6 antibody were purchased from Millipore (Billerica, MA, USA). The densities of the bands were measured using ImageJ software, and values were normalized to the densitometric values of the internal control in each sample. The relative fold changes of the PCR product and protein amounts were calculated for the wild-type or truncated LMP1 expression plasmid alone, or in combination with the asLMP1/pcDNA3.1(−) plasmid or dominant negative STAT3 mutant set in the presence or absence of U0126 treatment as compared with the vector control.

Cell fractionation

Cells were harvested and subjected to cytoplasmic and nuclear protein isolation using a ProteoJET™ Cytoplasmic and Nuclear Protein Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Luciferase activity assay

Cells were co-transfected with both luciferase reporter plasmid and pRL-TK vector (Promega, Madison, WI, USA) plus various expression plasmids. The luciferase activity of each sample was measured using a Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions and calculated by dividing the value of luciferase activity by the value of *Renilla* luciferase activity. The relative luciferase activity was represented as the fold of activation via the experimental set over either the luciferase reporter alone or the vector control.

5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) treatment

Cells were grown to 70–80% confluence and treated with 60 μM DRB (Sigma-Aldrich, St Louis, MO, USA), a RNA polymerase II inhibitor, in the presence or absence of LMP1, TTP or PUM2 expression plasmid. Cells were then harvested at the indicated time and subjected to RT-PCR analysis as described above.

Ribonucleoprotein immunoprecipitation (RNP-IP)

Briefly, cells were lysed in lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM DTT, protease and RNase inhibitor) followed by centrifugation. The supernatants were then incubated with antibody-coated protein G beads and the immunoprecipitates were eluted with elution buffer (100 mM Tris, pH 8.0, 10 mM EDTA, and 1% SDS) containing proteinase K. Subsequently, RNA was isolated and subjected to RT-PCR and quantitative real-time RT-PCR analysis. Conventional PCR was performed with a primer set (Supplementary Table S1) for the HIF-1α mRNA 3′-UTR (GenBank accession no. U22431) to amplify the PUM2- (1950/2392 bp) and the HuB-binding regions (2549/2876 bp), respectively. Real-time PCR for the HIF-1α transcript (forward primer 5′-GTTAGTT CAATTTTGATCCCCTTTCT-3′ and reverse primer 5′-GCTACTGCAATGCAATGGTTTAA-3) was carried out on an Applied Biosystems 7300 Real-Time PCR system using 2× Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. The cycling conditions were 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 60 s, and a dissociation stage. All PCR reactions were performed in triplicate. The fold enrichment of HIF-1α mRNA in each RNA sample was calculated using the comparative Ct method and normalized to GAPDH.

Chromatin immunoprecipitation (ChIP)

A ChIP assay was carried out using an EZ ChIP kit (Millipore) according to the manufacturer's instructions. Briefly, transfected cells were cross-linked with form-

aldehyde, followed by lysis and sonication. The supernatant was incubated with anti-NF-κB p65, anti-NF-κB p50 or anti-HIF-1α antibody, and then with Protein G agarose beads. Subsequently, DNA was eluted from the protein/DNA complex and subjected to PCR analysis with primers (Supplementary Table S1) for the *HIF-1A* promoter flanking the NF-κB binding site (-197/-188 bp) and the HRE spanning -538 to -139 bp from the transcription start site.

Statistical analysis

Statistical differences were analyzed using the unpaired *t*-test and were considered to be significant at values of $P < 0.05$.

Results

Elevation of HIF-1α transcript by LMP1 in NPC cells

Not only is the protein stability of HIF-1α increased by LMP1 in NPC cells [9,10]; in the current study, we showed that elevation of the HIF-1α mRNA level, but not HIF-2α or HIF-1β mRNA, was obviously induced by LMP1 in EBV-negative NPC cells or in LMP1 variant N-LMP1 (clone 1510)-transformed 3T3 cells (Fig. 1A, Supplementary Fig. S1A and B, and data not shown), yet was mitigated via knock-down of the expression of LMP1 (Fig. 1B). Intriguingly, LMP1-mediated elevation of HIF-1α mRNA was a distinctive manifestation in NPC cells, but not in hypopharyngeal carcinoma cells FaDu, immortalized keratinocytes HaCaT, or hypoxia-mimetic agent-treated C6 glioma cells (Supplementary Fig. S1A). To corroborate our observations, two EBV-positive NPC cell lines, NA and HA, which lack LMP1 at the latent stage [28,29], were used to assess the effects of EBV latent products on *HIF-1A* transcription. In contrast to the untransfected sample or the vector control, the amount of HIF-1α transcript was remarkably increased upon LMP1 excitation (Supplementary Fig. S1B and C). Consistently, the nuclear localization and transcriptional activity of HIF-1α were also enhanced by LMP1 (Fig. 1C and D). Therefore, these results suggested that augmentation of the amount of HIF-1α mRNA by LMP1 contributes to the elevated protein level and transactivation ability of HIF-1α in NPC cells.

The stability of HIF-1α mRNA is enhanced by LMP1

To explore the mechanism underlying up-regulation of *HIF-1A* transcription by LMP1, RNA turnover was first evaluated. Via blockade of *de novo* mRNA synthesis by RNA polymerase II inhibitor (DRB), the HIF-1α mRNA level was prolonged for up to 12 hours in the presence of LMP1, whereas it declined after 6 hours in the untransfected control (Fig. 2A). We thereby verified that LMP1 indeed modulated the stability of HIF-1α mRNA. A surrogate luciferase reporter of the HIF-1α mRNA 3′-UTR (pLuc-HIF-1α-3′UTR) was introduced into EBV-negative or derivative EBV-positive NPC cells with or without ectopic expression of LMP1. In line with our hypothesis, luciferase activity was positively regulated by LMP1 (Fig. 2B and Supplementary Fig. S2A). Comparably, the EGFP transcript level from another reporter with the HIF-1α mRNA 3′-UTR downstream of the EGFP coding region (pEGFP-C1-Hif-1α-3′UTR) was also elevated

