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Tpl2/AP-1 Enhances Murine Gammaherpesvirus 68 Lytic Replication▽

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How cellular factors regulate gammaherpesvirus lytic replication is not well understood. Here, through functional screening of a cellular kinase expression library, we identified mitogen-activated protein kinase kinase kinase 8 (MAP3K8/Tpl2) as a positive regulator of murine gammaherpesvirus 68 (MHV-68 or γHV-68) lytic gene expression and replication. Tpl2 enhances MHV-68 lytic replication by upregulating lytic gene expression and promoter activities of viral lytic genes, including RTA and open reading frame 57 (ORF57). By screening a cellular transcription factor library, we identified the Fos AP-1 transcription factor as a downstream factor that is both necessary and sufficient for mediating the enhancement of MHV-68 lytic replication by Tpl2. In addition, Tpl2 stimulates the promoter activities of key viral lytic genes, including RTA and ORF57, in an AP-1-dependent manner. We identified an AP-1-responsive element on the MHV-68 RTA promoter as the *cis* element mediating the upregulation of RTA promoter activity by Tpl2. MHV-68 lytic infection upregulates Fos expression, AP-1 activity, and RTA promoter activity in a Tpl2-dependent manner. We constructed a mutant MHV-68 virus that abolished this AP-1-responsive element. This mutant virus exhibited attenuated lytic replication kinetics, indicative of a critical role of this AP-1-responsive element during lytic replication. Moreover, Tpl2 knockdown inhibited the lytic replication of wild-type MHV-68 (MHV-68-WT) but not that of the MHV-68 mutant virus, indicating that endogenous Tpl2 promotes efficient virus lytic replication through AP-1-dependent upregulation of RTA expression. In summary, through tandem functional screens, we identified the Tpl2/AP-1 signaling transduction pathway as a positive regulator of MHV-68 lytic replication.

Gammaherpesviruses are a family of large, membrane-enveloped, double-stranded DNA viruses, including Epstein-Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV), herpesvirus saimiri, and murine gammaherpesvirus 68 (MHV-68 or γHV-68). Human gammaherpesviruses EBV and KSHV are associated with a number of malignancies. EBV is associated with Burkitt's lymphoma, nasopharyngeal carcinoma, gastric carcinoma, and Hodgkin's disease (14). KSHV is the etiological agent of three types of human tumors: Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and a plasmablastic variant of multicentric Castleman disease (MCD) (7, 8, 17).

Gammaherpesviruses, like other herpesviruses, have two phases in their life cycles, i.e., latency and lytic replication. Although latent infection is essential for gammaherpesvirus-associated tumorigenesis, lytic reactivation and lytic replication are also believed to play important roles in the persistent infection by gammaherpesviruses and their associated pathogeneses (15). Upon de novo lytic infection or reactivation from latency, a cascade of viral lytic genes is expressed. Herpesvirus lytic genes are classified as immediate early (IE), early (E), and late (L) (28). Viral IE transcription factors, including ZTA and RTA in EBV (13, 19, 38) and RTA in KSHV (38, 52) and MHV-68 (62), control the transcription of other viral lytic genes and are therefore important for initiating the whole lytic cascade. Early genes encode proteins important for viral genomic DNA replication, which is required for the expression of late genes, many of which encode structural proteins (27, 40). Virus assembly and egress complete the virus lytic replication cycle.

Many questions still remain unanswered regarding the regulation of gammaherpesvirus lytic replication, one critical aspect of which is the roles that cellular genes play. As with all other viruses, gammaherpesviruses rely on cellular machineries for replication and propagation. For example, several cellular genes have been shown to mediate KSHV entry in different types of cells (1, 31, 46). Other cellular genes, such as those for topoisomerase I, topoisomerase II, and poly(ADP-ribose) polymerase 1 (PARP-1), were shown to function during KSHV lytic DNA replication (58).

Gammaherpesviruses have a complex life cycle and therefore critically depend on their ability to sense specific cellular contexts to undergo different phases of their life cycle accordingly. Thus, cellular factors may play an even bigger role in influencing the fate of gammaherpesviruses than they do for other viruses that have simpler life cycles. Because of the critical role that RTA and/or ZTA plays in initiating the whole lytic replication cascade, a number of studies have focused on cellular factors that regulate RTA and ZTA. For example, several cellular factors, such as NF-κB, PARP-1, and KSHV-RTA-binding protein (K-RBP), were shown to inhibit gammaherpesvirus lytic replication through inhibiting RTA expression or activity (4, 24, 64), whereas RBP-Jκ (CSL or CBF1), CREB-binding protein (CBP), SWI/SNF, and CCAAT/enhancer-binding protein-α (C/EBPα) have been found to up-

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regulate RTA's transcriptional activity and lytic replication (22, 23, 35, 36, 57).

Despite the progress made, there is little doubt that the majority of the cellular genes that regulate gammaherpesvirus lytic replication have yet to be discovered. The identification and study of such cellular factors are hampered by the lack of a cell culture system that can support robust lytic replication of EBV or KSHV. MHV-68, which shares sequence homology with EBV and KSHV, is able to undergo efficient lytic replication in a number of common cell lines, including those of human origin, and therefore provides a system to effectively study gammaherpesvirus lytic replication *in vitro* and *in vivo* (44, 49, 51, 55).

Kinases and transcription factors are critical cellular proteins that regulate many aspects of cell homeostasis, including cell survival, proliferation, differentiation, and metabolism. Therefore, gammaherpesviruses are likely to be regulated by kinases and transcription factors. Here, we utilized tandem functional genetic screens to identify cellular kinases and transcription factors regulating MHV-68 lytic replication, establishing the role of the Tpl2/AP-1 pathway in regulating MHV-68 lytic gene expression and lytic replication.

MATERIALS AND METHODS

Cells. 293T cells, 293 cells, BHK-21 cells, and Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin. All cell lines were cultured in a humidified 5% CO_2 atmosphere at 37°C.

