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Latent membrane protein 1 of Epstein–Barr virus sensitizes cancer cells to cisplatin by enhancing NF-κB p50 homodimer formation and downregulating NAPA expression

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ABSTRACT

Expression of the oncogenic latent membrane protein 1 (LMP1) of Epstein–Barr virus is involved in the pathogenesis of nasopharyngeal carcinoma (NPC) and lymphoma. In previous studies, we found that expression of LMP1 was sufficient to transform BALB/c-3T3 cells. In contrast, other studies have shown that LMP1 induces apoptosis in a NF-κB-dependent manner and also inhibits the growth of tumors in mice, thereby indicating that LMP1 may produce various biological effects depending on the biological and cellular context. Still, the mechanism underlying the pro-apoptotic activity of LMP1 remains unclear. In the present study, we found that LMP1 inhibits the expression of NAPA, an endoplasmic reticulum SNARE protein that possesses anti-apoptotic properties against the DNA-damaging drug cisplatin. Accordingly, LMP1-transformed BALB/c-3T3 cells were sensitized to cisplatin-induced apoptosis, whereas no sensitization effect was noted following treatment with the mitotic spindle-damaging drugs vincristine and taxol. Knockdown of LMP1 with antisense oligonucleotides restored NAPA protein level and rendered the cells resistant to cisplatin. Similarly, overexpression of NAPA reduced the effect of LMP1 and induced resistance to cisplatin. LMP1 was shown to upregulate the NF-κB subunit p50, leading to formation of p50 homodimers on the *NAPA* promoter. These findings suggest that the viral protein LMP1 may sensitize cancer cells to cisplatin chemotherapy by downregulating NAPA and by enhancing the formation of p50 homodimers which in turn inhibit the expression of NF-κB regulated anti-apoptotic genes. These findings provide an explanatory mechanism for the pro-apoptotic activity of LMP1 as well as new therapeutic targets to control tumor growth.

Abbreviations: AP-1, activator protein-1; AS, antisense; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus; CTAR, C-terminal-activating region; DFF, DNA fragmentation factor; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EBV, Epstein–Barr virus; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERK, extracellular response kinase; FBS, fetal bovine serum; FL, full length; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione S-transferase; HA, haemagglutinin; HBSS, Hank's balanced salt solution; HBx, hepatitis B virus protein X; HDAC, histone deacetylase; HURP, hepatoma upregulated protein; ICAD, inhibitor of caspase activated DNase; IFN-α, interferon-alpha; IP, immunoprecipitation; JAK, Janus kinase; JNK, c-Jun-N-terminal kinase; LMP1, latent membrane protein 1; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAPA, NSF attachment protein α; NC, negative control; Neo, neomycin; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NPC, nasopharyngeal carcinoma; NSF, N-ethylmaleimide-sensitive factor; ORF, open-reading frame; PARP, poly-ADP ribose polymerase; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3 kinase; PVDF, polyvinylidene fluoride; P6, pyridone 6; RF, resistance factor; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SF, sensitization factor; shRNA, short-hairpin RNA; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; STAT, signal transducers and activators of transcription; TNF-α, tumor necrosis factor-alpha.

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1. Introduction

Epstein–Barr virus (EBV) is typically found in a latent state in undifferentiated nasopharyngeal carcinoma (NPC) cells. EBV encodes a number of proteins with transforming activity, including the latent membrane protein 1 (LMP1). The oncogenic LMP1 protein was shown to transform primary B lymphocytes [1] and rodent fibroblasts [2], and to inhibit terminal differentiation of human epithelial cells [3]. The importance of LMP1 in the development of NPC can be seen from the observation that thirty-to-sixty percents of EBV-associated NPCs are positive for LMP1 [4,5]. EBV is also associated with human lymphoid tumors, such as Burkitt's lymphoma, indicating that LMP1 may also be involved in the pathogenesis of this disease. Previous studies have shown that LMP1 confers resistance to apoptosis in B cells by regulating the transcription of the anti-apoptotic protein Bcl-2 [6]. In addition, LMP1 was shown to upregulate the expression of several genes that have opposing effects in regulating cell proliferation and apoptosis in B cells [7]. LMP1 also affects apoptosis in epithelial cells but the final cellular response appears to vary according to the stimulus and the cellular context [8–11]. For example, expression of LMP1 reduced apoptosis induced by tumor necrosis factor-alpha (TNF-α) but enhanced apoptosis triggered by other forms of cellular stress, such as treatment with the topoisomerase II inhibitor etoposide. In addition, the sensitization effect of LMP1 to cisplatin in epithelial cells was shown to be mediated by the JNK and NF-κB signaling pathways [12]. Besides, LMP1 was shown to repress DNA repair and enhance sensitivity to DNA-damaging agents in several NPC cell lines [13]. However, whether other mediators are needed for LMP1 activity and whether LMP1 has additional biological effects remain unclear.

LMP1 is a membrane protein composed of a short extracellular N-terminal domain, six transmembrane domains, and a long cytoplasmic C-terminus. Most LMP1-mediated signals implicated in gene regulation are regulated by three C-terminal-activating region (CTAR) domains termed CTAR1, CTAR2, and CTAR3 [14,15]. The cellular effects of LMP1 are mostly attributed to CTAR1 and CTAR2 [15]. CTAR1 has been shown to contribute to 30% of NF-κB activation and to the cell proliferation activity of LMP1 in some cell types [16]. CTAR1 also activates the phosphatidylinositol 3 kinase (PI3K) [17] and extracellular response kinase (ERK) 1/2 pathways [18]. CTAR1 is also involved in ERK1/2 activation which is necessary for LMP1-induced transformation of Rat-1 fibroblasts [19]. In comparison, CTAR2 contributes to 70% of NF-κB activation and to c-Jun-N-terminal kinase (JNK) activation [20], while the interaction between CTAR3 and Janus kinase 3 (JAK3) is required for activation of signal transducers and activators of transcription (STAT) proteins [14]. The regulation of apoptosis by LMP1, which occurs upstream of caspase-dependent mitochondrial perturbation, was also attributed to CTAR2 [11].

Activation of NF-κB represents a hallmark of the biological activity of LMP1 [21]. NF-κB is a critical regulator of many cellular processes, including cell survival and inflammation. NF-κB functions as a hetero or homodimer that can be formed from five NF-κB subunits: RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100). Among the various combinations of NF-κB subunits involved in gene regulation, the most studied combination is the p50/p65 heterodimer, which is activated by the classical pathway and usually promotes gene expression. The p50/p50 homodimer predominantly binds DNA in unstimulated or resting cells [22]. While the transcriptionally inactive NF-κB may comprise homodimers of p65 or p50 that are complexed with HDAC-1 in unstimulated cells, only the p50/p50 homodimer was shown to bind DNA and repress NF-κB-dependent gene transcription [23]. Following stimulation of cells with either LPS or TNF-α, NF-κB dimers containing phosphorylated p65 in complex with CBP/p300 are recruited to the DNA-binding site and displace the p50/p50/HDAC-1 complex, leading to initiation of NF-κB-dependent gene transcription (reviewed in Ref. [24]).

Cisplatin is a chemotherapeutic agent that targets chromosomal DNA and induces apoptosis in actively replicating cells [25,26]. Cisplatin is widely used for the treatment of various human cancers including NPC. However, the development of cisplatin resistance is a serious problem that limits the efficacy of this cancer treatment. The mechanisms responsible for chemoresistance have been investigated intensively in the past [27–29]. These mechanisms include inadequate drug exposure and changes in the micro-environment of cancer cells. While several cellular targets of cisplatin have been identified in the past, less is known about the molecules implicated in cell death and cisplatin resistance. Using bioinformatics and functional analyses, we have recently identified a panel of genes whose upregulation is associated with cellular resistance to DNA-damaging stress such as cisplatin treatment [30]. Among the genes identified, NAPA (also known as α-SNAP), an endoplasmic reticulum (ER) SNARE component, is an important regulator of cisplatin sensitivity that acts in both p53-dependent and p53-independent manners [31]. Thus, cisplatin not only targets nuclear DNA but also affects nucleus-independent cellular compartments involved in apoptotic signaling [32]. Consistent with the possibility that some ER proteins regulate apoptosis, other investigators have demonstrated that forced expression of NAPA delayed staurosporine-induced apoptosis in HeLa cells [33]. In addition, we found that the molecules and apoptotic pathways involved in regulating the sensitivity of cancer cells to cisplatin are affected by other biological stimuli, such as protein X of hepatitis B virus (HBx) [34,35]. Earlier, we transformed BALB/c-3T3 fibroblasts with LMP1 and successfully produced xenograph transplant tumors by injecting the LMP1-transformed cells in both immunocompetent BALB/c mice and T-cell-deficient nude mice [36]. In the present study, we investigated whether LMP1 and NAPA are involved in regulating the sensitivity of cancer cells to cisplatin. Notably, LMP1 was shown to sensitize cancer cells to cisplatin chemotherapy by suppressing the expression of NAPA. LMP1 was also shown to induce the accumulation of NF-κB p50 homodimers on the *NAPA* promoter. Our results provide a plausible explanation for the pro-apoptotic role of LMP1 in sensitizing EBV-positive tumors to cisplatin.

2. Materials and methods

2.1. Cell lines and reagents

Primary 3T3-LMP1 cells were derived from N-LMP1 transformed tumors in BALB/c mice (see below) and maintained in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml; Gibco), and streptomycin (100 μg/ml; Gibco) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The tumor was initially established from BALB/c-3T3 cells that were transformed with NPC-derived EBV-encoded LMP1 (referred to as N-LMP1) [36]. 3T3-Neomycin (Neo) cells containing the vector alone were established as described previously [37]. LMP1 and Neo expression in 3T3 cells was driven by the cytomegalovirus (CMV) promoter. The chemotherapeutic agents cisplatin, vincristine and taxol were purchased from Bristol-Myers Squibb (New York, NY, USA). The kinase inhibitors used in this study included Bay11-7082 (Sigma, St. Louis, MO, USA), SB203580, JNKII, PD98059 (Calbiochem, San Diego, CA, USA), and pyridone 6 (P6, also known as the JAK inhibitor I; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Commercial reagents also included interferon-α (IFN-α; Schering-Plough Corporation, Kenilworth, NJ, USA) and tumor necrosis factor-α (TNF-α; Sigma). The other chemicals were purchased

from Sigma. All reagents were prepared according to the instructions provided by the supplier.

2.2. Semi-quantitative real-time reverse transcription-PCR

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR, or in short qPCR) was performed on total RNA extracted with the Trizol reagent (Invitrogen, Carlsbad, CA, USA) using 200 nM of primers as before [38]. Primers for NAPA (GenBank sequence number: NM_025898.3) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; NM_008084.2) were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA). The designed primers were as follows: NAPA, forward, 5′-GCTGGAGCAGTACCAGAAGG-3′; reverse, 5′-GTCAATGCAGAAGTGGCAGA-3′; and GAPDH, forward, 5′-CCCACTAACATCAAATGGGG-3′; reverse, 5′-CCTTCCACAATGCCAAAGTT-3′. All samples and controls were prepared in triplicate on the same plate. Relative quantification was calculated using the ΔΔCt method with normalization against GAPDH. Namely, the ΔCt for each candidate was calculated as ΔCt (candidate) = [Ct (candidate) − Ct (GAPDH)]. The relative abundance of the candidate gene X was calculated as $2^{\Delta Ct(X)\_\Delta Ct(GAPDH)}$.