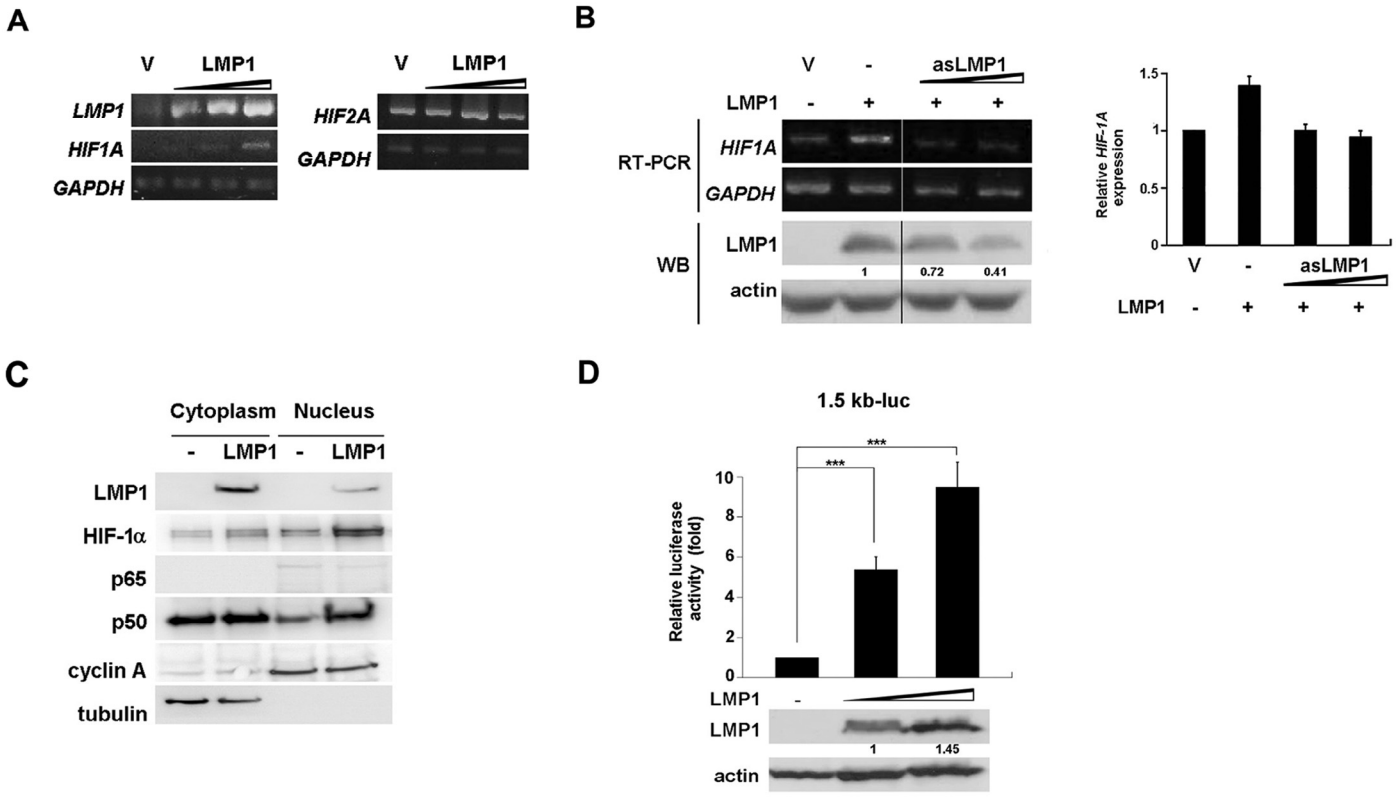

Fig. 1. HIF-1α mRNA level was up-regulated by LMP1. HONE-1 cells were transfected with (A) LMP1 expression plasmid or (B) plus asLMP1 expression plasmid. RNA and cell lysates were then extracted and subjected to RT-PCR for LMP1, HIF-1α or HIF-2α mRNA, in addition to Western blot analysis for LMP1. Transfection with pcDNA3 and pcDNA3.1(–) was used as a vector control in panels A and B, respectively. The RT-PCR product fold change was calculated as described in *Materials and Methods*, and is shown as the mean ± SD of three independent experiments (B, *right*). (C) For the cell fractionation assay, nuclear and cytoplasmic proteins were extracted from NPC-TW01 cells with or without LMP1 expression plasmid transfection and then subjected to Western blot analysis for LMP1, HIF-1α, NF-κB p65, NF-κB p50, cyclin A (nuclear protein marker) or tubulin (cytosol protein marker). (D) HONE-1 cells were transfected with luciferase reporter driven by VEGF promoter (1.5 kb-luc) alone or plus LMP1 expression plasmid. The luciferase activity of each sample was measured and the relative luciferase activity was calculated as described in *Materials and Methods*, and is shown as the mean ± SD of three independent experiments. Asterisks indicate the significance at $^{***}P < 0.001$. Expression of LMP1 was detected by Western blot analysis. The protein expression fold change was calculated as described in *Materials and Methods*. All RT-PCR or Western blot analysis data shown are representative of three independent experiments.