Full-length cDNA expression library and plasmids. The MHV-68 RTA promoter reporter construct pBLRP was derived by cloning a 1,127-bp region upstream of the RTA start codon into the pBL plasmid. Serial 5′ deletion clones of the reporter construct were derived from pBLRP. RP-M1 and RP-M2 were constructed by mutating the AP-1 element on the RTA promoter using the QuikChange II XL site-directed mutagenesis kit (Stratagene). The promoter reporter plasmid for open reading frame 57 (ORF57) (57pLuc) was a kind gift from S. Speck (Emory University) (37). pGL3/M3promoter-LUC was described earlier (39). Tpl2 and Fos expression plasmids contain cDNA of each human gene. Short hairpin RNA (shRNA) lentiviral vectors were constructed by annealing two DNA oligonucleotides containing target sequences and cloning them into the pLKO.1-TRC vector (Addgene) following the instructions of the manufacturer. The sequences of the oligonucleotides are CCG GGC TGG TAG TTA GTA GCA TGT TCT CGA GAA CAT GCT ACT AAC TAC CAG CTT TTT G (shFos-1 forward), AAT TCA AAA AGC TGG TAG TTA GTA GCA TGT TCT CGA GAA CAT GCT ACT AAC TAC CAG C (shFos-1 reverse), CCG GGC GGA GAC AGA CCA ACT AGA ACT CGA GTT CTA GTT GGT CTG TCT CCG CTT TTT G (shFos-2 forward), AAT TCA AAA AGC GGA GAC AGA CCA ACT AGA ACT CGA GTT CTA GTT GGT CTG TCT CCG C (shFos-2 reverse), CCG GCA CTG CTT ACA CGT CTT CCT TCT CGA GAA GGA AGA CGT GTA AGC AGT GTT TTT G (shFos-3 forward), AAT TCA AAA ACA CTG CTT ACA CGT CTT CCT TCT CGA GAA GGA AGA CGT GTA AGC AGT G (shFos-3 reverse), CCG GGC GCC TTT GGA AAG GTA TAC TCT CGA GAG TAT ACC TTT CCA AAG GCG CTT TTT G (shTpl2 forward), AAT TCA AAA AGC GCC TTT GGA AAG GTA TAC TCT CGA GAG TAT ACC TTT CCA AAG GCG C (shTpl2 reverse), CCG GCA ACA AGA TGA AGA GCA CCA ACT CGA GTT GGT GCT CTT CAT CTT GTT GTT TTT G (shCtrl forward), and AAT TCA AAA ACA ACA AGA TGA AGA GCA CCA ACT CGA GTT GGT GCT CTT CAT CTT GTT G (shCtrl reverse). Full-length kinase and transcription factor cDNA expression libraries were constructed by OriGene Technologies. Tam-67 was constructed as described previously (5).

Cell transfection. An appropriate number of cells was seeded onto each well of a 48-well plate so that cells were 80 to 90% confluent at the time of transfection. Cells were transfected with expression plasmids, reporter plasmids, or shRNA vectors using Lipofectamine 2000 (Invitrogen) following the manufacturer's recommendations.

Viruses and plaque assays. MHV-68 was originally obtained from the American Type Culture Collection (VR1465). The recombinant MHV-68-M3FL virus was constructed by S. Hwang by integrating to the left side of the wild-type MHV-68 (MHV-68-WT) viral genome a firefly luciferase gene driven by the viral M3 promoter (29). The recombinant viral bacterial artificial chromosome (BAC) plasmid MHV-68-AP1M containing mutations in the AP-1-responsive element on the RTA promoter was constructed through a two-step allelic exchange described earlier (50, 60). The successful incorporation of mutations was verified by PCR and DNA sequencing. The integrity of the mutant BAC plasmid was verified by comparing the restriction digestion pattern of the mutant BAC plasmid with that of the wild-type BAC plasmid. The mutant virus was reconstituted by transfecting 293T cells with the recombinant MHV-68-AP1M BAC plasmid. Virus stocks were grown by infecting BHK-21 cells at 0.1 PFU per cell. The titers of the produced viruses were determined by plaque assays, using a monolayer of BHK-21 or Vero cells overlaid with 1% methylcellulose. After 5 days of infection, the cells were fixed and stained with 2% crystal violet in 20% ethanol, and plaques were counted to determine the titers.

Quantitation of MHV-68-M3FL virus titer by firefly luciferase assay. A total of 1.5×10^3 293T cells were seeded in 70 μl of medium into each well of a 96-well plate 1 day prior to infection, and 50 μl of different dilutions of samples containing MHV-68-M3FL virus was added to each well. Luciferase activities were determined using the Bright-Glo luciferase assay system (Promega).

shRNA lentiviral vector production and knockdown of genes. To produce shRNA lentiviruses, shRNA vector was cotransfected with pCMV-dR8.2 dvpr (Addgene) and pCMV-VSV-G (Addgene) into 293T cells using the calcium phosphate transfection method. Lentiviruses were collected at 48 and 72 h posttransfection, filtered through 0.45-μm filters, and concentrated by centrifugation at 15,000 rpm using a Sorvall SA-600 rotor for 4 h at 2°C. Supernatant was removed, and virus pellets were resuspended with phosphate-buffered saline (PBS). To transduce cells, virus was added to cells seeded in medium containing 4 μg/ml Polybrene. When selection of transduced cells was required, 293T or 293 cells were subjected to 3-μg/ml puromycin selection at 24 h posttransduction for 1 day.

Luciferase reporter assay. 293T or 293 cells were seeded onto 48-well plates at 24 h prior to transfection so that cells were ~90% confluent by the time of transfection. Cells were then transfected with a DNA mixture containing 1 ng MHV-68 RTA promoter firefly luciferase reporter plasmid (Rp) or 0.2 ng ORF57 promoter reporter plasmid (57pLuc) together with 0.1 ng pRL-SV40 *Renilla* luciferase plasmid and cDNA expression and/or shRNA plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The total amount of DNA transfected for each well was adjusted to 0.4 μg using appropriate control plasmids. Cells were cultured for an additional 24 or 48 h, followed by dual-luciferase assays using a kit provided by Promega.

Reverse transcription-quantitative PCR (RT-Q-PCR) analysis. Cells were transfected with expression plasmids as described above. At 24 h posttransfection, cells were infected with MHV-68 at a multiplicity of infection (MOI) of 1. Cells were collected at different time points postinfection, and RNA was purified using a kit provided by Invitrogen, after which first-strand cDNA was synthesized using Superscript III polymerase (Invitrogen). Sybr green PCR was performed using probes specific for MHV-68 RTA and the human β-actin gene. The primers used for assaying ORF50 were previously described (30).