2.3. Plasmids, transfection, cell extracts, and immunoblot analysis

pcDNA3-NAPA, the expression plasmid containing the NAPA open-reading frame (ORF; NM_025898.3), was constructed as before [31]. In some experiments, the NF-κB subunit p50 (NM_008689.2) in fusion form was also expressed in cells. To construct fusion p50, we prepared the DNA sequence that covers the p50 full-length ORF with Primer Express 3.0, and amplified the sequence by RT-PCR using the primers 5′-GAATTCGGTCGTGAGCTGCGCATCTTCACC-3′ (forward) and 5′-CTCGAGGGGCTTTGGTTTACACAGTGTGGG-3′ (reverse; the underlined sequences correspond to the restriction sites EcoRI and XhoI, respectively) as well as an existing GST-p50 template (a generous gift from Dr. Ming-Zong Lai, Academia Sinica, Taipei, Taiwan). The isolated p50 DNA sequence was cloned into the hemagglutinin (HA)-tagged vector pcDNA3-HA by using the restriction enzymes EcoRI and XhoI to obtain pcDNA3-HA-p50. Cells were transfected with plasmid DNA to express either NAPA or the p50 fusion proteins as described [38]. Total protein extracts for immunoblotting were prepared as before [39]. Fifty μg of protein extract were separated by 10% SDS-PAGE, followed by transfer to PVDF membranes, and incubation with primary antibodies. The antibodies included PARP (H-250; Santa Cruz Biotechnology), ICAD/DFF (K-17), GAPDH (FL-335), JNK1/2 (C-17), ERK1/2 (C-16), p38 (H-147), IκB-α (C-21), NF-κB p65 (C-20), NF-κB p50 (C-19), c-Rel (C), p-c-Jun (KM-1), STAT1 (B-9), p-STAT1 (Tyr-701), HA (F-7), GST (B-14), GFP(B-2), α-tubulin (H-300), Lamin-A/C (636), cleaved caspase-3 (D175; 5AIE; Cell Signaling Technology, Danvers, MA, USA), p-JNK1/2 (Thr183/Tyr185; G9), p-ERK1/2 (p44/42; Thr202/Tyr204; E10), p-p38 (Thr180/Tyr182; 28B10), NAPA (4E4; Abcam, Cambridge, MA, USA), and LMP1 (Dako, Carpinteria, CA, USA). The membranes were incubated with the secondary antibodies goat anti-mouse or goat anti-rabbit-horseradish peroxidase (Amersham, Buckinghamshire, UK). The resulting signal was visualized by enhanced chemiluminescence according to specifications from the supplier (Pierce, Rockford, IL, USA). Intensity of the protein bands was determined by scanning X-ray films with a densitometer (GS 300; Hoefer, Holliston, MA, USA).

2.4. Knockdown of NAPA and LMP1 gene expression

To knockdown NAPA, we used the pLKO.1 plasmid to express short-hairpin RNA (shRNA; National RNAi Core Facility, Academia Sinica). A recombinant plasmid that expressed luciferase-shRNA (shLuc; TRCN0000072244) was used as a negative control. Five recombinant plasmid clones that expressed shNAPA were tested for knockdown efficiency in HEK293 cells. The shNAPA-expressing plasmid (TRCN0000111543) that was the most effective in inhibiting NAPA expression was used in this study. Transient transfection of shRNA plasmids was performed by treating cells (1.5×10^4 cells/well in six-well plates) with 2 μg/well of plasmid and 3 μl/well of Lipofectamine (Invitrogen) according to instructions from the manufacturer. The stable cell clones that were inefficient for plasmid transfection were infected with recombinant lentivirus that expressed shNAPA. The recombinant lentivirus carried a puromycin-resistance gene for selection (National RNAi Core Facility).

To knockdown LMP1, we synthesized fifteen-mer unmodified oligodeoxynucleotides based on established protocols [40]. The LMP1 antisense sequence was derived from published results [41]. Two oligodeoxynucleotides were used in the present study: LMP1 antisense, 5′-AAGGTCGTGTTCCAT-3′ and scrambled LMP1 antisense, 5′-ACGTCATGCTAGTGT-3′. Oligodeoxynucleotides at a concentration of 50 μM were transfected in the cells (2×10^5 cells/ml) with 50 μg/ml of Lipofectamine in serum-free medium. After 24 h of culture, the serum-free medium was replaced with DMEM containing 10% FBS as before [40].

2.5. Overexpression of recombinant plasmid DNA

3T3-LMP1 cells were transfected with either the pcDNA3-NAPA or pcDNA3 vector, and cell extracts were prepared as described [31]. In some experiments, the cells were co-transfected with GST-p50 and HA-p50 expression plasmids. Unless indicated otherwise, the transfected cells were incubated for 48 h, and cell extracts were prepared at 4 °C.

2.6. Co-immunoprecipitation (co-IP) assay

For co-IP assay, cells were grown to near confluence in 15-cm plates. The cells were transfected with expression plasmids, and were treated or not with cisplatin, prior to harvesting in lysis buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, or SDS, and protease inhibitor cocktail; BD Biosciences, San Jose, CA, USA). IP experiments were performed with antibodies against p50, GST, or HA. Immunoprecipitated complexes were separated by 12% SDS-PAGE, and subjected to Western blot.

2.7. Chromatin immunoprecipitation (ChIP) assays

Formaldehyde cross-linking and ChIP assays of tissue culture cells were performed as described [42] with modifications as below. Briefly, ChIP was performed using a commercial kit (Upstate Biotechnology, Lake Placid, NY, USA). Cells were cross-linked with 1% formaldehyde that was added directly to cell culture medium for 10 min at room temperature. Cross-linking was stopped by adding glycine to a final concentration of 125 mM for 5 min. The cells were washed three times with ice-cold 1× PBS containing the protease inhibitor cocktail, and were collected by centrifugation ($700 \times g$ for 4 min). Cell pellets were resuspended in cell lysis buffer, and incubated for 10 min on ice. Nuclei were pelleted by centrifugation using a microfuge at $2000 \times g$ for 5 min), and resuspended in the nuclear lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS, and the protease inhibitor cocktail) for 10 min on ice. Sonication was performed on power setting five using a Vibra Cell Sonifier (Sonics & Materials, Danbury, CT, USA) with 20-s bursts for 3 min, followed by 1-min incubation on ice. This protocol resulted in DNA fragment sizes of 0.3–1.5 kb. Chromatin was

sonicated on ice to obtain an average length of 600 bp. Cellular debris were cleared by centrifugation at 15,000 × *g* for 10 min at 4 °C. Supernatants were diluted five-fold in the ChIP dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl, pH 8.1, and protease and phosphatase inhibitor cocktails). To reduce nonspecific background, we pre-cleared the samples with 80 μl of salmon sperm DNA and protein-A agarose beads for 30 min at 4 °C with agitation. Twenty percent of total supernatant was used as a total-input control. The eluted immunoprecipitates were processed for the cross-linking reversal step. Equal amounts of chromatin from each sample were incubated with 5 μg of antibodies at 4 °C overnight with mixing by rotation. Immunocomplexes were collected with 60 μl of salmon sperm DNA and protein-A/G agarose beads for 1 h at 4 °C with rotation. Beads were washed successively with low-salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl, pH 8.1), high-salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris–HCl, pH 8.1), and LiCl washing buffer (0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.1) for 10 min each and twice in 1× TE buffer. Complexes were eluted twice with 250 μl of freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$). To reverse formaldehyde crosslinks, we added 1 μl of RNase (10 mg/ml) and NaCl to a final concentration of 0.3 M to the eluates, followed by incubation in a water bath at 65 °C for 4 h. Volumes of 2.5-fold of 100% ethanol were added to precipitate chromatin DNA overnight at −20 °C. Two microliters of 0.5 M EDTA, 4 μl of 1 M Tris pH 6.5, and 1 μl of 20 mg/ml Proteinase K were added to the sample, prior to incubation for 2 h at 45 °C. DNA was recovered using QiaQuick spin columns (Qiagen, Valencia, CA, USA), and eluted with 80 μl of 10 mM Tris (pH 8.0). Two microliters of the recovered DNA was subjected to 35 PCR cycles and the amplified products were separated on a 1.5%-agarose gel and visualized by ethidium bromide staining. NF-κB and AP-1-binding sites on the endogenous *NAPA* promoter in chromosome 7 (NC_000073.5) were determined by using TFSEARCH (Searching transcription factor binding sites; http://www.cbrc.jp/research/db/TFSEARCH.html; accessed 04-20-2011). The primers were designed by Primer 3.0. The primers used included: primer 1 for NF-κB-binding site, forward, 5′-CTCCTTACCTGTGGGTCTTTATG-3′ and reverse, 5′-GGATCTAGTGGGAAGGAAGATAC-3′, which yielded a 231-bp product; primer 2 for AP-1-binding site, forward, 5′-TTGCAGTGGTGACTTCTTGC-3′ and reverse, 5′-CCCACATGACTGCAGAGCTA-3′, which produced a 187-bp product.

2.8. Cell clonogenicity, cell viability, and apoptosis analysis

Cell clonogenicity and cell viability was determined respectively by colony formation [43] and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assays [39]. Cells were treated with cisplatin, vincristine, or taxol in serum-free medium for 2 h, and cultured in drug-containing and serum-containing medium for either 3 days for the MTT assay or 14 days for the colony formation assay. The percentage of cell clonogenicity, or cell survival, was calculated as the number of colonies stained with Coomassie blue for the treated cells divided by the number of colonies for the mock-treated cells. The percentage of cell viability was calculated as the ratio of total number of viable cells divided by the total number of cells counted. Cells with apoptotic nuclear phenotype were assessed as described [39]. The percentage of apoptotic cells was calculated as the ratio of dead cells divided by the total number of cells counted. To analyze drug-induced apoptosis, we prepared cell extracts for Western blot with antibodies against cleaved caspase-3 and cleaved PARP [39]. Unless indicated otherwise, three independent experiments were performed. The data were reported as mean values ± standard deviation (SD). Statistical significance (*P* value) was calculated with a two-tailed Student's *t* test for single comparison. The symbol * denotes $P < 0.05$ while ** denotes $P < 0.01$.

2.9. Maintenance of the LMP1-dependent tumor model

LMP1-dependent tumors were maintained in BALB/c mice as before [36]. To prepare tumor single cells, we cut tumor masses into small pieces and immersed them in Hank's balanced salt solution (HBSS) medium (Invitrogen) containing collagenase D (1 mg/ml; Roche, Mannheim, Germany). Samples were incubated 45 min at 37 °C, grounded, and filtered through a 53-mm-mesh nylon filter (Spectra Mesh, Houston, TX, USA) to exclude undigested tissue fragments. The resulting tumor suspension was centrifuged at 225 × *g* for 5 min, and the cell pellet was washed twice. For tumor grafting, tumors were grown to 1 cm^3, dissected, cut into small pieces of 9 mm^3, and transplanted subcutaneously into normal BALB/c mice. Tumor grafting was routinely performed every 4–6 weeks for maintenance of the tumors in vivo.