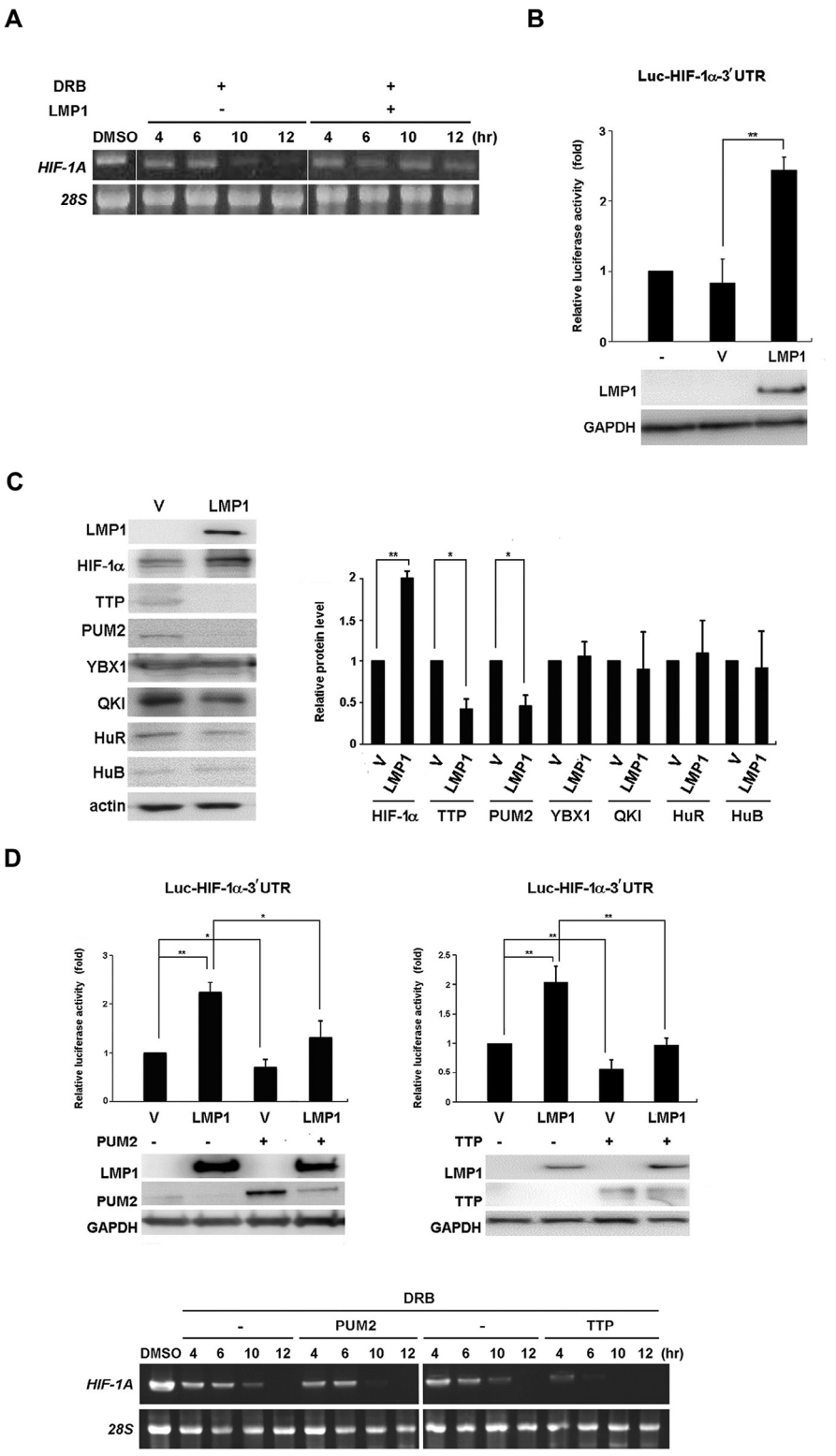

Fig. 2. Reduction in levels of RNA-destabilizing RBPs participated in elevation of stability of HIF-1α mRNA by LMP1. (A) HONE-1 cells were treated with RNA polymerase II inhibitor (DRB) in the presence or absence of LMP1 expression plasmid transfection. RNA was then extracted at the indicated time and subjected to RT-PCR analysis for HIF-1α transcript as described in *Materials and Methods*. (B) A surrogate luciferase reporter of the HIF-1α mRNA 3′-UTR alone or plus LMP1 expression plasmid was transfected into HONE-1 cells. The luciferase activity of each sample was measured and the relative luciferase activity was calculated as described in *Materials and Methods*, and is shown as the mean ± SD of three independent experiments. Asterisks indicate the significance at $^{**}P < 0.01$. Immunoblot analysis was utilized to determine the expression of LMP1. (C) NPC-TW01 cells were transfected with LMP1 expression plasmid. Cell lysate was extracted and then subjected to Western blot analysis for LMP1, HIF-1α, TTP, PUM2, YBX1, QKI, HuR and HuB. The protein expression fold change was calculated as described in *Materials and Methods*, and is shown as the mean ± SD of three independent experiments. Asterisks indicate the significance at $^{*}P < 0.05$ or $^{**}P < 0.01$. (D) *Upper:* in the presence of luciferase reporter of HIF-1α mRNA 3′-UTR, HONE-1 cells were transfected with LMP1 expression plasmid alone or plus either PUM2 (*left*) or TTP (*right*) expression plasmid. The relative luciferase activity was calculated as shown in panel B. Asterisks indicate the significance at $^{*}P < 0.05$ or $^{**}P < 0.01$. Expressions of LMP1, PUM2 and TTP were examined using immunoblot analysis. pcDNA3 transfection was used as a vector control. *Lower:* cells were treated with DRB in the presence or absence of PUM2 or TTP expression plasmid transfection. Expression of HIF-1α mRNA was determined as described in panel A. All RT-PCR or Western blot analysis data shown are representative of three independent experiments.

under LMP1 induction (Supplementary Fig. S2B). These results thus suggested a stabilizing effect of LMP1 on HIF-1α mRNA activity.

LMP1 down-regulates expressions and binding affinities of RNA-destabilizing RBPs

Much evidence has shown that interaction between RNA binding proteins (RBPs) and conserved *cis*-acting AU-rich elements (AREs) of the HIF-1α mRNA 3′-UTR precisely influences post-transcription and translation of the HIF-1α gene [20–22,31–33]. Accordingly, to delineate features of HIF-1α mRNA stabilization by LMP1, using computer analysis (RNA-Binding Protein DataBase (RBPDB), http://rbpdb.ccbr.utoronto.ca) [34] and gene function survey (http://www.genecards.org), RBPs acting on mRNA decay, such as TTP (binding to ARE-containing mRNAs and promoting degradation), YBX1 (stabilizing cytoplasmic mRNAs), QKI (promoting stability of mRNAs), HuB and HuR (stabilizing ARE-containing mRNAs), as well as PUM2 (post-transcriptional repression and promoting mRNA degradation), interactions with the HIF-1α mRNA 3′-UTR were predicted (Supplementary Fig. S3A) and selected to investigate the role of LMP1 stabilization of HIF-1α mRNA. In contrast to the unchanged expressions of YBX1, QKI, HuB and HuR, the amounts of TTP and PUM2 were explicitly reduced due to the presence of LMP1 along with increased luciferase activity of the HIF-1α mRNA 3′-UTR reporter (Fig. 2C and D). However, in spite of the presence of LMP1, the reporter activity was disturbed by restoration of TTP and PUM2 (Fig. 2D). Consistently, elevated expression of PUM2 or TTP also obviously facilitated degradation of HIF-1α mRNA (Fig. 2D).

Via delivery of wild-type or various CTAR-deletion mutants of LMP1 into NPC cells, we identified that the expressions of TTP or PUM2 were negatively regulated by LMP1 through CTAR1 or CTAR3 (Fig. 3A and B, Supplementary Fig. S3B and C). However, the amounts of TTP and PUM2 were conversely increased through interruption of the CTAR1- and CTAR2-recruitment pathways utilizing ERK1/2 inhibitor U0126 treatment and dominant negative mutant STAT3 introduction, respectively (Fig. 3C and D, Supplementary Fig. S3D–F) [1,5]. In addition to suppression of the expressions of TTP and PUM2, ribonucleoprotein immunoprecipitation (RNP-IP) analysis revealed that the intensity of PUM2 binding to the HIF-1α mRNA 3′-UTR in the LMP1 transfection set was lower than in the vector control, indicating that the ability of PUM2 to target HIF-1α mRNA was also diminished by LMP1 (Fig. 3E).