Antibodies and Western blotting. Fos (9F6) rabbit monoclonal antibody (MAb) was purchased from Cell Signaling Technology. Tpl2 (M-20) antibody was purchased from Santa Cruz Biotechnology. Rabbit hyperimmune serum against MHV-68-infected rabbit cells (53) and anti-ORF52 polyclonal serum (2) were described earlier. Anti-rabbit or anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (Cell Signaling Technology) was used as a secondary antibody. The proteins were detected by chemiluminescence detection (ECL Plus system; Amersham Pharmacia Biotech) and analyzed using a Storm imaging system (Molecular Dynamics).

RESULTS

Tpl2 enhances MHV-68 lytic replication. To systematically study the effects of cellular gene expression on MHV-68 lytic replication, we screened a library of 353 kinase genes for those that can enhance or inhibit MHV-68 lytic replication. The overall scheme of the screen is illustrated in Fig. 1A. Briefly, we first reverse transfected 60 ng of individual kinase expression plasmid predotted in wells of a 384-well plate into 293T cells. Since plasmid DNA had been predotted into wells of the

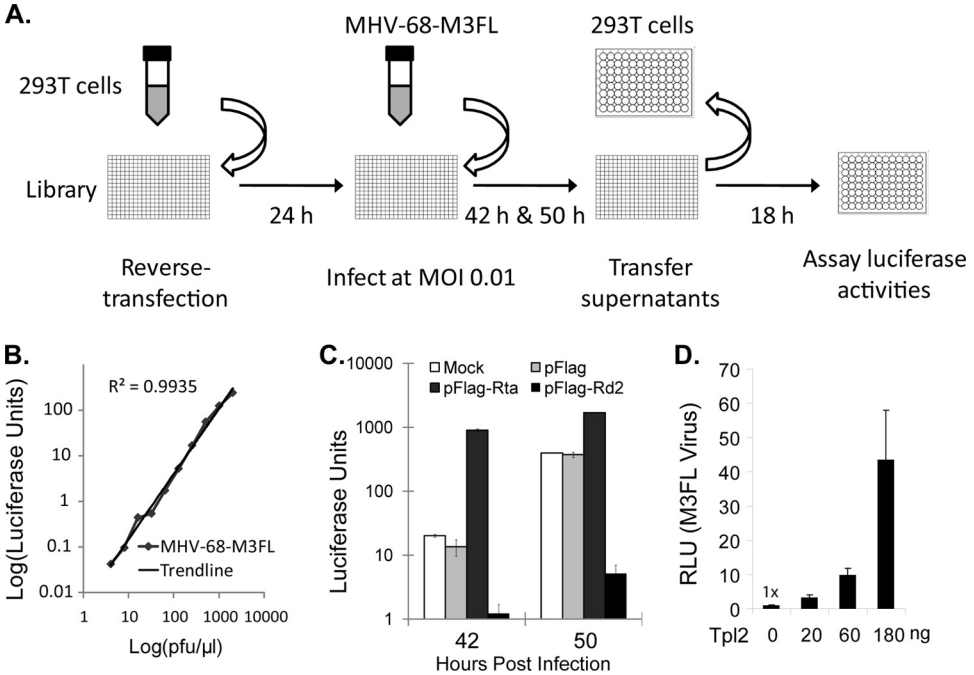

FIG. 1. Tpl2 enhances MHV-68 lytic replication. (A) Overall scheme of the screen. The individual kinase expression plasmids predotted on a 384-well plate were reverse transfected into 293T cells. Cells were infected with MHV-68-M3FL at an MOI of 0.01 at 24 h posttransfection. At 42 and 50 h postinfection, 10 μl of supernatants was transferred to naive 293T cells that were seeded on 96-well plates (referred to as destination plates) 1 day prior to transfer. Luciferase activities of 293T cells on the destination plates were measured at 18 h posttransfer. (B) Luciferase activities as indicators of MHV-68-M3FL titers. 293T cells were infected with serially diluted MHV-68-M3FL viruses. Luciferase activities were determined at 18 h postinfection and plotted against titers determined by plaque assays. Representative results are presented as the means for triplicates. (C) Effects of RTA and a dominant negative mutant of RTA on MHV-68-M3FL lytic replication. 293T cells were mock transfected or transfected with the indicated plasmids, followed by MHV-68-M3FL infection, supernatant transfer, and luciferase assays of cells on the destination plate as described for panel A. Error bars indicate standard deviations. (D) Dose response of MHV-68-M3FL lytic replication to MAP3K8 transfection. Virus production at 42 h postinfection from 293T cells transfected with the indicated amounts of the MAP3K8 plasmid was determined as for panel C.

384-well plate, reverse transfection was more suitable for our screen than conventional transfection due to the reverse order of addition of DNA and adherent cells. We used 293T cells for the screen because 293T cells can be transfected at high efficiency using typical liposomal transfection reagents (>90% cells as determined by enhanced green fluorescent protein [EGFP] fluorescence microscopy after cells were transfected with a plasmid encoding EGFP [pEGFP-N1] [data not shown]). More importantly, 293T cells support robust replication of MHV-68. We then infected cells that had been transfected with individual cDNA clones with a recombinant MHV-68 reporter virus, MHV-68-M3FL, that encodes firefly luciferase under control of the viral lytic gene M3 promoter (29). Cells were infected at a multiplicity of infection (MOI) of 0.01. We did not use the luciferase activities from the original cells (hereafter referred to as producer cells) that were transfected with cDNA clones as indicators of virus lytic replication, because the expression of cellular kinases may directly affect the activity of the M3 promoter driving the firefly luciferase gene, independent of the kinases' effects on virus replication. Instead, at 42 and 50 h after infection, we transferred supernatants containing viruses produced and released by kinase-expressing producer cells to naive 293T cells (hereafter referred to as destination cells) that had not been transfected with any cDNA clone. The relative amounts of viruses in the transferred supernatants were then determined by assaying luciferase activities at 18 h after supernatant transfer. Because luciferase activities representing virus titers were assayed in destination cells that did not express any exogenous cDNA kinase, the potential confounding effect of kinase expression on M3FL activity in producer cells was avoided. The luciferase activities assayed in naive cells have a near-linear relationship with virus titers determined by plaque assays across a wide range of virus titers and were therefore used to indicate the relative amounts of virus (Fig. 1B). The use of MHV-68-M3FL in combination with luciferase assays enabled us to quickly (in 1 day instead of 6 days, as required for conventional plaque assays) determine the relative amounts of viruses in the supernatants in a much less laborious way and was therefore suitable for our medium-throughput screen. We used RTA and Rd2 (61), a dominant negative version of RTA, as positive and negative regulators of MHV-68 lytic replication, respectively, to optimize the screen conditions. Under the optimized conditions, RTA and Rd2 significantly increased and decreased the luciferase readings, respectively, indicating that RTA enhanced, whereas Rd2 inhibited, virus lytic replication and production (Fig. 1C). RTA and Rd2 were subsequently used as controls in our screen.