3. Results

3.1. LMP1-transformed 3T3 cells are hypersensitive to cisplatin

We first investigated whether primary 3T3-LMP1 cells that were derived from LMP1-transformed mouse tumors are sensitive to various chemotherapeutic drugs. We observed that the 3T3-LMP1 cells that stably expressed the LMP1 protein were hypersensitive to cisplatin compared to control 3T3-Neo cells (Fig. 1A). To quantify the level of sensitization, we used a sensitization factor (SF) corresponding to the ratio of IC_{50} for 3T3-Neo cells divided by the IC_{50} of 3T3-LMP1 cells as assessed by the cell viability assay. In this case, the SF of 3T3-LMP1 was calculated at 2.38 (Fig. 1A). Notably, no sensitization effect was found for 3T3-LMP1 cells that were treated with either vincristine or taxol, indicating that LMP1 sensitized cells specifically to cisplatin. The hypersensitivity of 3T3-LMP1 cells to cisplatin was confirmed by the decreased cell survival of these cells in the presence of cisplatin as monitored by the colony formation assay (Fig. 1B) and the corresponding quantified results (Fig. 1C). The decreased cell survival of LMP1-expressing cells was obvious at a low dose of cisplatin, while the effect was more modest at a higher dose (Fig. 1C; 1 μM vs. 10 μM). The hypersensitivity of LMP1-expressing cells to cisplatin was further confirmed by apoptotic assays as assessed by nuclear phenotype (Fig. 1D) and apoptotic protein markers (Fig. 1E). As such, LMP1-expressing cells showed a higher level of apoptotic nuclear phenotype (Fig. 1D), an increased level of cleaved caspase-3 and cleaved PARP, and a reduced level of the apoptosis-inhibitory proteins DFF/ICAD compared to control cells (Fig. 1E).

3.2. Downregulation of NAPA sensitizes 3T3-Neo cells to cisplatin

We have recently identified several proteins that are associated with cisplatin resistance in tumor and non-tumor cell lines [30]. Downregulation of these proteins, including the ER-resident protein NAPA, significantly sensitized cells to cisplatin [30]. Moreover, these proteins were not implicated in resistance to mitotic spindle damage induced by other drugs, such as vincristine or taxol [30]. To explore the possibility that LMP1 affects proteins implicated in cisplatin resistance, we examined the expression level of these proteins in 3T3-LMP1 cells. Among the five proteins examined, the mRNA and protein levels of NAPA and CABIN1 were downregulated in LMP1-expressing cells (Fig. S1). Since NAPA was shown earlier to possess the highest activity in inducing resistance

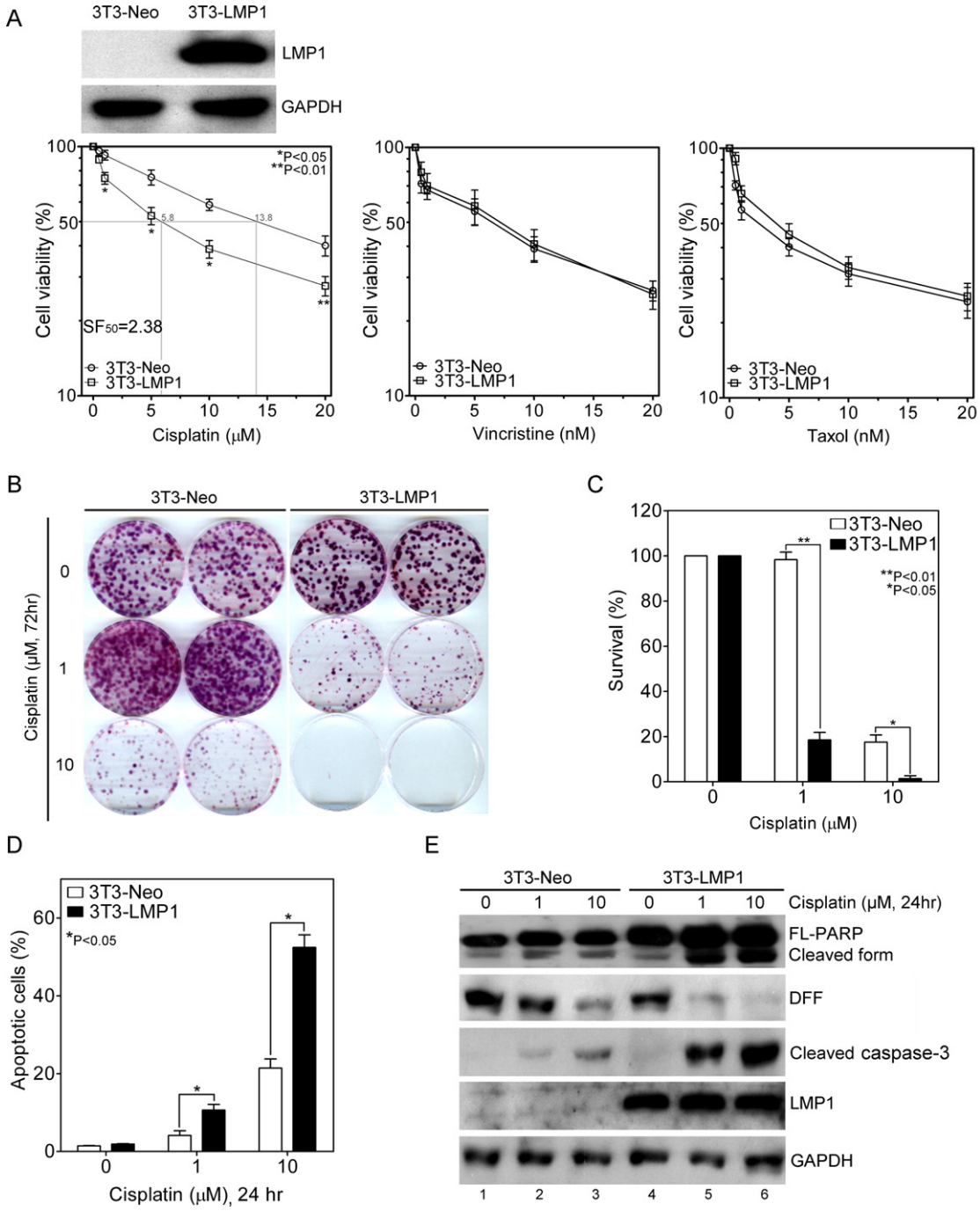

Fig. 1. LMP1-transformed mouse cells are hypersensitive to cisplatin chemotherapy. (A) Enhanced viability of 3T3-LMP1 cells in response to cisplatin. Cell viability was determined by the MTT assay (see Section 2). No difference was noted in cell viability between 3T3-LMP1 and 3T3-Neo cells following treatment with either vincristine or taxol. (B) Representative cell colony assays showing reduced survival of 3T3-LMP1 cells in response to cisplatin. Colonies were stained with Coomassie blue. Cisplatin concentrations were indicated. (C) Quantitative data of the results shown in (B) showing reduced survival of 3T3-LMP1 cells in response to cisplatin. The data were reported as mean values ± standard deviation (SD) done in triplicate. The symbol '*' denotes $P < 0.05$; '**' denotes $P < 0.01$. (D) Increased apoptosis of 3T3-LMP1 cells compared to 3T3-Neo cells in response to cisplatin. The data were reported as mean values ± SD. (E) Apoptotic markers in 3T3-Neo and 3T3-LMP1 cells treated with cisplatin. FL-PARP, full length PARP; DFF, DFF/ICAD.

to cisplatin [31], we focused on this protein in the present study. qPCR analysis confirmed the reduced expression of NAPA mRNA in 3T3-LMP1 cells (Fig. 2A). 3T3-LMP1 cells also showed reduced NAPA protein levels as monitored by Western blots (Fig. 2B) and quantification of the protein bands by densitometry (Fig. 2C). To assess the role of NAPA in the response of 3T3 cells to cisplatin, we downregulated NAPA expression by expressing shRNA in these cells (Fig. 2D). 3T3-Neo cells that expressed shNAPA were hypersensitive to cisplatin compared to cells that expressed negative control shRNA (Fig. 2E; shNC; SF = 2.95). Expression of shNC did not affect sensitivity to cisplatin in negative-control cells (Fig. 2E). Consistent with these results, 3T3-Neo cells that expressed shNAPA also displayed a higher level of apoptotic nuclear phenotypes in response to cisplatin (Fig. 2F). Notably, NAPA knockdown without cisplatin treatment also increased apoptosis compared to control cells (Fig. 2F). On the other hand, NAPA knockdown did not sensitize 3T3-Neo cells to vincristine or taxol (Fig. 2G and H).