On the other hand, to characterize the responsive *cis*-elements of the HIF-α mRNA 3′-UTR, luciferase reporter with full-length or progressive deletion of the HIF-1α mRNA 3′UTR (F1–F8) was transfected into cells in the presence or absence of LMP1. As shown in Fig. 4A, in contrast to the basal activity in the reporter alone, the F4 construct with 291 base deletion still showed a luciferase activity elevated by LMP1; however, this activation was hampered due to further truncation to 325 base (F5 construct), indicating a dominant role of the fragment within b291 to b325 in response to LMP1. Computer analysis predicted that this region was potentially occupied by TTP and HuB (Supplementary Fig. S3A). As mentioned above, HuB serves as an RNA-stabilizing RBP; however, the expression level and association with the HIF-1α mRNA 3′-UTR were not facilitated by LMP1 (Figs. 2C and 4B). Of interest, compared with other deletion constructs, the basal luciferase activity was increased in F7, yet reduced in F8 (Fig. 4A), implying a negative and a positive effect of *cis*-elements within b553 to b704 and b704 to b1033 on the maintenance of the HIF-1α transcript, respectively. Taken together, these data indicated that under LMP1 manipulation, an increased HIF-1α mRNA level can be ascribed to improvement of the HIF-1α mRNA stability through down-regulation of RNA-destabilizing RBPs such as TTP and PUM2.

Up-regulation of HIF-1A promoter activity by LMP1 through NF-κB and a positive feedback loop of HIF-1α

In addition to increasing the stability of HIF-1α mRNA, we investigated whether regulation of *HIF-1A* transcription also contributed to LMP1-mediated HIF-1α mRNA augmentation. As shown in Fig. 5A, compared with the vector control, we found that exogenously-expressed LMP1 obviously promoted *HIF-1A* transcription in EBV-negative or -positive NPC cells using a luciferase reporter driven by the HIF-1α gene promoter, which contains several putative *cis*-acting elements including SP1, AP-1, NF-κB, and HRE binding sites [23]. Furthermore, via introduction of different CTAR deletion mutants of LMP1, *HIF-1A* promoter activity was unequivocally induced by LMP1 through CTAR1, regardless of the presence of CTAR2 and CTAR3 (Fig. 5B). CTAR1 and CTAR2 of LMP1 have been found to activate NF-κB via interaction with adaptor proteins, followed by recruitment of NF-κB-inducing kinase (NIK) and IκB kinases (IKKs), leading to activation of canonical and non-canonical NF-κB pathways [1,5]. Notably, sequence analysis and accumulating evidence have also revealed that NF-κB, such as the p50-p65 heterodimer, serves as a principle regulator to control *HIF-1A* transcription under hypoxic conditions [21,23,35–39]. These results suggested that NF-κB may participate in the induction of HIF-1α gene transcription by LMP1 through CTAR1 recruitment. In line with our assumption, LMP1 CTAR1-induced *HIF-1A* transcription was evidently repressed by constitutive expression of IκB or a dominant negative IκB mutant (Fig. 5C), indicating that the canonical NF-κB pathway could be involved in excitation of *HIF-1A* transcription by LMP1. To further discriminate the respective roles of NF-κB subunits p65 and p50, p65 and p50 expression plasmids were transfected into NPC cells in the presence of various CTAR deletion mutants of LMP1. Via analysis of luciferase reporter activity, as shown in Fig. 5D, *HIF-1A* promoter activity was apparently rescued by p50 but not p65, even with the loss of the CTAR1 motif of LMP1. Concurrently, the nuclear localization of p50 and its DNA binding ability on the NF-κB responsive element of the HIF-1α gene promoter also increased under LMP1 excitation (Figs. 1C and 5E).

From the results of previous studies [1,5] and the current results (Supplementary Fig. S3F), we inferred that NF-κB was a downstream target of ERK1/2 through triggering by LMP1 CTAR1. In contrast to the solvent control, we showed that LMP1-excited HIF-1α gene promoter activity was suppressed under ERK1/2 inhibitor U0126 treatment (Fig. 5F). Moreover, reduced activity of ERK1/2 not only hampered the NF-κB transcriptional activity, but diminished the HIF-1α mRNA level, despite the existence of wild-type or a functional CATR1 mutant of LMP1 (Fig. 5G). In conclusion, these data indicate a “pipeline” in the regulation of HIF-1α gene transcription by LMP1 through CTAR1-recruited ERK1/2 activating the NF-κB pathway.

Intriguingly, TRANSFAC database (http://www.gene-regulation.com/index2) prediction revealed a consensus core HRE (5′-RCGTG-3′) on the HIF-α gene promoter, suggesting an auto-regulation activity of HIF-1α [23,25,26,35]. Via chromatin immunoprecipitation (ChIP) analysis, HIF-1α actually showed a basal DNA binding activity on its own gene promoter, which was obviously facilitated by ectopic expression of LMP1 (Fig. 6A). By utilizing *HIF-1A* promoter-dependent luciferase reporter as a template and employing site-directed mutagenesis to introduce point mutations on the HRE (Fig. 6B), the luciferase activity was significantly abolished, despite expression of LMP1 (Fig. 6C). Importantly, in the presence of HIF-1α, the activity of the reporter driven by the wild-type *HIF-1A* promoter was apparently elevated, yet was impaired due to HRE mutations (Fig. 6D). Consequently, in line with our assumption, an auto-regulation loop by HIF-1α also plays a positive role in LMP1-induced *HIF-1A* transcription.