Through this screen, we found that several members of the mitogen-activated protein kinase (MAPK) family, including

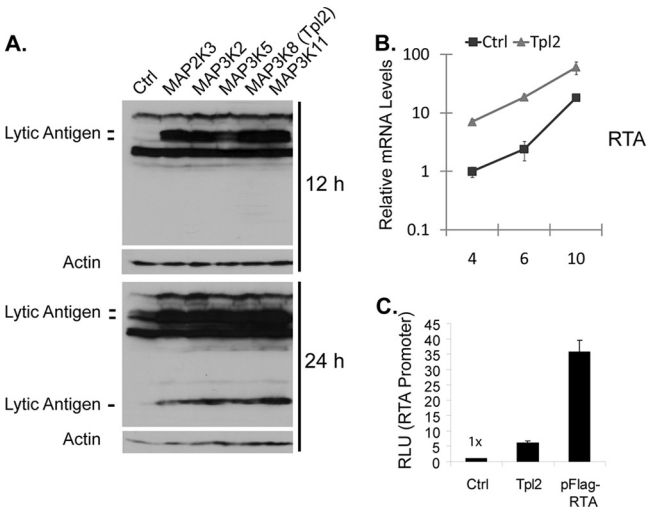

FIG. 2. Tpl2 upregulates viral lytic gene expression. (A) Western blotting analysis of MHV-68 lytic protein expression in cells expressing various kinases. 293T cells were transfected with individual kinase expression plasmids and infected with MHV-68 at an MOI of 1. At 12 or 24 h postinfection, whole-cell lysates were analyzed by Western blotting for MHV-68 lytic antigen expression using rabbit hyperimmune serum against MHV-68-infected rabbit cells. An actin immunoblot is shown as a loading control. (B) Tpl2 increases the mRNA levels of RTA. 293T cells were transfected with Tpl2 or the backbone control plasmid and infected with MHV-68 at an MOI of 1 at 24 h posttransfection. The relative mRNA levels of RTA normalized to actin were determined by reverse transcription-quantitative PCR at the indicated time points postinfection. (C) Tpl2 activates the MHV-68 RTA promoter. 293T cells were cotransfected with pBLRP, a luciferase reporter construct containing approximately 1.1 kb of upstream sequence of RTA, the control plasmid SV40-RL (*Renilla* luciferase), and Tpl2, pFlag-RTA, or the control backbone plasmid. Luciferase activity was determined at 24 h posttransfection. Results are presented as the means for triplicates with their standard deviations.

MAP2K3, MAP3K2, MAP3K5, MAP3K8/Tpl2/COT, and MAP3K11, enhanced MHV-68 lytic replication when overexpressed (Fig. 2A and data not shown). We focused on one of the strongest stimulators, MAP3K8/Tpl2/COT (hereafter referred to as Tpl2) to study the mechanism by which it enhances MHV-68 lytic replication. We confirmed the ability of Tpl2 expression to promote viral lytic replication in a secondary screen of the hits from our primary screen and showed that Tpl2 expression enhanced MHV-68 lytic replication in a dose-dependent manner (Fig. 1D).

Tpl2 upregulates viral lytic gene expression. To determine which step(s) of virus replication is affected by Tpl2 expression, we first examined the effect of Tpl2 expression on MHV-68 lytic protein expression during virus lytic replication (Fig. 2A). Cells were transfected with plasmids encoding kinases or the control backbone plasmid. At 24 h posttransfection, cells were infected with MHV-68 at an MOI of 1. At both 12 h and 24 h postinfection, the expression of Tpl2 as well as other kinases examined enhanced MHV-68 lytic protein expression (Fig. 2A). These results suggest that Tpl2 may promote virus replication through increasing the levels of viral lytic proteins. We then asked if Tpl2 enhanced virus lytic gene expression at the mRNA level. RTA, a transcription factor encoded by MHV-68, is necessary and sufficient to initiate MHV-68 lytic replication during both de novo infection and lytic reactivation (61, 62). We therefore examined the effects of Tpl2 expression on the mRNA levels of RTA during MHV-68 lytic replication (Fig. 2B). Cells transfected with a Tpl2 expression plasmid or the control plasmid were infected with MHV-68 at an MOI of 1. Cells were collected at 4, 6, and 10 h postinfection. The mRNA levels of RTA were then measured by reverse transcription-quantitative PCR (RT-Q-PCR). Tpl2 increased the mRNA levels of RTA 7-fold at as early as 4 h postinfection (Fig. 2B). The mRNA levels of RTA in Tpl2-transfected cells remained significantly higher than those from the control transfection at 6 and 10 h postinfection. RTA transactivates the expression of other viral genes, including viral early genes which are required for viral DNA replication and the expression of late genes. Not surprisingly, the mRNA levels of open reading frame 6 (ORF6), a viral early gene, and M9 (or ORF65), a viral late gene, also increased in cells transfected with Tpl2 compared with cells transfected by the control plasmid (data not shown).

We then asked if Tpl2 enhanced RTA mRNA levels through upregulating RTA transcription. We used luciferase reporter assays to determine if Tpl2 expression affects RTA promoter activity (Fig. 2C). Cells were cotransfected with Tpl2 or the control plasmid together with an RTA promoter luciferase reporter plasmid. Luciferase activities were determined at 24 h posttransfection. Tpl2, as well as RTA, significantly enhanced MHV-68 RTA promoter activity (Fig. 2C), indicating that Tpl2 enhances RTA mRNA levels through activating RTA transcription. We concluded that Tpl2 enhances MHV-68 lytic gene expression and enhances viral lytic replication.

Tpl2 enhances MHV-68 lytic replication through upregulating AP-1 activities. Tpl2 may regulate transcription factors through mechanisms such as activation of downstream MAPK pathways. We hypothesized that some of those downstream transcription factors might mediate the enhancement of MHV-68 lytic replication by Tpl2. To identify them, we screened a library of approximately 700 transcription factors for the ones that can enhance MHV-68 lytic replication using the same procedure we used for our kinase library screen. Our initial screen identified several transcription factors, including the AP-1 transcription factor Fos, that enhanced MHV-68 lytic replication when overexpressed. Verification experiments confirmed that the overexpression of Fos enhanced MHV-68 lytic replication (Fig. 3A).