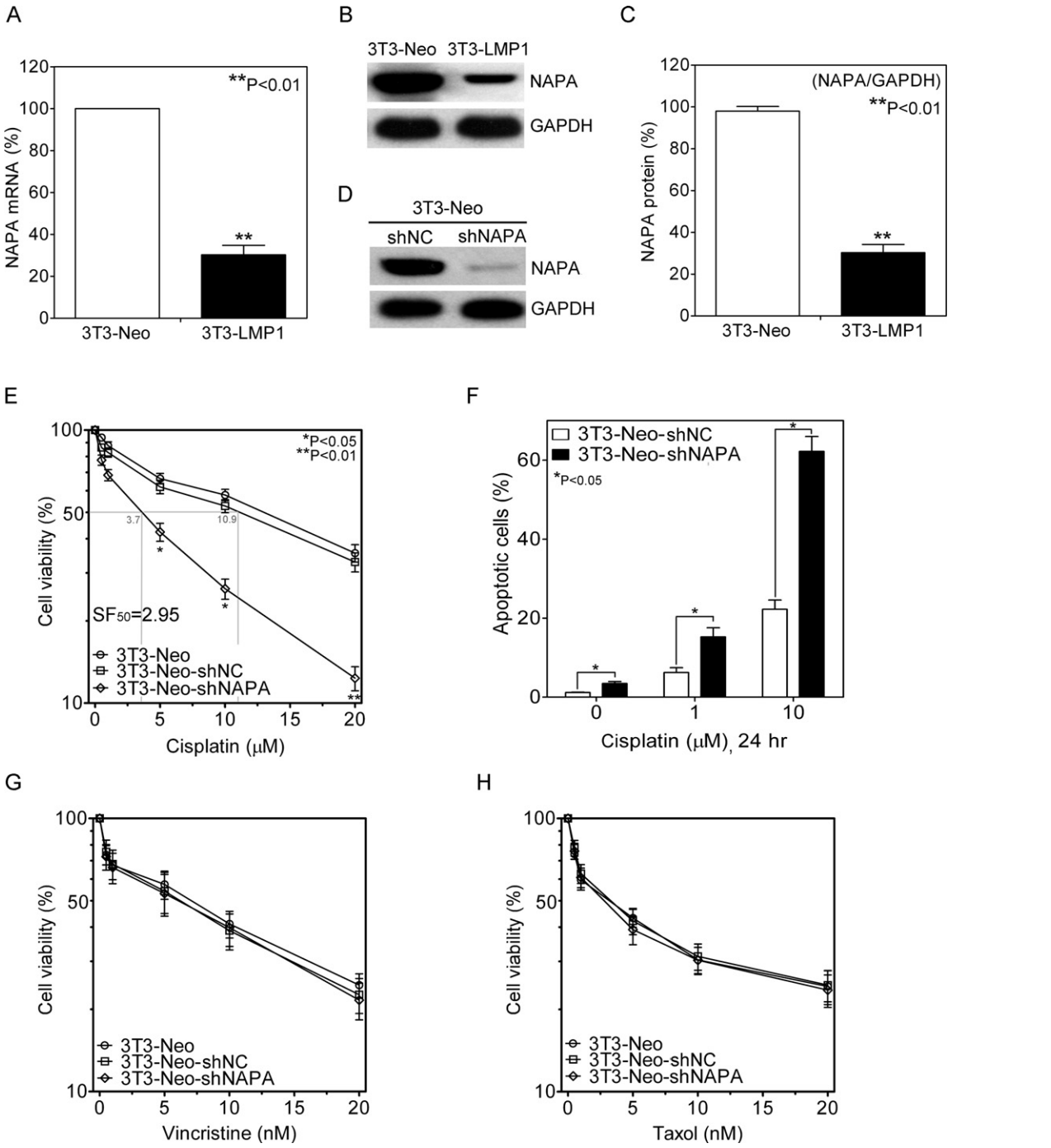

Fig. 2. Downregulation of NAPA sensitizes 3T3-Neo cells to cisplatin. (A) Reduced NAPA mRNA in 3T3-LMP1 cells as measured by qPCR. (B) Reduced NAPA protein level in 3T3-LMP1 cells. (C) Quantification of the protein bands seen in B as measured by densitometry. (D) Knockdown of NAPA by shRNA (shNAPA) in 3T3-Neo cells. shNC, negative control shRNA. (E) Reduced viability of 3T3-Neo cells expressing shNAPA and treated with cisplatin as monitored by the MTT assay. (F) Increased apoptosis of 3T3-Neo cells expressing shNAPA and treated with cisplatin as assessed by nuclear phenotype. (G and H) No difference in cell viability between shNAPA and shNC 3T3-Neo cells following treatment with either vincristine or taxol.

3.3. LMP1 sensitizes cells to cisplatin by downregulating NAPA expression

To assess the role of NAPA in LMP1-mediated sensitization to cisplatin, we treated 3T3-LMP1 cells with antisense (AS) oligonucleotides that target LMP1 (LMP1AS). Following LMP1 knockdown by LMP1AS, NAPA mRNA and protein levels were restored to the levels observed in 3T3-Neo cells (Fig. 3A). Knockdown of LMP1 with AS rendered 3T3-LMP1 cells highly resistant to cisplatin compared to control cells (Fig. 3B; resistance factor, or RF, of 3.60). The induction of resistance following knockdown of LMP1 was not observed in cells treated with either vincristine or taxol (data not shown). Consistent with these results, LMP1 knockdown dramatically reduced cisplatin-induced apoptotic marker activation as seen by the decreased level of cleaved caspase-3 and PARP, and the increased level of DFF/ICAD (Fig. 3C). Cisplatin-induced apoptosis was reduced in LMP1AS-treated cells compared to scrambled AS-treated cells (Fig. 3D, $P < 0.05$). To further confirm the role of NAPA in LMP1-induced sensitization to cisplatin, we prepared 3T3-LMP1 cells that

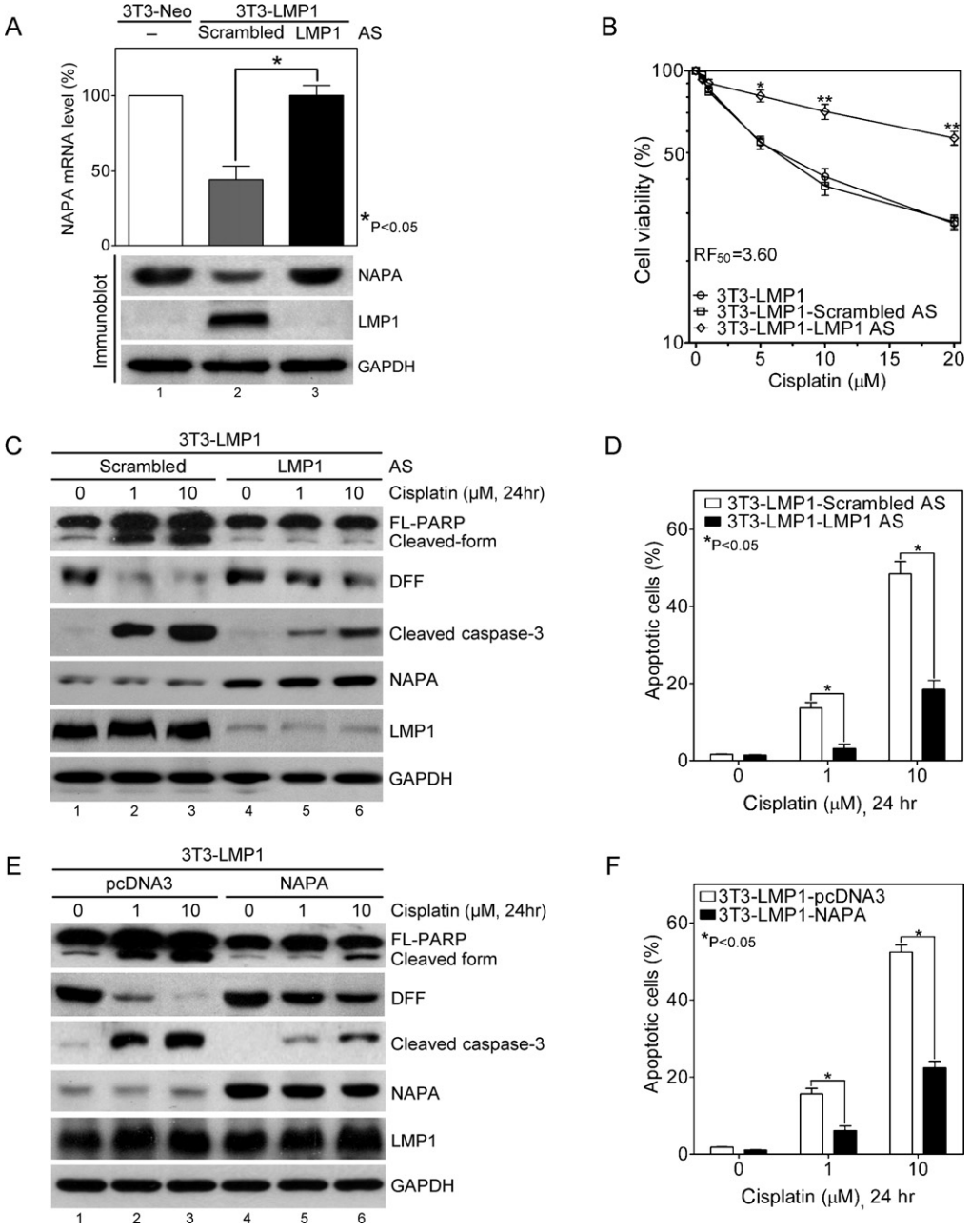

Fig. 3. LMP1 sensitizes cells to cisplatin by downregulating NAPA expression. (A) Reversal of the inhibition of NAPA mRNA and protein levels by LMP1 antisense (AS) oligonucleotides. The difference is significant when compared with scrambled AS ($P < 0.05$). (B) Resistance to cisplatin in 3T3-LMP1 cells treated with LMP1AS. The resistance factor (RF = 3.60) is indicated. (C) Reduction of cisplatin-induced caspase-3 activation and substrate cleavage by LMP1 knockdown in 3T3-LMP1 cells. (D) Reduction of cisplatin-induced apoptosis by LMP1 knockdown in 3T3-LMP1 cells. (E) Reduction of cisplatin-induced caspase-3 activation and substrate cleavage by NAPA overexpression in 3T3-LMP1 cells. Transfection of the cells with the empty vector pcDNA3 was used as a control. (F) Reduction of cisplatin-induced apoptosis by NAPA overexpression in 3T3-LMP1 cells. The *P*-values were indicated.

overexpressed exogenous NAPA. As expected, overexpression of NAPA dramatically decreased the cleavage of caspase-3 and PARP and increased the level of DFF/ICAD (Fig. 3E). Notably, cisplatin-induced apoptosis was greatly reduced in NAPA-overexpressing 3T3-LMP1 cells (Fig. 3F). These results suggest that LMP1 sensitizes 3T3 cells to cisplatin at least in part by downregulating NAPA expression.

3.4. Downregulation of NAPA by LMP1 is independent of MAPK, NF-κB and JAK/STAT signaling pathways

To assess the effect of LMP1 on the upstream signaling pathways that are known to regulate gene expression, we compared the levels of MAPK, NF-κB and JAK/STAT kinases in 3T3-LMP1 and 3T3-Neo cells. In the absence of cisplatin, the level of phosphorylated MAPKs (p-JNK1/2, p-ERK1/2, and p-p38) was higher in 3T3-LMP1 cells (Fig. 4A, compare lanes 1 and 4). On the other hand, the level of the NF-κB-inhibitor IκB-α was reduced, consistent with the observation that LMP1 activates the NF-κB pathway [21]. In the presence of cisplatin, most of these signals (p-JNK1/2, p-ERK1/2, and NF-κB) increased in a time-dependent manner in 3T3-LMP1 cells whereas p-p38 decreased during this period (Fig. 4A, lanes 2 and 3 vs. lanes 5 and 6). To verify whether these kinase pathways are involved in LMP1-induced down-regulation of NAPA, we verified the effect of the NF-κB signaling inhibitor Bay11-7082. As expected, the Bay11-7082 inhibitor