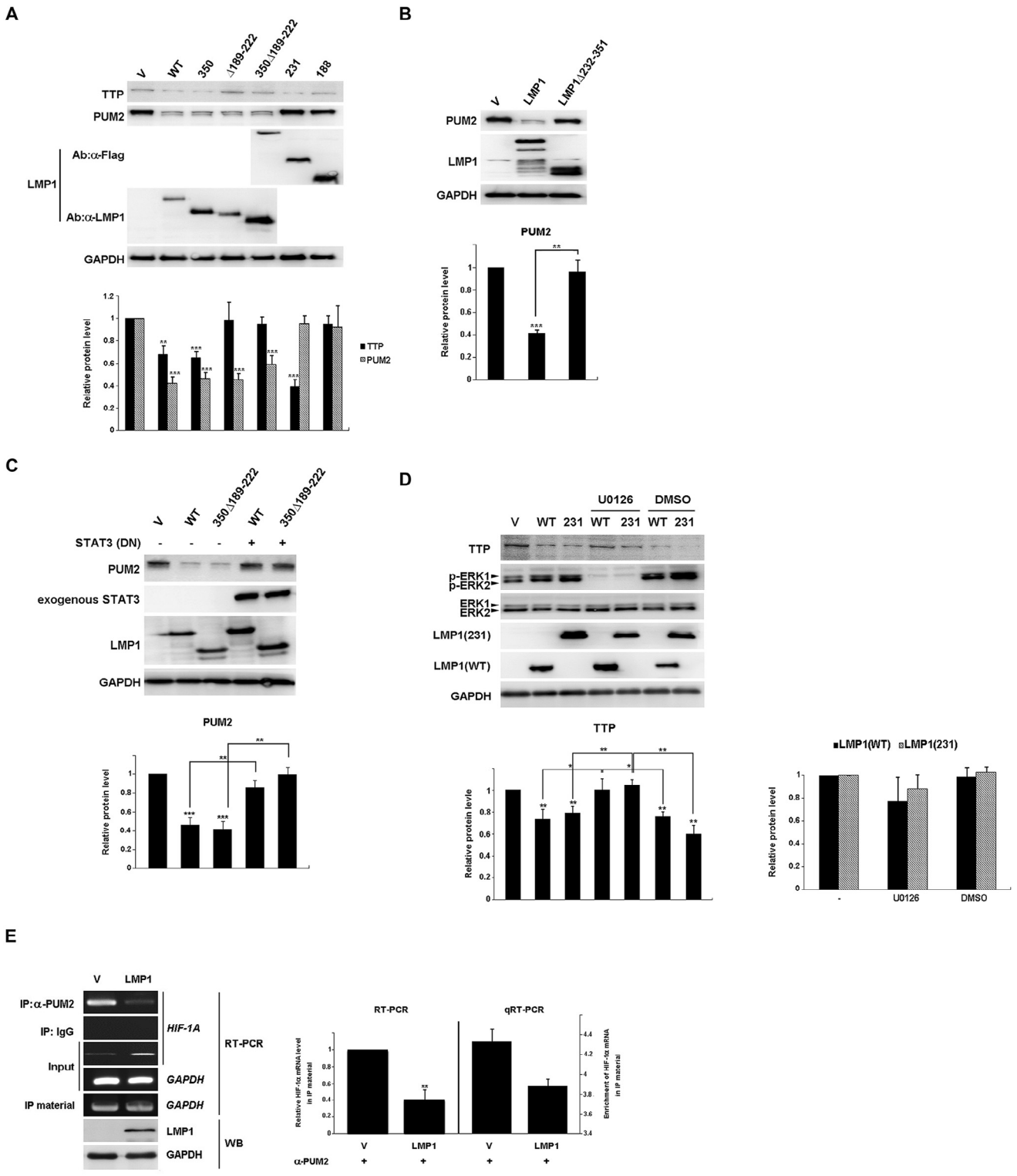

Fig. 3. Expression of TTP and PUM2 was down-regulated by LMP1 through the CTAR1/ERK1/2 and CATR3/STAT3 signaling pathways, respectively. (A) HONE-1 cells were transfected with wild-type LMP1 expression plasmid or a truncated mutant of LMP1 in CTAR2 (350), CTAR1 (Δ189-222), CTAR1/2 (350Δ189-222), CTAR2/3 (231) or the entire C-terminal region (188). Cell lysates were extracted and then subjected to Western blot analysis for TTP and PUM2, as well as wild-type or truncated LMP1. pIRESpuro2 transfection was used as a vector control. (B) Cell lysates from wild-type LMP1 or LMP1-deleted CTAR3 mutant (Δ232-351)-transfected cells were collected and subjected to Western blot analysis for PUM2, LMP1 or LMP1 mutant. Transfection with pSG5 was utilized as a vector control. (C) HONE-1 cells were transfected with wild-type LMP1 expression plasmid or LMP1 mutant with CTAR1/2 deletion in the presence or absence of dominant negative mutant STAT3 plasmid (DN). Subsequently, cell lysates were extracted and subjected to Western blot analysis for PUM2, STAT3, LMP1 and LMP1 mutant. (D) Wild-type LMP1 or LMP1-deleted CTAR2/3 mutant-transfected cells were treated with or without ERK1/2 inhibitor U0126. Cell lysates were collected, and expressions of TTP, phospho-ERK1/2, ERK1/2, LMP1 and truncated LMP1 were measured using immunoblot analysis. (E) NPC-TW01 cells were transfected with LMP1 expression plasmid. The association of PUM2 with the HIF-1α mRNA 3′-UTR was determined by RNP-IP analysis. The fold enrichment of HIF-1α mRNA in the IP material was calculated as described in *Materials and Methods*, and is shown as the mean ± SD of three independent experiments. Expression of LMP1 was analyzed using Western blot analysis. pcDNA3 transfection was used as a vector control. The protein expression fold change was calculated as described in *Materials and Methods*, and is shown as the mean ± SD of three independent experiments. Asterisks indicate the significance at $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$. All RT-PCR or Western blot analysis data shown are representative of three independent experiments.