Overexpression of Tpl2 has been shown to activate MAPK pathways through MEK1 and MEK4, as well as through MEK6 and MEK5, leading to the activation of downstream extracellular signal-regulated kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs), and p38 kinases (11, 48). Other studies demonstrated that the ERK pathway may enhance Fos transcriptional activity through multiple mechanisms (9, 41, 42, 54, 59), suggesting a role for Fos in mediating the enhancement of MHV-68 lytic replication by Tpl2. Indeed, the Fos protein level was significantly increased by Tpl2 transfection, suggesting that increased Fos protein may contribute to the enhancement of MHV-68 lytic replication by Tpl2 (Fig. 3B). To test whether Fos upregulation is required for Tpl2-induced enhancement of MHV-68 lytic replication, we cotransfected shFos-1 or shFos-2, two small hairpin RNA (shRNA) lentiviral vectors targeting Fos, with the Tpl2 ex-

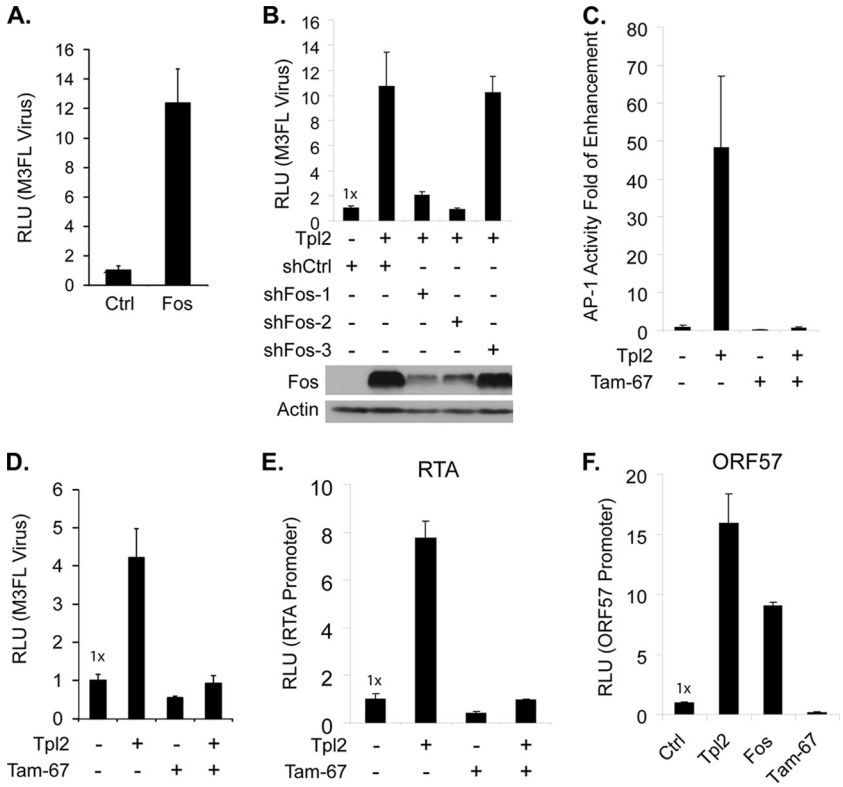

FIG. 3. Tpl2 enhances MHV-68 lytic replication through upregulation of AP-1 activities. (A) Effects of Fos on MHV-68-M3FL replication. 293T cells were transfected with a Fos expression plasmid or control plasmid, followed by MHV-68-M3FL infection, supernatant transfer at 42 h postinfection, and luciferase assays of cells on the destination plate as described for Fig. 1C. Error bars indicate standard deviations. (B) shRNA against Fos inhibits Fos upregulation and MHV-68 lytic replication induced by Tpl2. 293T cells were cotransfected with Tpl2 or the control backbone plasmid with shRNA vectors targeting Fos or a control shRNA vector, shCtrl. For Western blotting analysis, whole-cell lysates were subjected to SDS-PAGE followed by Western blotting and analyzed for Fos expression at 48 h posttransfection. An actin immunoblot is shown as a loading control. For MHV-68 lytic replication assay, transfected cells were infected with MHV-68-M3FL, followed by supernatant transfer to fresh 293T cells and luciferase analysis. (C) Effects of Tpl2 or Tam-67 on AP-1 activities. 293T cells were cotransfected with an AP-1 reporter construct, SV40-RL, and Tpl2, Tam-67, or Tpl2 plus Tam-67. Luciferase activities were determined at 24 h posttransfection. (D) Tam-67 inhibits the enhancement of virus replication by Tpl2. 293T cells were transfected with the indicated plasmids, followed by MHV-68-M3FL infection, supernatant transfer at 42 h postinfection, and luciferase assays of cells on the destination plate as described for Fig. 1C. (E) Tam-67 inhibits the activation of the RTA promoter by Tpl2. The RTA promoter reporter plasmid, pBLRP, and SV40-RL were cotransfected with the indicated expression plasmids into 293T cells. Luciferase activities were determined at 24 h posttransfection. (F) Tpl2 and Fos activate the ORF57 promoter. 57pLuc and SV40-RL were cotransfected with the indicated expression plasmids into 293T cells. Luciferase activities were determined at 24 h posttransfection.

pression plasmid to inhibit the upregulation of Fos by Tpl2 (Fig. 3B). Both shFos-1 and shFos-2 significantly inhibited the enhancement of MHV-68 replication by Tpl2, whereas shCtrl, an shRNA vector that does not target any cellular genes, or shFos-3, which was designed to target Fos but did not efficiently knock down Fos expression, failed to inhibit MHV-68 replication (Fig. 3B), indicating that Fos upregulation is required for Tpl2 to enhance MHV-68 lytic replication.