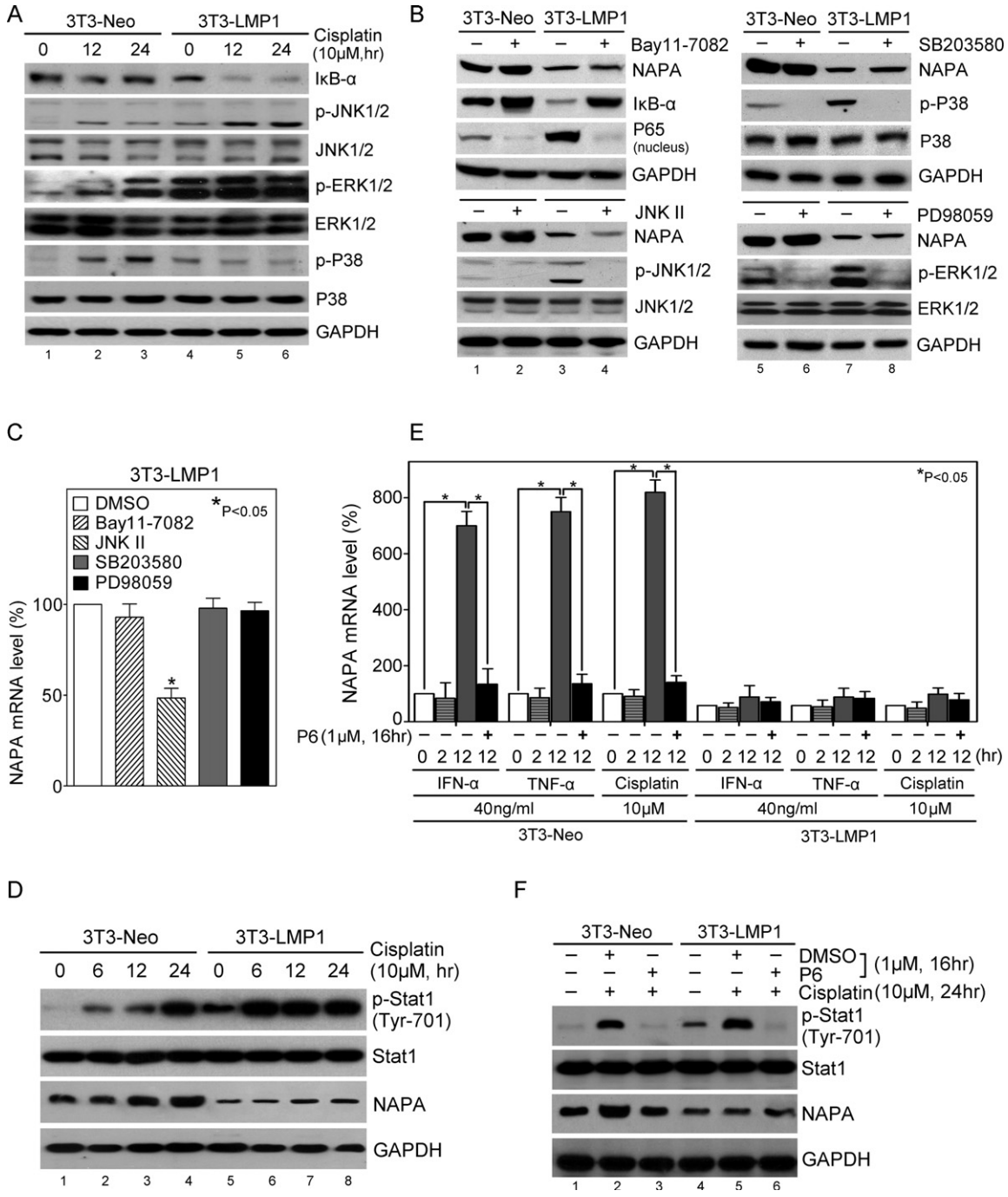

Fig. 4. Downregulation of NAPA by LMP1 is independent of kinase signaling pathways. (A) Enhanced MAP kinase and NF-κB signaling pathways in 3T3-LMP1 cells. Kinase activation was reflected by the phosphorylated form of the indicated kinases. NF-κB activity was reflected by the level of its inhibitor IκB. (B) Lack of increase of NAPA protein level by MAP and NF-κB kinase inhibitors. Bay11-7082, NF-κB inhibitor; JNKII, JNK inhibitor; SB203580, p38 inhibitor; PD98059, ERK inhibitor. (C) Lack of increase of NAPA mRNA expression by MAP and NF-κB kinase inhibitors. DMSO, which was used to dissolve the inhibitors, was used as control. (D) Enhanced STAT1 signaling in 3T3-LMP1 cells. The representative phosphorylation of STAT1 at Tyr-701 was used to reflect kinase activity. The enhancement was found in unstimulated and cisplatin-stimulated cells. (E) Reduced NAPA mRNA level in 3T3-LMP1 cells following treatment with stimulators of STAT1 signaling. IFN-α, TNF-α, cisplatin and the STAT1 inhibitor P6 were used at the indicated concentrations and incubation time. (F) Lack of increase of NAPA protein level by STAT1 kinase inhibitor in both cell lines.

caused the accumulation of IκB-α in both cell lines and a concomitant reduction in the nuclear localization of the NF-κB member p65 (Fig. 4B; upper left panel, compare lanes 1 and 3 with lanes 2 and 4). In these conditions, NAPA protein level was not significantly increased by Bay11-7082. Next, we observed that the p38/MAPK signaling inhibitor SB203580 did not increase NAPA (Fig. 4B; upper right panel, compare lanes 7 and 8). Treatment with the JNK/MAPK signaling inhibitor JNKII also did not affect the level of NAPA in 3T3-LMP1 cells (Fig. 4B; lower left panel, compare lanes 3 and 4). Similarly, NAPA protein level was not affected by the ERK1/2/MAPK signaling inhibitor PD98059 (Fig. 4B; lower right panel, compare lanes 5 and 7 with lanes 6 and 8). Furthermore, NAPA mRNA level was not increased in 3T3-LMP1 cells treated with the kinase inhibitors tested (Fig. 4C). These results suggest that LMP1-downregulated NAPA expression in 3T3-LMP1 cells is not mediated by MAPK and NF-κB signaling pathways.

We observed that p-STAT1 level was enhanced in 3T3-LMP1 cells compared to 3T3-Neo cells, both with and without cisplatin

treatment (Fig. 4D). To assess the involvement of the JAK/STAT signaling pathway in LMP1-induced downregulation of NAPA, we used IFN-α and TNF-α as activators of these pathways. While NAPA mRNA level was highly induced in 3T3-Neo cells following treatment with either IFN-α or TNF-α, only a slight or no induction of NAPA mRNA was detected in 3T3-LMP1 cells (Fig. 4E). In the presence of the inhibitor of STAT phosphorylation pyridone 6 (P6), we observed that the induced NAPA mRNA level was almost entirely inhibited. Next, we treated the cells with P6 in the presence of cisplatin for protein study. In the absence of stimuli, p-STAT1 was higher in 3T3-LMP1 cells than in 3T3-Neo cells (Fig. 4F, compare lanes 1 and 4). While p-STAT1 was considerably increased in both cell lines in the presence of cisplatin, P6 almost entirely suppressed p-STAT1 in both cell lines (Fig. 4F). NAPA protein level did not increase under these conditions; instead, it slightly decreased (Fig. 4F). These results indicate that the MAPK, NF-κB and JAK/STAT signaling pathways may not be involved in the downregulation of NAPA by LMP1.

3.5. Enhanced nuclear accumulation of NF-κB p50 in LMP1-transformed cells

The NF-κB signaling inhibitor Bay11-7082 used above is known to inhibit IκB-α phosphorylation [44] and p65/p50 heterodimerization [45]. Although Bay11-7082 affects other signaling pathways such as JNK and p38, NF-κB p50/p50 homodimers are not affected. Several putative binding sites have been found for the transcription factors NF-κB and AP-1 upstream of the *NAPA* promoter (Fig. 5A). The p50/p50 homodimer which inhibits gene expression may negatively regulate *NAPA* gene expression by accumulating in the nucleus under LMP1 expression. To explore this possibility, we compared the level of NF-κB subunits (p65, p50, and c-Rel) in nuclear fractions of 3T3-LMP1 and 3T3-Neo cells. In the absence of cisplatin, the level of the NF-κB subunit c-Rel was similar in the nucleus of both cell lines, but p65 and p50 appeared to increase in the nucleus of 3T3-LMP1 cells compared to that of 3T3-Neo cells (Fig. 5B, compare lanes 3 and 7). This observation was not due to the upregulation of both genes in 3T3-LMP1 cells since p65 and p50 mRNA levels were not increased upon LMP1 expression as assessed by real-time PCR (Fig. S4A). In the presence of cisplatin, all NF-κB subunits accumulated in the nucleus in both cell lines (Fig. 5B, lane 4 vs. lane 8). The average nuclear fraction of these proteins indicated that all three NF-κB subunits accumulated more in the nucleus of both cisplatin-treated cell lines (Fig. 5C). p50 was abundantly increased in the nucleus following cisplatin treatment. Even without cisplatin treatment, most of p50 was already localized in the nucleus of 3T3-LMP1 cells (Fig. 5C). Using co-IP experiments, we observed that nuclear p65 or c-Rel was brought down together with p50 when immunoprecipitated with p50 antibody in both cell lines, either with or without cisplatin (Fig. 5D, lanes 3 and 7 and lanes 4 and 8, respectively). The level of each protein loading (20% of total) was also shown (Fig. 5D, lanes 1 and 2 and lanes 5 and 6 for 3T3-Neo and 3T3-LMP1, respectively). To assess the formation of p50/p50 homodimers, GST-p50 and HA-p50 fusion proteins were overexpressed in 3T3-LMP1 cells. GST-p50 that was immunoprecipitated with GST antibody also interacted with HA-p50 in cells that were co-transfected with both plasmids (Fig. 5E, lane 4). In contrast, HA-p50 was not detected in cells transfected with GST-p50 and pcDNA3-HA (without HA-p50) as a negative control (Fig. 5E, lane 3). The same results were found in parallel experiments in the presence of Bay11-7082 (Fig. 5E, compare lanes 3 and 4 with lanes 5 and 6), supporting the notion that the NF-κB inhibitor did not affect the formation of nuclear p50/p50 homodimers in these cells. Consistent with these findings, we observed that HA-p50 immunoprecipitated with HA antibody also interacted with GST-p50 in cells that were co-transfected with both plasmids compared to transfection with single plasmid (Fig. 5F, lane 3 vs. lane 4). Similarly, Bay11-7082 did not affect the formation of p50/p50 homodimers in this system (Fig. 5F, compare lanes 3 and 4 with lanes 5 and 6).

To further investigate the role of LMP1 in the nuclear translocation of NF-κB subunits, we downregulated LMP1 expression by using AS oligonucleotides (LMP1AS) (Fig. S2). The initial nuclear accumulation of p50 was reduced to minimal level by LMP1AS in 3T3-LMP1 cells (Fig. S2B). In the presence of cisplatin, nuclear accumulation of p65 and c-Rel was reduced by LMP1AS by 30–40%, whereas nuclear p50 was reduced by 80% compared to the scrambled AS control. The average percentage of co-IP NF-κB subunits with p50 in either scrambled-AS or LMP1AS-treated cells in the absence of cisplatin was approximately 20% and 10%, respectively (Fig. S2D). In the presence of cisplatin, p50-bound NF-κB subunits were slightly increased. Notably, the NF-κB subunits bound to p50 appeared to be similar. Taken together, these results indicate that LMP1 appears to preferentially promote the nuclear translocation of p50. In addition, the interaction between p50 and NF-κB subunits (p65, c-Rel, or p50) is not clearly altered in the nuclear extracts of LMP1-expressing cells.