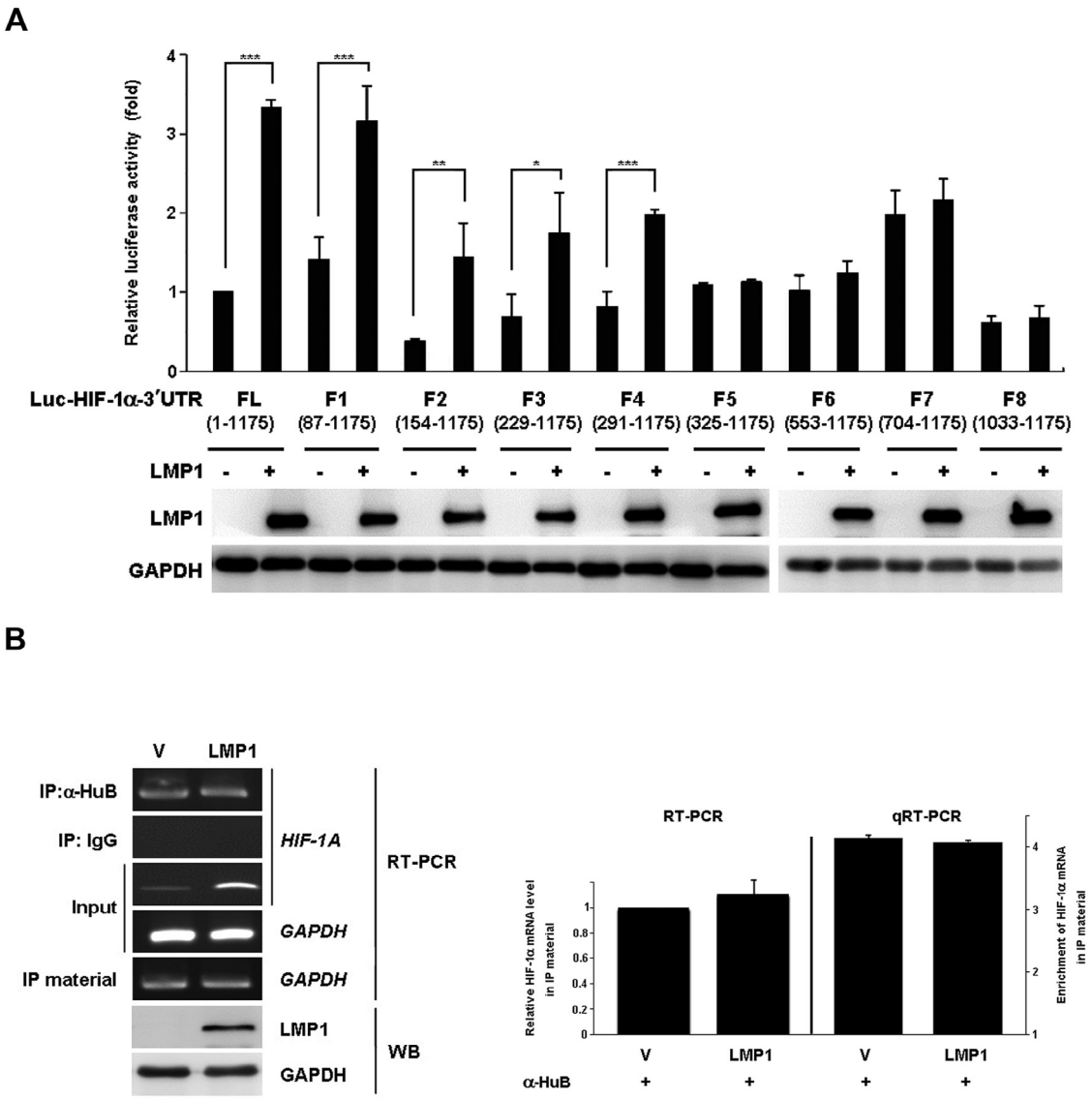

Fig. 4. Characterization of responsive *cis*-elements within HIF-1α mRNA 3′-UTR upon LMP1 induction. (A) Full-length or variously truncated HIF-1α mRNA 3′-UTR downstream luciferase gene reporters were transfected into NPC-TW01 cells in the presence or absence of LMP1 expression plasmid. The luciferase activity of each sample was measured and the relative luciferase activity was calculated as described in *Materials and Methods*, and is shown as the mean ± SD of three independent experiments. Asterisks indicate the significance at $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$. Expression of LMP1 was examined using Western blot analysis. (B) RNP-IP analysis was utilized to evaluate the association of HuB with the HIF-1α mRNA 3′-UTR as described in the figure legend of Fig. 3E. All RT-PCR or Western blot analysis data shown are representative of three independent experiments.

Discussion

During tumorigenesis, up-regulation of the stability of HIF-1α has been characterized as a rapid response force to counteract excessive oxygen and energy consumption; that is, a hypoxic microenvironment is a prerequisite for HIF-1α induction. However, this feature does not apply as a rule to all tumors. Consequently, it is worth clarifying the relationship between HIF-1α and the development of tumors under normoxic conditions. In the current study, we drew a cell type-specific and new picture of HIF-1α gene regulation by EBV oncoprotein LMP1 in NPC cells, especially in relation to transcription and post-transcription processes (Fig. 6E). In addition to non-redundant transcription activity and different hypoxic-responsive patterns in HIF-1α and HIF-2α [40], with regard to gene expression, our results also indicated a distinct regulatory effect of LMP1 on the HIF-1α gene, but not the HIF-2α gene, in NPC cells, which could be due to the absence of significant similarity between HIF-1α and HIF-2α mRNAs in the 5′-regulatory region and 3′-UTR (data not shown). Intriguingly, via an EBV-positive NPC cell model in which the EBV infection status and gene expression profile resembled previous observations in NPC biopsies [29,41], the dominant role of LMP1 in modulation of the HIF-1α gene expression was further corroborated.

Dysregulation of ARE-containing mRNA turnover and association with RBPs are involved in many human diseases; for example, a deficient amount of TTP and a reverse of elevation of expression of ARE-encoding mRNAs have been demonstrated in a variety of human malignancies, which are also correlated with poor survival of tumor patients or promotion of multiple tumor behaviors [42–44]. Recent study also suggested a suppressive role of PUM2 in maintaining genomic integrity [45]. In agreement with these findings, our data showed a de-repression mechanism of TTP- and PUM2-mediated destabilization of HIF-1α mRNA by LMP1 in NPC cells. Mechanistically, we identified that reductions of TTP and PUM2 were