Previous studies have shown that Fos can bind to Jun to form a heterodimeric AP-1 factor and that Tpl2 activates the activity of Jun through enhancing both its expression and phosphorylation (11, 48). We therefore asked if Fos/Jun AP-1 activity is required for the enhancement of MHV-68 lytic replication by Tpl2. Tam-67 is a transactivation domain deletion mutant of Jun and can function in a dominant negative manner to repress AP-1-dependent transcription by forming dimers with wild-type AP-1 factors (5). As expected, Tam-67 when cotransfected with Tpl2 largely abolished the enhancement of AP-1 activity by Tpl2 in a luciferase reporter assay (Fig. 3C). In addition, Tam-67 cotransfection inhibited the enhancement of virus replication by Tpl2 (Fig. 3D). Interestingly, the transfection of Tam-67 alone without Tpl2 also inhibited MHV-68 lytic replication (Fig. 3D), suggesting that endogenous Fos/Jun AP-1 activity may also be important for efficient MHV-68 lytic replication in the absence of Tpl2 overexpression.

Because Tpl2 expression increased RTA mRNA levels during virus lytic replication (Fig. 2B), we next asked whether AP-1 promotes RTA transcription downstream of Tpl2. Tpl2 transfection alone increased MHV-68 RTA promoter activity over 7-fold (Fig. 3E). Tam-67 cotransfection abolished the activation of the RTA promoter by Tpl2 (Fig. 3E). Notably, Tam-67 transfection alone caused an >50% drop of RTA promoter activity (Fig. 3E), mimicking the inhibition of AP-1 activity (Fig. 3C) and virus replication (Fig. 3D) by Tam-67 transfection. Thus, it appears that endogenous AP-1 activity is important for both RTA expression and virus replication.

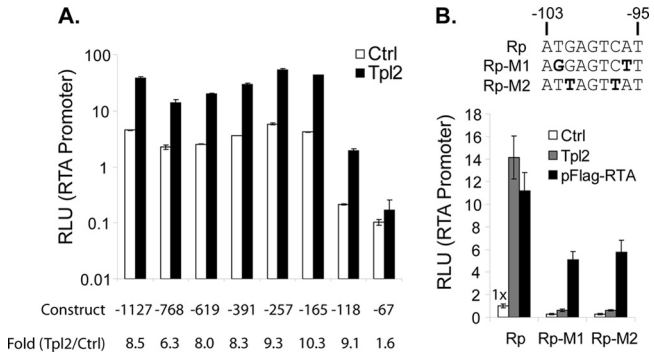

FIG. 4. Tpl2 activates the MHV-68 RTA promoter via an AP-1-responsive element. (A) 293T cells were cotransfected with RTA promoter luciferase construct pBLRP with serial 5′ deletions and SV40-RL, a control *Renilla* luciferase plasmid, and Tpl2 or the control backbone plasmid. Luciferase activity was determined at 24 h post-transfection. (B) 293T cells were cotransfected with mutant pBLRP with nucleotides in the AP-1 elements mutated and SV40-RL, a control *Renilla* luciferase plasmid, and Tpl2, pFlag-RTA, or the control backbone plasmid. Luciferase activity was determined at 24 h post-transfection. The upper panel shows the mutations introduced into pBLRP. Results are presented as the means for triplicates with their standard deviations.

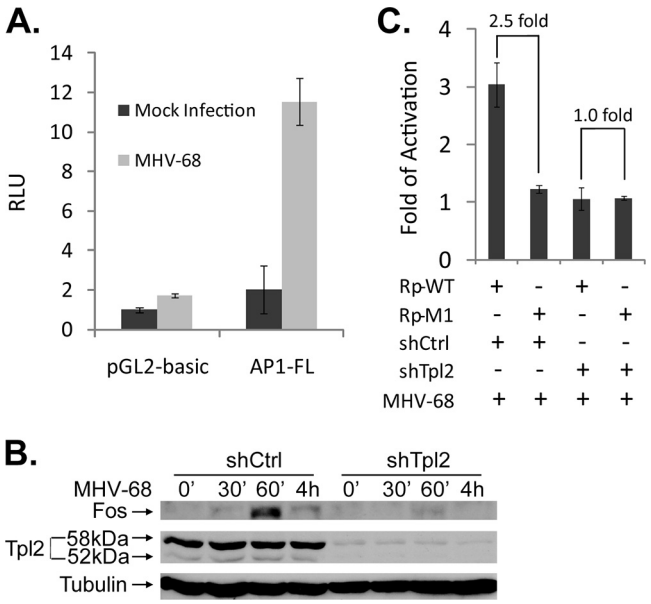

FIG. 5. Endogenous Tpl2 promotes AP-1-dependent activation of the RTA promoter during virus lytic replication. (A) 293 cells were transfected with AP-1 luciferase reporter plasmid AP1-FL or the parental vector pGL2-basic. At 12 h posttransfection, cells were mock infected or infected with MHV-68; the cells were subjected to luciferase reporter assay at 24 h postinfection. Error bars indicate standard deviations. (B) 293 cells were transduced with shCtrl or shTpl2. At 3 days posttransduction, cells were infected with MHV-68; cells were collected at different time points postinfection for Western blotting analysis. (C) 293 cells were transduced with shCtrl or shTpl2. At 3 days posttransduction, cells were transfected with Rp-WT or Rp-M1 reporter plasmid. At 12 h posttransfection, cells were mock infected or infected with MHV-68; cells were subjected to luciferase reporter assay at 24 h postinfection. The fold activation (infection/mock infection) was calculated.

In addition to promoting RTA expression, Tpl2 might also directly enhance the expression of other viral lytic genes through activating AP-1. Previous studies showed that KSHV ORF57 is essential for infectious virion production, and an AP-1 binding motif has been identified in the KSHV ORF57 promoter region (6, 25, 56). We found that transfection of either Tpl2 or Fos enhanced the ORF57 promoter activity, whereas Tam-67 transfection inhibited ORF57 promoter activity (Fig. 3F), suggesting that Tpl2 expression may also enhance ORF57 expression through upregulating AP-1. Interestingly, the AP-1-responsive element previously identified in the KSHV ORF57 promoter (56) is conserved in the MHV-68 ORF57 promoter at a similar position relative to the ORF57 start codon (data not shown). Therefore, we concluded that Tpl2 enhances both the transcription of viral lytic genes such as RTA and ORF57 and MHV-68 lytic replication through the upregulation of AP-1 activity.