3.6. Enhanced binding of p50/p50 and c-Jun to NAPA promoter in LMP1-transformed mouse cells

The co-IP experiments of nuclear extracts described above could not distinguish whether the NF-κB p50 subunits were in free form or bound to chromatin DNA. To address this question, we performed ChIP assays. Chromatin prepared by sonication was processed for immunoprecipitation with specific antibodies (see Section 2). The PCR product (231-bp) obtained using primer pair 1, which covers a putative NF-κB binding site (Fig. 5A), was detected in p50 IP complexes from 3T3-LMP1 cells, but not in 3T3-Neo cells (Fig. 6A, lanes 4 and 3, respectively). There was no clear PCR product detected in p65, c-Rel, or IgG IP complexes from 3T3-LMP1 cells (Fig. 6A, lanes 6, 8, and 10). This observation was not due to the antibody used since p65 was detected when we assessed the TNF-α-mediated recruitment of p65 on the IκBα promoter using ChIP assays (Fig. S4B). Input proteins (20%) were also shown as reference (Fig. 6A, lanes 1 and 2). Similar amounts of PCR product was detected in p50 IP complexes prepared from mock or cisplatin-treated 3T3-LMP1 cells (Fig. 6B, lanes 3 and 4) but no PCR product was detected in other IP complexes. We did not exclude the possibility that a trace amount of other NF-κB subunits, such as p65, was also bound to the DNA sequence as appeared to be the case from the slightly higher amount of PCR product in cisplatin-treated cells (Fig. 6B, compare lanes 6 and 5). These results suggest that p50 may bind to the *NAPA* promoter in 3T3-LMP1 cells without cisplatin stress.

The presence of a PCR product in p50 IP complexes also suggests that the complex of NF-κB subunits is unlikely to represent a heterodimer. To assess this possibility, we used plasmids of p50 with GST or HA tag for two-step IP. 3T3-LMP1 cells were co-transfected with these plasmids, and the first IP was performed with GST antibody, followed by the second IP of the re-suspended pellet. The final IP complexes were processed for PCR using primer pair 1. A PCR product was clearly detected in cells that were co-transfected with the two plasmids, but no PCR product was detected in the cells transfected with GST-p50 and control pcDNA3-HA plasmids (Fig. 6C, compare lanes 4 and 3). To confirm that the enhanced p50/p50 binding to *NAPA* promoter was due to LMP1, we performed the same experiments in 3T3-LMP1 cells that expressed LMP1AS. The PCR product was considerably reduced in double IP complexes in cells treated with LMP1AS compared to the cells treated with scrambled AS control (Fig. 6D, lanes 4 vs. lane 2). These results demonstrate that LMP1 expression induces the formation of p50/p50 homodimer on the *NAPA* promoter.

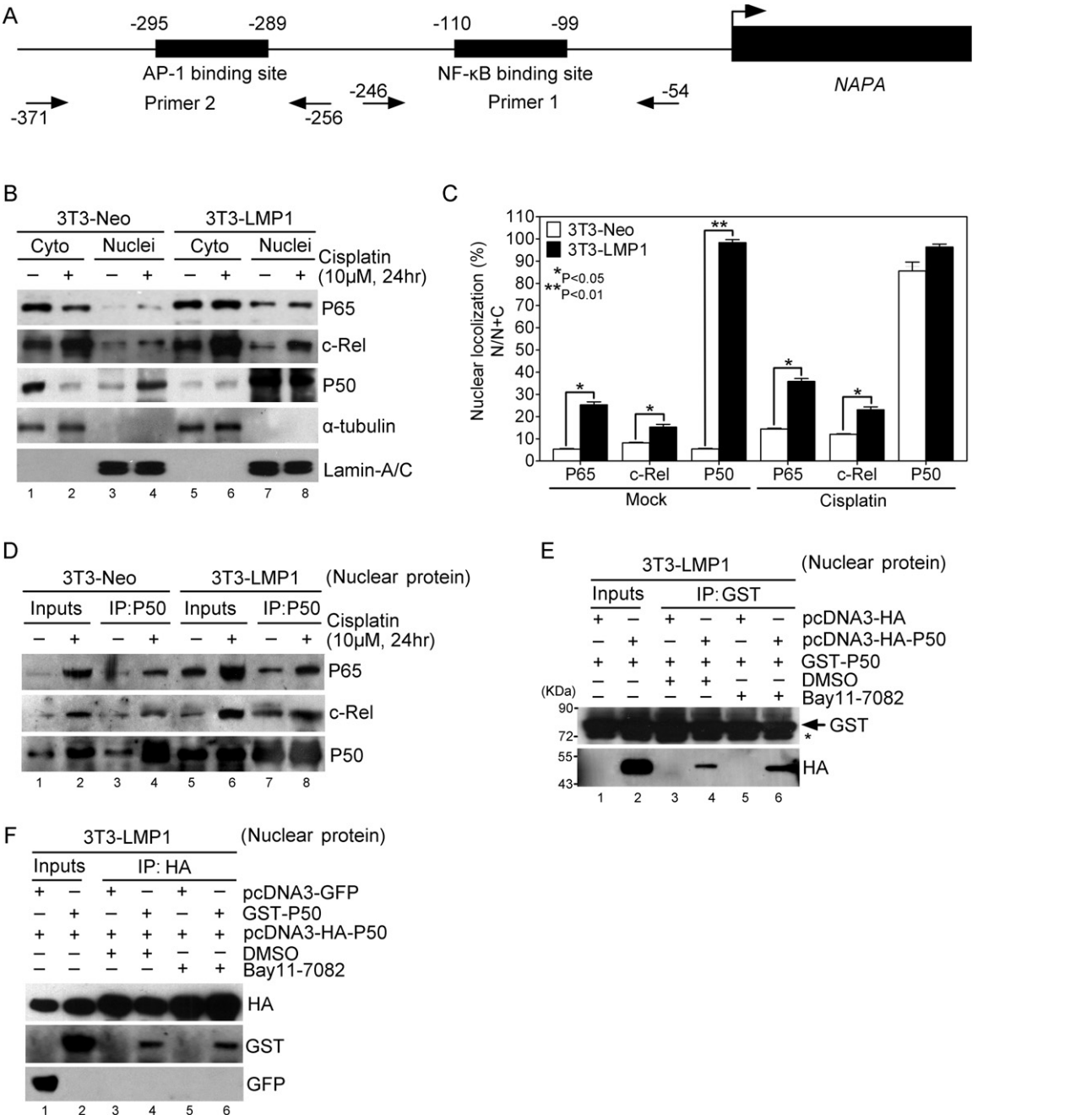

Fig. 5. Enhanced nuclear accumulation of NF-κB subunits and formation of p50/p50 homodimers in LMP1-transformed mouse cells as revealed by co-IP experiments. (A) Diagram of the NAPA promoter with putative transcription factor-binding sites. The position of potential binding sites for AP-1 and NF-κB are indicated. The positions of the two primer pairs used are also shown. (B) Enhanced nuclear accumulation of NF-κB subunits p65, p50 and c-Rel in 3T3-LMP1 cells. (C) Statistical analysis of the nuclear localization of NF-κB subunits in 3T3-LMP1 cells compared to 3T3-Neo cells in unstimulated (mock) and cisplatin-stimulated cells. The data were reported as mean values ± SD for experiments performed in triplicate. Mean values were calculated as nuclear proteins N divided by total proteins C + N and multiplied by 100 to obtain percentage values. The *P*-values were indicated. (D) Enhanced co-IP of p50 with p50 in the nuclear extracts of unstimulated 3T3-LMP1 cells. The p50 level which co-IP with p50 is about same in both stimulated cell lines (compare lanes 4 and 8). (E) Formation of p50 homodimers in nuclear proteins of unstimulated 3T3-LMP1 cells. p50 with either HA or GST tag (see Section 2) were co-expressed in cells, and IP was performed from nuclear extracts with GST antibody, followed by detection of the complex with HA antibody. Note that the NF-κB inhibitor did not affect the formation of two-fusion p50 (compare lanes 4 and 6). (F) Formation of p50 homodimers in nuclear proteins of unstimulated 3T3-LMP1 cells. The experimental design was the same as panel E except that HA antibody bound HA-p50 was used as bait.

In addition, we noted the presence of a putative AP-1-binding site upstream of the *NAPA* promoter (Fig. 5A). To explore whether LMP1 regulates the binding of the AP-1 component c-Jun on the *NAPA* promoter, we performed ChIP assays with 3T3-LMP1 cells as done above for NF-κB subunits. We detected an increase of PCR product by using primer pair 2 (Fig. 5A) from p-c-Jun IP complexes of 3T3-LMP1 cells when compared to that of 3T3-Neo cells (Fig. 6E, compare lanes 4 and 3), suggesting that p-c-Jun interacts with the *NAPA* promoter in 3T3-LMP1 cells. This observation was not due to a reduction of c-Jun in 3T3-Neo cells since the PCR products from both inputs were similar (Fig. 6E, compare lanes 1 and 2). Surprisingly, the same PCR product was not detected in the presence of cisplatin (Fig. 6F, compare lanes 3 and 4). Again, this result was not due to a lack of p-c-Jun expression since equal amounts of PCR products were detected in the samples with and without cisplatin (Fig. 6F, compare lanes 1 and 2). These results