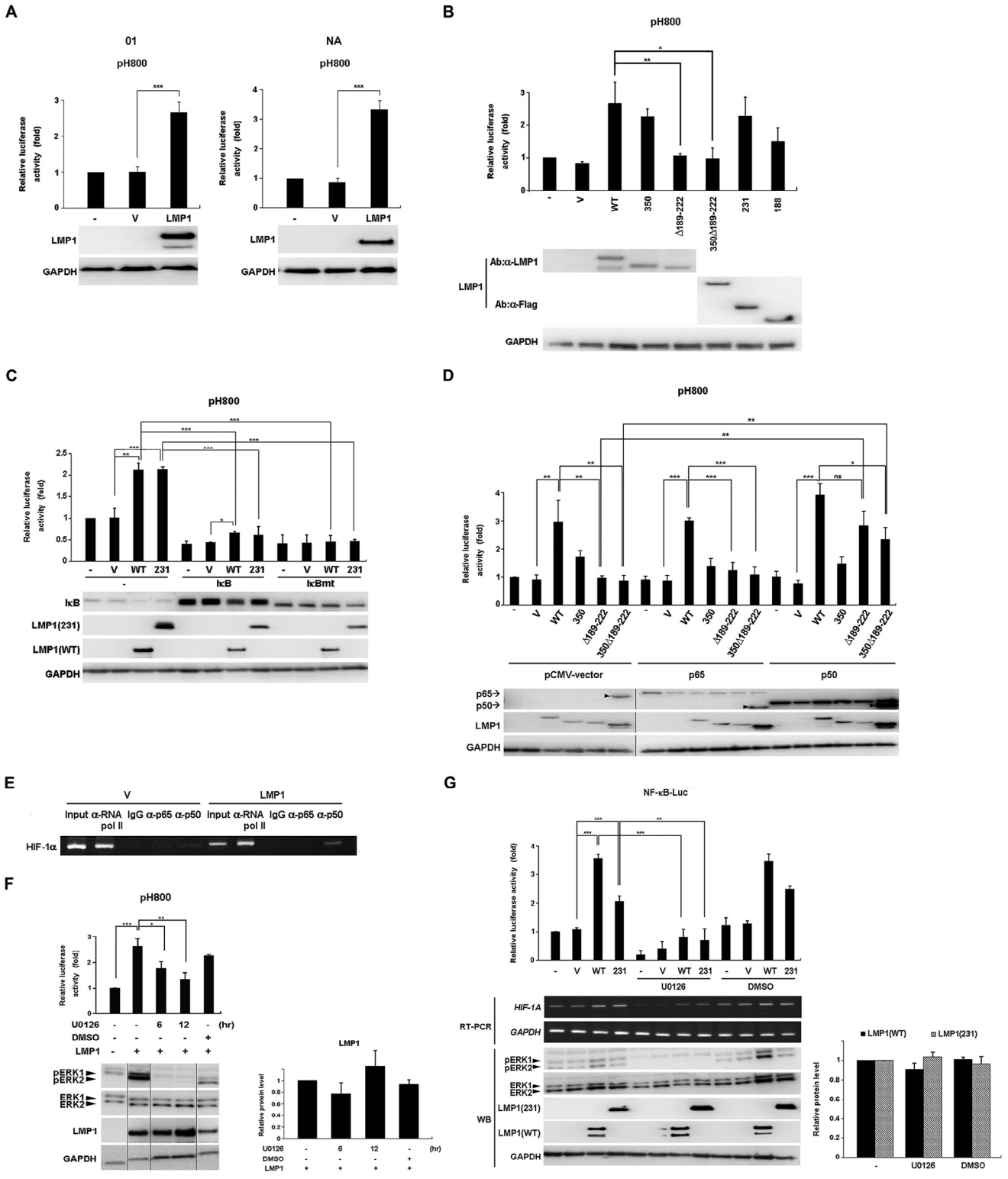

Fig. 5. *HIF-1A* promoter activity was enhanced by LMP1 through the CTAR1/ERK1/2/NF-κB pathway. A luciferase reporter driven by *HIF-1A* promoter plus (A) wild-type LMP1 expression plasmid or (B) various CTAR deletion mutants of LMP1 in the absence or (C) presence of IκB, dominant negative IκB mutant (IκBmt), (D) NF-κB p50 or NF-κB p65 expression plasmid were co-transfected into NPC-TW01 cells (A, *left*, B–D) or EBV-positive NA cells (A, *right*). The relative luciferase activity was calculated in *Materials and Methods* and is shown as the mean ± SD of three independent experiments. Asterisks indicate the significance at $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$. Expressions of LMP1, LMP1 mutant, IκB, and exogenous p50 and p65 were determined by Western blot analyses. The arrow-head indicates the expression of the truncated CTAR1/2 (350Δ189-222) mutant of LMP1 using anti-Flag antibody (D). (E) The ability of the NF-κB subunit to target the HIF-1α gene promoter was measured using ChIP analysis as described in *Materials and Methods*. (F) *HIF-1A* promoter- or (G) NF-κB-dependent luciferase reporter plasmid was transfected into NPC-TW01 cells in the absence or presence of wild-type LMP1 expression plasmid or truncated CTAR2/3 (231) mutant of LMP1 under ERK1/2 inhibitor U0126 treatment. RNA and cell lysates were extracted and then subjected to RT-PCR for HIF-1α transcript and Western blot analysis for LMP1, LMP1 mutant, ERK1/2, and phospho-ERK1/2. The relative luciferase activity was calculated as described in *Materials and Methods* and is shown as the mean ± SD of three independent experiments. Asterisks indicate the significance at $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$. The protein expression fold change was calculated as described in *Materials and Methods*, and is shown as the mean ± SD of three independent experiments. All conventional PCR, RT-PCR or Western blot analysis data shown are representative of three independent experiments.