Tpl2 activates the MHV-68 RTA promoter via an AP-1-responsive element. Because of the critical role of RTA in controlling the whole cascade of viral lytic gene expression, we asked if a *cis* element in the RTA promoter region mediates the enhancement of RTA promoter activity by Tpl2. We measured the effects of Tpl2 expression on a series of RTA promoter luciferase reporter constructs with increasing lengths of deletions from the 5′ end (Fig. 4A). We found that the deletion of a region encompassing bp 67 to 118 upstream of the RTA ATG start codon greatly reduced the activation of the RTA promoter by Tpl2, from 9.1-fold to 1.6-fold (Fig. 4A). Examination of this region revealed an AP-1 motif (ATGAGTCAT) spanning the region from bp −103 to −95 (Fig. 4B, upper panel). We previously showed that Tpl2 expression enhanced AP-1 activity (Fig. 3C) and activated the RTA promoter in an AP-1-dependent manner (Fig. 3E). Therefore, this AP-1 motif may mediate the activation of the RTA promoter by Tpl2. To examine this, we constructed two RTA promoter reporter constructs, each of which has two nucleotides within the AP-1 motif mutated (Fig. 4B, upper panel). Both sets of double mutations largely abolished the ability of Tpl2 to activate the RTA promoter (Fig. 4B, lower panel), indicating that this AP-1 motif indeed mediates the activation of the RTA promoter by Tpl2. We also note that the mutant promoters showed lower activities than the wild-type promoter when cells were transfected with either the control plasmid or the RTA expression plasmid (Fig. 4B, lower panel). However, the relative fold activation by RTA did not decrease when the AP-1 motif was mutated, indicating that the AP-1 motif specifically mediates the activation of the RTA promoter by Tpl2 but not by RTA (Fig. 4B, lower panel).

Endogenous Tpl2 contributes to AP-1 activation during MHV-68 lytic infection. Mindful of the possibility that the roles attributed to overexpressed Tpl2 in our previous experiments might not reflect the real function of Tpl2 at a more physiologically relevant level, we performed a series of experiments exploring the role of endogenous Tpl2 during MHV-68 lytic replication (Fig. 5 and 6). Having shown that overexpression of Tpl2 activates the RTA promoter in an AP-1-dependent manner, we asked if endogenous Tpl2 plays a role in the activation of AP-1 and the RTA promoter during MHV-68 lytic replication. We first determined if AP-1 activity is upregulated in cells infected with MHV-68 in the absence of overexpression of

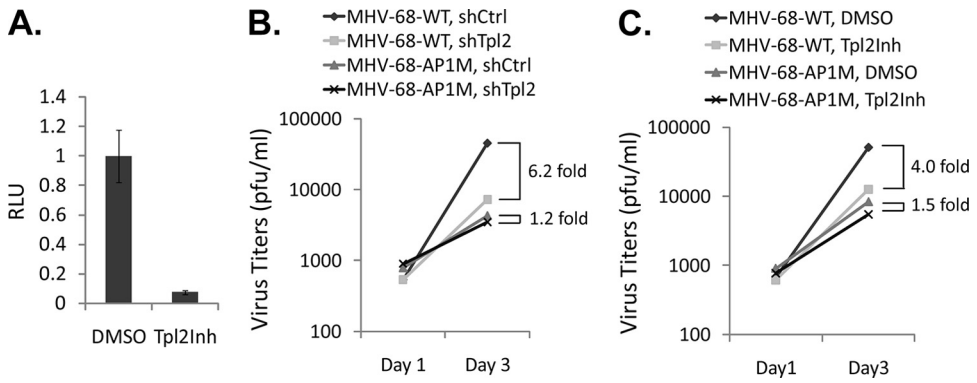

FIG. 6. Endogenous Tpl2 promotes MHV-68 lytic replication through facilitating AP-1-dependent activation of the RTA promoter. (A) 293 cells were pretreated with Tpl2 kinase inhibitor (Tpl2Inh) or dimethyl sulfoxide (DMSO) vehicle control for an hour and were then infected with MHV-68-M3FL in the presence of Tpl2Inh or DMSO, followed by supernatant transfer to fresh 293T cells and luciferase analysis. Error bars indicate standard deviations. (B) 293 cells were transduced with shCtrl or shTpl2. At 3 days posttransduction, cells were infected with the indicated viruses at an MOI of 0.01. Cell lysates and supernatants were collected at day 1 and day 3 after infection. Virus titers were determined by plaque assays on Vero cells. (C) 293 cells were pretreated with 5 μM Tpl2Inh or DMSO vehicle control for an hour. Cells were then infected with the indicated viruses at an MOI of 0.01 in the presence of Tpl2Inh or DMSO. Cell lysates and supernatants were collected at day 1 and day 3 after infection. Virus titers were determined by plaque assays on Vero cells.

Tpl2 (Fig. 5A). Cells were transfected with an AP-1 luciferase reporter plasmid or the control reporter plasmid, pGL2-basic. Transfected cells were then infected with MHV-68 at 12 h posttransfection. Results from reporter assays carried out at 24 h postinfection indicated that MHV-68 infection activated the AP-1 reporter over 10-fold, whereas the control reporter was activated less than 2-fold (Fig. 5A). We then asked if endogenous Tpl2 plays a role in the activation of AP-1. Since Tpl2 overexpression upregulated Fos (Fig. 3B), we examined Fos protein levels in cells in which endogenous Tpl2 was depleted through RNA interference (RNAi). We used an shRNA vector against Tpl2, shTpl2, to deplete endogenous Tpl2 (Fig. 5B). Cells were transduced with shTpl2 or shCtrl lentiviral vector 3 days prior to MHV-68 infection to allow sufficient time for endogenous Tpl2 to be significantly reduced (Fig. 5B). Cells were then infected with MHV-68 and collected at different time points postinfection for Western blotting analysis (Fig. 5B). In cells transduced with shCtrl, Fos was upregulated following MHV-68 infection, with the Fos protein level peaking at approximately 60 min postinfection (Fig. 5B). In contrast, the Fos protein level increased to a much smaller extent in cells transduced with shTpl2 (Fig. 5B), suggesting that endogenous Tpl2 is important for Fos upregulation during virus lytic infection.