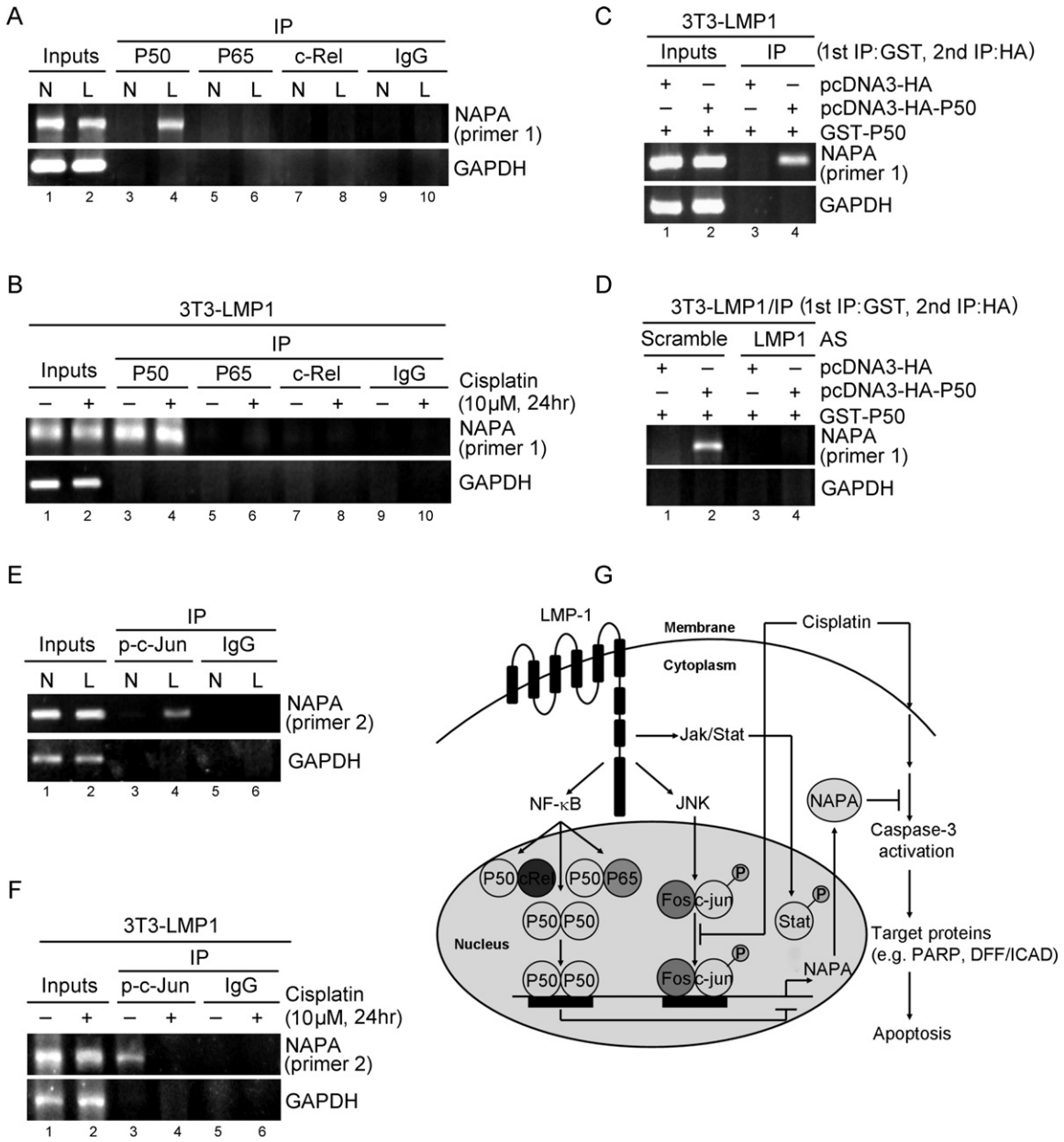

Fig. 6. Enhanced binding of p50/p50 homodimers and c-Jun to *NAPA* promoter in LMP1-transformed mouse cells as revealed by ChIP experiments. (A) Detection of p50 binding to NAPA promoter in unstimulated 3T3-LMP1 cells. N, 3T3-Neo cells; L, 3T3-LMP1 cells. (B) Detection of p50 binding to NAPA promoter in cisplatin-stimulated 3T3-LMP1 cells. (C) Formation of p50 homodimers on *NAPA* promoter in 3T3-LMP1 cells. Physical interaction between HA-p50 and GST-p50 was detected by double IP, with the first IP being done with GST antibody and the second with the supernatant of resuspended pellet and HA antibody. The pellet was then processed for PCR (see Section 2). (D) Inhibition of p50 homodimer formation by LMP1AS. Physical interaction between HA-p50 and GST-p50 was also detected by double IP as described in panel C. (E) Detection of enhanced p-c-Jun binding to NAPA promoter in unstimulated 3T3-LMP1 cells. Methods and symbols are same as for A except that primer pair 2 was used for PCR. (F) Reduction of p-c-Jun binding to NAPA promoter in cisplatin-stimulated 3T3-LMP1 cells. (G) Proposed model for the activity of LMP1 in downregulating NAPA expression and sensitizing cells to cisplatin-induced apoptosis. For simplification, the epigenetic regulators HDAC and CBP/p300 are not included.

demonstrate that the transcription factor AP-1 binds to the *NAPA* promoter in 3T3-LMP1 cells, and that the formation of this complex is reduced in the presence of cisplatin.

4. Discussion

In this study, we found that LMP1 sensitizes cancer cells to cisplatin chemotherapy by downregulating NAPA expression. This conclusion is supported by the observation that LMP1 knockdown caused accumulation of NAPA and rendered 3T3-LMP1 cells resistant to cisplatin. Furthermore, restoration of NAPA expression in 3T3-LMP1 cells rendered the cells resistant to cisplatin. Importantly, LMP1-mediated suppression of NAPA expression affected the response of mouse cells to cisplatin, but not to mitotic spindle damagers like vincristine and taxol. These results are consistent with our previous findings that NAPA plays a critical role in modulating the response of human cancer cells to DNA damagers in a p53-dependent manner [30,31]. Based on these results, NAPA may be downregulated by EBV-encoded LMP1, thereby rendering the cells pro-apoptotic and vulnerable to cisplatin.

We also found that NAPA expression at the transcriptional level was dramatically suppressed in LMP1-expressing cells. As shown by other authors [46], LMP1 was sufficient to activate kinase

signaling in unstressed BALB/c-3T3 primary cells. Surprisingly, these cells did not show increased NAPA expression following pre-treatment with inhibitors of various kinase pathways, including NF-κB. These results suggest that the regulation of the *NAPA* gene by LMP1 is independent of NF-κB. On the other hand, LMP1 induced the accumulation of NF-κB p50 subunits in the nucleus. This observation suggests that LMP1 stabilizes p50 by preventing its degradation since we found that p50 is more stable in 3T3-LMP1 cells than in 3T3-Neo cells (Fig. S4C and D). Furthermore, p50 homodimers prominently interacted with the putative NF-κB-binding site of the *NAPA* promoter in LMP1-transformed 3T3 cells. Although other NF-κB subunits also accumulated in the nucleus of these cells, these subunits did not bind to the *NAPA* promoter as revealed by ChIP assays. Our main findings can be summarized by the model presented in Fig. 6G, which, for simplification, do not include epigenetic regulators like HDAC and CBP/p300. This postulated model shows the binding of p50 homodimers to DNA and the resulting repression of *NAPA* gene transcription in unstimulated cells. Following stimulation with cisplatin, NF-κB dimers translocate to the nucleus, whereas in the presence of LMP1 the recruitment of p50 homodimers to DNA is dominant over that of other NF-κB dimers. However, our results (Fig. 4B) do not appear to support a role for p65 in initiating NF-κB-dependent *NAPA* gene transcription in 3T3-Neo cells. Furthermore, NAPA protein level is only minimally affected, if at all, by cisplatin in the treated cell lines (Figs. 3C and 4D). These observations may explain the negligible effect of LMP1 on NAPA level in cisplatin-treated cells (Fig. 3C). Our results also indicate that LMP1 promotes AP-1 (c-Jun/Fos) binding to the *NAPA* promoter in unstimulated cells. Surprisingly, binding of AP-1 was decreased in cisplatin-stimulated cells, leaving p50 homodimers bound at the promoter. The basal expression of the *NAPA* gene may be repressed by LMP1 through its unique conformation and the formation of complexes with p50 homodimers on the promoter. Furthermore, p50 homodimers may be stably bound to the promoter, a process that may prevent the inducible expression of the *NAPA* gene by AP-1. Furthermore, we also detected abundant LMP1 and low NAPA protein level in CG1 cells, which represent EBV-positive epithelial cells that were derived from a NPC patient in Taiwan [47]. LMP1 knockdown with LMP1 AS increased NAPA protein level and rendered the cells resistant to cisplatin (Fig. S3). These results suggest that the experimental model of 3T3 fibroblasts used in this study may be suitable to study the response of cancer cells to LMP1 expression and cisplatin. Notably, this experimental model can be used to explain the mechanism of expression of NF-κB-regulated genes in LMP1-transformed cancer cells. Following stimulation with cisplatin, the NF-κB dimers containing phosphorylated p65 that are in complex with CBP/p300 may be recruited to DNA where they displace p50 homodimers and HDAC-1 and initiate NF-κB-dependent gene transcription.

It has been demonstrated by other authors that the complex biological effects of LMP1-TRAF-mediated activation of signaling pathways are important for controlling the survival and proliferation of EBV-infected cells [46]. Reducing the expression of LMP1 in NPC cells promoted apoptosis and enhanced cell radiosensitivity by blocking signaling pathways that are activated by LMP1, including the NF-κB, AP-1, and STAT3 pathways [48]. Previously, LMP1 was considered to be a tumorigenic protein since it can transform primary B lymphocytes [1] and rodent fibroblasts [2], and it can also inhibit terminal differentiation of human epithelial cells [3]. LMP1 also affects apoptosis in epithelial cells, but the cellular outcome appears to vary according to the biological stimulus and cellular context. Furthermore, LMP1 upregulates the expression of multiple genes which induce opposing effects on the proliferation and apoptosis of B cells [7]. We recently also found that LMP1 induces the NF-κB and p38 MAPK signaling pathways and leads to the induction of thymidine phosphorylase, a marker of poor prognosis in NPC patients [49]. According to our model, the extent of nuclear translocation of p50 and binding of its dimer form to the promoter of the thymidine phosphorylase gene may be prevented by p65. The same idea is applicable to the investigation of two cisplatin resistance genes, *CABIN1* and *ADM*, whose products are dysregulated by LMP1 and reversed by the NF-κB inhibitor Bay11-7082 (Fig. S5). These findings may partly explain the contrasting observations made on the role of LMP1 in apoptosis. We also found that NF-κB (p65) inhibitor did increase the viability of 3T3-Neo cells to cisplatin (data not shown), and that p65 is not involved in NAPA induction. These results suggest that NAPA-independent factors such as CABIN1 may represent targets of LMP1-induced NF-κB pathway. Going beyond our proposed model (Fig. 6G), other yet-to-be-found factors that are also regulated by LMP1 through NF-κB (p65) kinase signaling may account for the apoptotic response observed in this study. For example, activation of Myc and NF-κB, possibly by EBV-encoded LMP1, in natural killer and T-cell lymphomas results in the upregulation of survivin which is responsible for apoptotic resistance in tumor cells [50]. In addition, LMP1 has been shown to repress DNA repair and enhance sensitivity to DNA-damaging agents in NPC cell lines [13], suggesting that disruption of DNA repair by LMP1 may contribute to genomic instability in epithelial cells. A proportion of the cells that receive a high level of LMP1-mediated stress may have unstable genomes and eventually undergo apoptosis.

In addition, it has been demonstrated that transmembrane domains 3–6 of LMP1 induce autophagy in a dose-dependent manner and thus this protein may modify the physiology of the host. Inhibition of autophagy in EBV-positive B cells leads to LMP1 accumulation and a decreased ability to form cell colonies [51]. Furthermore, ER-associated degradation (ERAD) pathways are often protective and promote cell survival but ER stress, when excessive, is thought to result in autophagic cell death [52]. Since NAPA has a protective effect against ER stresses induced by cisplatin [31], it is possible that suppression of NAPA expression by LMP1 may amplify autophagic apoptosis. Taken together, these observations suggest that various extrinsic stimuli including chemical and biological agents regulate cell signaling, a process which in turn modifies apoptotic regulator such as NAPA and, in concert with other molecules, leads to cell apoptosis or resistance to apoptosis.

Although the CTAR domains of LMP1 are responsible for most of the intracellular signaling that are associated with a variety of cell responses (such as cell proliferation, apoptosis, and inflammation), downstream effector molecules that participate or regulate cell responses remain poorly understood. NF-κB and JNK signaling pathways are believed to mediate LMP1-enhanced apoptosis induced by cisplatin in epithelial cells [12]. In this study, we identified NAPA as an LMP1 target in the regulation of cisplatin-induced apoptosis. Our findings also suggest that NF-κB p50 enrichment in the nucleus and binding of its homodimer form to the *NAPA* gene promoter may be involved in regulating gene expression. These results suggest a new therapeutic target for the treatment of LMP1-associated cancers, as well as novel targets for the development of biomarkers for early detection and new endpoints for therapeutic efficacy and toxicity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.09.010.