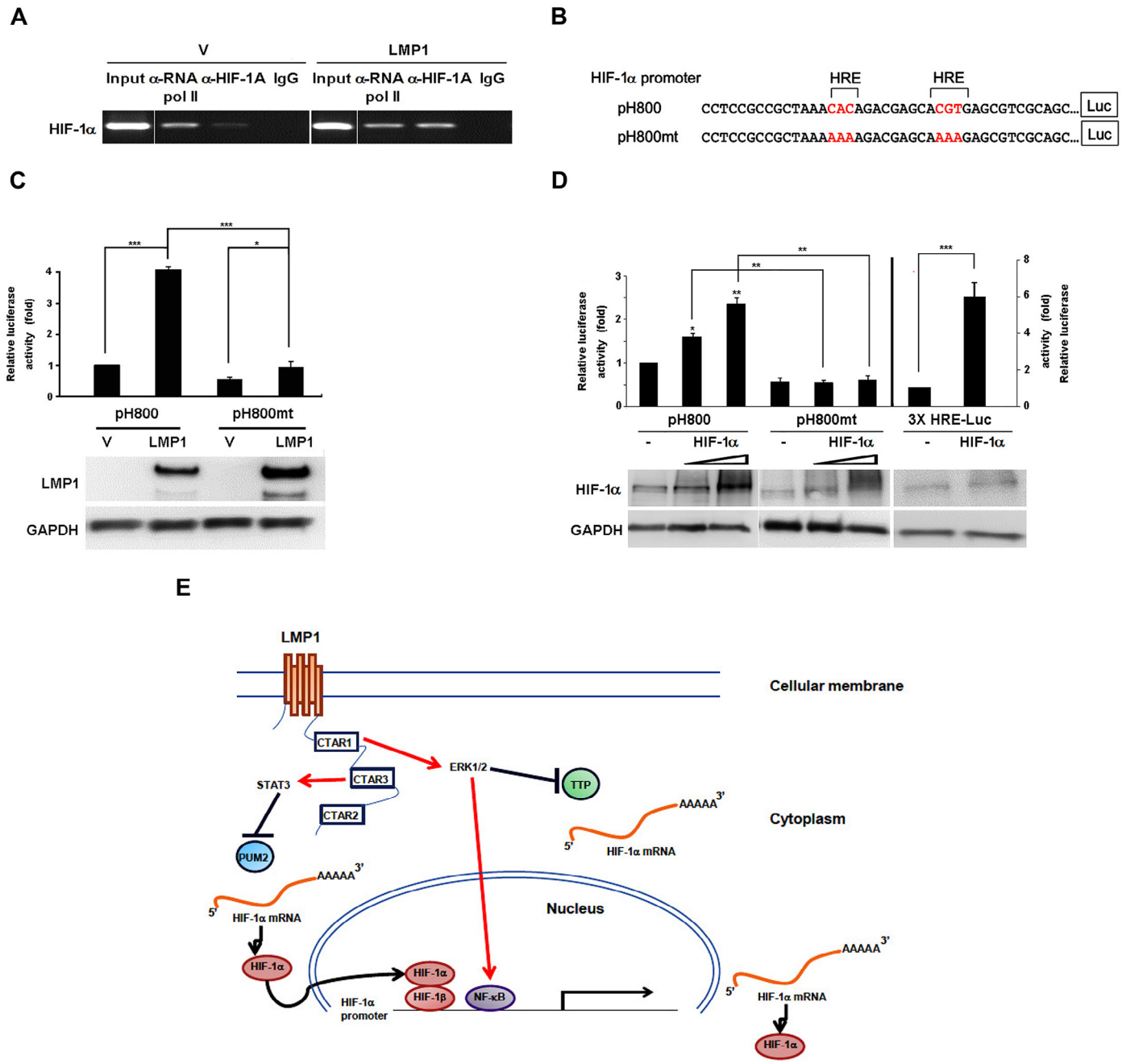

Fig. 6. A positive feedback loop of HIF-1α was involved in LMP1-induced *HIF-1A* transcription. (A) NPC-TW01 cells were transfected with either LMP1 expression plasmid or pcDNA3 vector. The ability of HIF-1α targeting of the HRE within its own gene promoter was examined by ChIP analysis as described in *Materials and Methods*. (B) Schematic representation of luciferase reporter driven by wild-type (pH800) or point mutations on the HRE (pH800mt) within the HIF-1α gene promoter. pH800 or pH800mt reporter was transfected into NPC-TW01 cells in the presence of (C) LMP1 or (D) HIF-1α expression plasmid. In addition, luciferase reporter containing three copies of the EPO HRE (3XHRE-Luc) plus HIF-1α expression plasmid transfection in NPC-TW01 cells was included to demonstrate HIF-1α transcriptional activity (D). The relative luciferase activity was calculated as described in *Materials and Methods* and is shown as the mean ± SD of three independent experiments. Asterisks indicate the significance at $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$. Expressions of LMP1 and HIF-1α were detected using immunoblot analysis. pcDNA3 transfection was used as a vector control. All conventional PCR or Western blot analysis data shown are representative of three independent experiments. (E) LMP1 activated transcription and post-transcription processes of the HIF-1α gene in NPC cells. In the post-transcription step, the stability of HIF-1α mRNA was enhanced via down-regulation of RNA-destabilizing TTP and PUM2 by LMP1 through CTAR1- and CTAR3-engaged ERK1/2, as well as the STAT3 signaling pathway. Concomitantly, the LMP1 CTAR1-recruited ERK1/2 signal was also involved in the facilitation of the *HIF-1A* promoter by LMP1 through activation of the canonical NF-κB pathway. Notably, elevated HIF-α further displayed a positive feedback loop of transcription regulation on its own gene promoter under LMP1 excitation.

mediated by LMP1 through CTAR1 and CTAR3 interaction with ERK1/2, as well as the STAT3 signaling pathway, respectively. Consistently, previous study has also shown post-transcriptional regulation of ARE-containing mRNA by ERK1/2 signaling and TTP, as well as PUM2 [46]. However, the linkages of TTP with ERK1/2 or PUM2 with the STAT3 pathway are still unclear. Via serial deletion within the HIF-1α mRNA 3′-UTR, we identified a fragment from b291 to b325 in response to LMP1 induction. Luciferase reporter analysis additionally indicated negative and positive effects of *cis*-elements from b553 to b704 and b704 to b1033 in terms of HIF-1α mRNA stability, respectively. Our results thus raised some questions: first, is there any direct or indirect effect of the ERK1/2 or STAT3 pathway on

down-regulation of TTP or PUM2? Second, what kinds of adaptor protein are involved in activation of ERK1/2 or STAT3 signaling by CTAR1 or CTAR3 of LMP1? Third, do other RBPs also contribute to HIF-1α mRNA decay? These are issues that should be further addressed.

Evidence shows that multiple kinases, such as ERK1/2, serve as upstream regulators of post-translational modification or activity of HIF-1α [19,47]. On the contrary, under LMP1 excitation, we demonstrated remarkable activity of ERK1/2 signaling in HIF-1α gene transcription through preponderant action of NF-κB p50. In accordance with our results, NF-κB p50 homodimer, p50/p50/Bcl-3 and p50/RelB complexes have also been identified in EBV-associated NPC cells [48], indicating a pivotal role of p50 in regulating transcription of the HIF-1α gene. Considering the relationships between NPC and EBV latent proteins, although an augmented HIF-1α mRNA level has been reported in EBV-encoded nuclear antigen (EBNA)-1-expressing NPC cells by increasing AP-1 activity under low oxygen tension [49], our data showed the absence of incitation of *HIF-1A* transcription by EBNA-1 in EBV-positive NPC cells in an aerobic environment.

Most oncogenic viruses, including human papillomavirus (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV), Kaposi's sarcoma-associated herpesvirus (KSHV) and human T-cell lymphotropic virus (HTLV-1), are capable of stabilizing or enhancing the transcriptional activity of HIF-1α, which thereby integrates a multitude of biological pathways to confer viral carcinogenesis [27,50]. The current study was the first to indicate novel modulation of HIF-1α gene expression in transcription and post-transcription by LMP1. Moreover, LMP1-induced HIF-1α revealed auto-regulation of its own gene expression, leading to amplification of LMP1 action. Furthermore, our data also provided evidence to corroborate up-stream regulation prior to *HIF*-1α mRNA translation by a tumor-associated virus under normoxic conditions.

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Conflict of interest

The authors declare no conflict of interest.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.08.021.

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