References

[1] Kaye KM, Izumi KM, Kieff E. Epstein–Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. Proc Natl Acad Sci USA 1993;90:9150–4.
[2] Wang D, Liebowitz D, Kieff E. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. Cell 1985;43:831–40.
[3] Dawson CW, Rickinson AB, Young LS. Epstein–Barr virus latent membrane protein inhibits human epithelial cell differentiation. Nature 1990;344:777–80.
[4] Young LS, Dawson CW, Clark D, Rupani H, Busson P, Tursz T, et al. Epstein–Barr virus gene expression in nasopharyngeal carcinoma. J Gen Virol 1988;69:1051–65.
[5] Fåhraeus R, Hu L, Ernberg I, Finke J, Rowe M, Klein G, et al. Expression of Epstein–Barr virus-encoded proteins in nasopharyngeal carcinoma. Int J Cancer 1988;42:329–38.
[6] Henderson S, Rowe M, Gregory C, Croom-Carter D, Wang F, Longnecker R, et al. Induction of bcl-2 expression by Epstein–Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. Cell 1991;65:1107–15.
[7] Dirmeier U, Hoffmann R, Kilger E, Schultheiss U, Briseno C, Gires O, et al. Latent membrane protein 1 of Epstein–Barr virus coordinately regulates proliferation with control of apoptosis. Oncogene 2005;24:1711–7.
[8] Fries KL, Miller WE, Raab-Traub N. Epstein–Barr virus latent membrane protein 1 blocks p53-mediated apoptosis through the induction of the A20 gene. J Virol 1996;70:8653–9.
[9] Kawanishi M. The Epstein–Barr virus latent membrane protein 1 (LMP1) enhances TNF-α-induced apoptosis of intestine 407 epithelial cells: the role of LMP1 C-terminal activation regions 1 and 2. Virology 2000;270:258–66.
[10] Zhang X, Hu L, Fadeel B, Ernberg IT. Apoptosis modulation of Epstein–Barr virus-encoded latent membrane protein 1 in the epithelial cell line HeLa is stimulus-dependent. Virology 2002;304:330–41.
[11] Zhang X, Uthaisang W, Hu L, Ernberg IT, Fadeel B. Epstein–Barr virus-encoded latent membrane protein 1 promotes stressinduced apoptosis upstream of caspase-2-dependent mitochondrial perturbation. Int J Cancer 2005;113:397–405.
[12] Zhang X, Sanmun D, Hu L, Fadeel B, Ernberg I. Epstein–Barr virus-encoded LMP1 promotes cisplatin-induced caspase activation through JNK and NF-κ B signaling pathways. Biochem Biophys Res Commun 2007;360:263–8.
[13] Liu MT, Chen YR, Chen SC, Hu CY, Lin CS, Chang YT, et al. Epstein–Barr virus latent membrane protein 1 induces micronucleus formation, represses DNA repair and enhances sensitivity to DNA-damaging agents in human epithelial cells. Oncogene 2004;23:2531–9.
[14] Gires O, Kohlhuber F, Kilger E, Baumann M, Kieser A, Kaiser C, et al. Latent membrane protein 1 of Epstein–Barr virus interacts with JAK3 and activates STAT proteins. EMBO J 1999;18:3064–73.
[15] Huen DS, Henderson SA, Croom-Carter D, Rowe M. The Epstein–Barr virus latent membrane protein-1 (LMP1) mediates activation of NF-(B and cell surface phenotype via two effector regions in its carboxy-terminal cytoplasmic domain. Oncogene 1995;10:549–60.
[16] Mosialos G, Birkenbach M, Yalamanchili R, VanArsdale T, Ware C, Kieff E. The Epstein–Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. Cell 1995;80:389–99.
[17] Dawson CW, Tramountanis G, Eliopoulos AG, Young LS. Epstein–Barr virus latent membrane protein 1 (LMP1) activates the phosphatidylinositol 3-kinase/Akt pathway to promote cell survival and induce actin filament remodeling. J Biol Chem 2003;278:3694–704.
[18] Roberts ML, Cooper NR. Activation of a ras-MAPK-dependent pathway by Epstein–Barr virus latent membrane protein 1 is essential for cellular transformation. Virology 1998;240:93–9.
[19] Mainou BA, Everly Jr DN, Raab-Traub N. Unique signaling properties of CTAR1 in LMP1-mediated transformation. J Virol 2007;81:9680–92.
[20] Eliopoulos AG, Blake SM, Floettmann JE, Rowe M, Young LS. Epstein–Barr virus-encoded latent membrane protein 1 activates the JNK pathway through its extreme C terminus via a mechanism involving TRADD and TRAF2. J Virol 1999;73:1023–35.
[21] Young LS, Rickinson AB. Epstein–Barr virus: 40 years on. Nat Rev Cancer 2004;4:757–68.
[22] Hou S, Guan H, Ricciardi RP. Phosphorylation of serine 337 of NF-kappaB p50 is critical for DNA binding. J Biol Chem 2003;278:45994–8.
[23] Zhong H, May MJ, Jimi E, Ghosh S. The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. Mol. Cell 2002;9:625–36.
[24] Pereira SG, Oakley F. Nuclear factor-κB1: Regulation and function. Intl J Biochem Cell Biol 2008;40:1425–30.
[25] Kaufmanns SH. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etopside, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res 1989;49:5870–8.
[26] Lowe SW, Ruley HE, Jacks T, Housman DE. p53-Dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993;74:957–67.
[27] Andrews PA, Howell SB. Cellular pharmacology of cisplatin: perspectives on.mechanisms of acquired resistance. Cancer Cells 1990;2:35–43.
[28] Chu G. Cellular responses to cisplatin. The role of DNA-binding proteins and DNA repair. J Biol Chem 1994;269:787–90.
[29] Chao CC. Molecular basis of cis-diamminedichloroplatinum(II) resistance: a review. J Formos Med Assoc 1996;95:893–900.
[30] Wu ZZ, Lu HP, Chao CC. Identification and functional analysis of genes which potentially confer resistance to cisplatin in tumor cells. Biochem Pharmacol 2010;80:262–76.
[31] Wu ZZ, Chao CC. Knockdown of NAPA using short-hairpin RNA sensitizes cancer cells to cisplatin: implications to overcome chemoresistance. Biochem Pharmacol 2010;80:827–37.
[32] Mandic A, Hansson J, Linder S, Shoshan MC. Cisplatin induces endoplasmic reticulum stress and nucleus-independent apoptotic signaling. J Biol Chem 2003;278:9100–6.
[33] Nakajima K, Hirose H, Taniguchi M, Kurashina H, Arasaki K, Nagahama M, et al. Involvement of BNIP1 in apoptosis and endoplasmic reticulum membrane fusion. EMBO J 2004;23:3216–26.
[34] Kuo TC, Chao CC. Hepatitis B virus X protein prevents apoptosis of hepatocellular carcinoma cells by upregulating SATB1 and HURP expression. Biochem Pharmacol 2010;80:1093–102.
[35] Chao CC-K. A Search for the genes involved in resistance to cisplatin chemotherapy: Review of the experimental evidence. Curr Top Pharmacol 2011;14:47–54.
[36] Chow KPN, Wu CC, Chang HY, Chang C, Chang YS. A simplified tumour model established via Epstein–Barr virus-encoded, nasopharyngeal carcinoma-derived oncogene latent membrane protein 1 in immunocompetent mice. Lab Anim 2008;42:193–203.
[37] Chen ML, Tsai CN, Liang CL, Shu CH, Huang CR, Sulitzeanu D, et al. Cloning and characterization of the latent membrane protein (LMP) of a specific Epstein–Barr virus variant derived from the nasopharyngeal carcinoma in the Taiwanese population. Oncogene 1992;7:2131–40.
[38] Sun CL, Chao CC. Cross-resistance to death ligand-induced apoptosis in cisplatin-selected HeLa cells associated with overexpression of DDB2 and subsequent induction of cFLIP. Mol Pharmacol 2005;67:1307–14.
[39] Kamarajan P, Sun NK, Sun CL, Chao CC. Apaf1 overexpression partially overcomes apoptotic resistance in a cisplatin-selected HeLa cell line. FEBS Lett 2001;505:206–12.
[40] Kenney JL, Guinness ME, Curiel T, Lacy J. Antisense to the Epstein–Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) suppresses LMP1 and bcl-2 expression and promotes apoptosis in EBV-immortalized B cells. Blood 1998;92:1721–7.
[41] Fennewald S, van Santen V, Kieff E. Nucleotide sequence of an mRNA transcribed in latent growth-transforming virus infection indicates that it may encode a membrane protein. J Virol 1984;61:411–9.
[42] Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M. Cofactor dynamics and sufficiency in estrogen receptor–regulated transcription. Cell 2000;103:843–52.
[43] Chao CC, Lee Y-L, Lin-Chao S. Phenotypic reversion of cisplatin resistance accompanies reduced host cell reactivation of damaged plasmid. Biochem Biophys Res Commun 1990;170:851–9.
[44] Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, et al. Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. J Biol Chem 1997;272:21096–103.
[45] Wilczynska KM, Gopalan SM, Bugno M, Kasza A, Konik BS, Bryan L, et al. A novel mechanism of tissue inhibitor of metalloproteinases-1 activation by interleukin-1 in primary human astrocytes. J Biol Chem 2006;281:34955–64.
[46] Soni V, Cahir-McFarland E, Kieff E, LMP1 TRAFficking activates growth and survival pathways. Adv Exp Med Biol 2007;597:173–87.
[47] Chang YS, Lin SY, Lee PF, Durff T, Chung HC, Tsai MS. Establishment and characterization of a tumor cell line from human nasopharyngeal carcinoma tissue. Cancer Res 1989;49:6752–7.
[48] Yang L, Lu Z, Ma X, Cao Y, Sun LQ. A therapeutic approach to nasopharyngeal carcinomas by DNAzymes targeting EBV LMP1 gene. Molecules 2010;15:6127–39.
[49] Chen CC, Chen LC, Liang Y, Tsang NM, Chang YS. Epstein–Barr virus latent membrane protein 1 induces the chemotherapeutic target, thymidine phosphorylase, via NF-(B and p38 MAPK pathways. Cell Signal 2010;22:1132–42.
[50] Ng SB, Selvarajan V, Huang G, Zhou J, Feldman AL, Law M, et al. Activated oncogenic pathways and therapeutic targets in extranodal nasal-type NK/T cell lymphoma revealed by gene expression profiling. J Pathol 2011;223:496–510.
[51] Lee DY, Sudgen B. The latent membrane protein 1 oncogene modifies B-cell physiology by regulating autophagy. Oncogene 2008;27:2833–42.
[52] Schleicher SM, Moretti L, Varki V, Lu B. Progress in the unraveling of the endoplasmic reticulum stress/autophagy pathway and cancer: Implications for future therapeutic approaches. Drug Resist Updates 2010;13:79–86.