Endogenous Tpl2 promotes AP-1-dependent activation of the RTA promoter during MHV-68 lytic infection. Given the presence of an AP-1 element on the RTA promoter and the contribution of endogenous Tpl2 to AP-1 activation during virus lytic infection (Fig. 4B and 5B), we asked whether endogenous Tpl2 plays a role in the activation of the RTA promoter in an AP-1-dependent manner. Cells were transduced with shTpl2 or shCtrl lentiviral vector 3 days prior to cotransfection with either the wild-type RTA promoter reporter Rp-WT or the AP-1 mutant RTA reporter Rp-M1 (Fig. 5C). Cells were infected with MHV-68 at 12 h posttransfection. Results from luciferase reporter assays carried out at 24 h postinfection indicated that in shCtrl-transduced cells, MHV-68 infection activated Rp-WT approximately 3-fold over mock infection (Fig. 5C). Mutation of the AP-1-responsive element (Rp-M1) inhibited promoter activation 2.5-fold (Fig. 5C), suggesting that AP-1 contributed to the activation of the RTA promoter during lytic infection. In contrast, the RTA promoter was not activated by MHV-68 lytic infection in cells transduced with shTpl2 (Fig. 5C), indicating that endogenous Tpl2 contributes to efficient activation of the RTA promoter during lytic replication. In addition, whereas in shCtrl-transduced cells, mutation of the AP-1-responsive element reduced the RTA promoter reporter activity 2.5-fold, the same mutation did not further reduce (1.0-fold) reporter activity in shTpl2-transduced cells (Fig. 5C), indicating that AP-1-dependent activation of the RTA promoter during MHV-68 lytic replication requires endogenous Tpl2.

Endogenous Tpl2 promotes MHV-68 lytic replication by mediating AP-1-dependent activation of the RTA promoter. Considering the contribution of endogenous Tpl2 to the activation of AP-1 and the RTA promoter, we asked if endogenous Tpl2 promotes MHV-68 lytic replication. To address this question, we used a chemical inhibitor of the Tpl2 kinase (21) in addition to the Tpl2 RNAi described above to inhibit the function of endogenous Tpl2 (Fig. 6). A virus replication assay similar to the assay used in our library screen was used to determine the effect of the inhibition of Tpl2 kinase on virus lytic replication (Fig. 6A). The lytic replication of the recombinant MHV-68 luciferase reporter virus MHV-68-M3FL was significantly inhibited by the chemical inhibitor of the Tpl2 kinase, Tpl2Inh (Fig. 6A), suggesting that endogenous Tpl2 kinase activity is required for efficient virus replication.

We then asked if endogenous Tpl2 promotes MHV-68 lytic replication through facilitating AP-1-dependent activation of RTA transcription. We constructed a recombinant MHV-68 virus, MHV-68-AP1M, that has a mutated AP-1-responsive element on the RTA promoter as in the Rp-M1 luciferase reporter. Cells were transduced with shTpl2 or shCtrl vector 3 days prior to infection with either wild-type MHV-68 (MHV-68-WT) or the mutant MHV-68 (MHV-68-AP1M) (Fig. 6B). Virus titers from cells collected at 1 day and 3 days postinfection were then

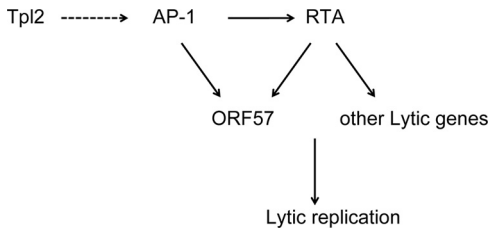

FIG. 7. Working model of the regulation of MHV-68 lytic gene expression and lytic replication by Tpl2 and AP-1 factors. Tpl2 activates AP-1 factors to upregulate RTA and ORF57 transcription. Upregulated RTA expression leads to enhanced expression of other lytic genes and lytic replication.

determined by plaque assays. At day 3 postinfection, lytic replication of MHV-68-AP1M in shCtrl-transduced cells (MHV-68-AP1M, shCtrl) was attenuated approximately 10-fold compared with that of MHV-68-WT in the same shCtrl-transduced cells (MHV-68-WT, shCtrl) (Fig. 6B), suggesting that AP-1-dependent RTA promoter activation is important for efficient virus replication. Moreover, whereas MHV-68-WT lytic replication was inhibited 6.2-fold at day 3 postinfection by Tpl2 knockdown (MHV-68-WT, shCtrl, versus MHV-68-WT, shTpl2), the replication of MHV-68-AP1M mutant virus was not significantly affected (1.2-fold) by Tpl2 knockdown (MHV-68-AP1M, shCtrl, versus MHV-68-AP1M, shTpl2) (Fig. 6B), suggesting that the full contribution of endogenous Tpl2 to MHV-68 lytic replication requires the presence of the AP-1 element on the RTA promoter. Similar results were obtained when endogenous Tpl2 was inhibited with the chemical inhibitor Tpl2Inh instead of by RNAi (Fig. 6C). Taking the results together, we concluded that endogenous Tpl2 promotes MHV-68 lytic replication through activating the RTA promoter in an AP-1-dependent manner.

DISCUSSION

In this study, through screening a cellular kinase and a transcription factor library, we demonstrated that Tpl2 promotes MHV-68 lytic replication through enhancing viral lytic gene expression, notably the expression of RTA in an AP-1 dependent manner (Fig. 7). AP-1 activity plays an important role during EBV and KSHV reactivation (18, 20, 63). Our results demonstrated the essential role of AP-1 activity in the lytic gene expression of a gammaherpesvirus during de novo infection. Tpl2 has been previously shown to be required for EGF-induced activation of Fos promoter and AP-1 activity (32), yet the exact mechanism of the activation of the Fos promoter downstream of Tpl2 is not clear. Overexpression of Tpl2 is known to activate MAPK pathways through MEK1, MEK2, MEK4, MEK5, and MEK6, leading to the activation of downstream extracellular signal-regulated kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs), and p38 kinases (11, 16, 48). The ERK pathway can enhance Fos transcriptional activity through multiple mechanisms (9, 41, 42, 54, 59). Therefore, it is possible that Tpl2 upregulates Fos expression and AP-1 activity through activating the ERK pathway. Alternatively, Tpl2 has been shown to be able to phosphorylate histone H3 and cooperate with phosphorylated H3 to activate the Fos promoter (12). Further studies probing these two possibilities may help us to further delineate the pathway linking Tpl2 and Fos.

Although we showed that the enhancement of viral replication by Tpl2 is in large part mediated by AP-1-dependent activation of the RTA promoter (Fig. 6B and C), it is entirely possible that Tpl2 also contributes to viral replication through RTA-independent upregulation of other viral lytic genes (Fig. 7). For example, we showed that overexpression of Tpl2 or Fos activated the ORF57 promoter, whereas overexpression of a dominant negative version of Jun, Tam-67, inhibited the ORF57 promoter (Fig. 3F). As noted previously, ORF57 is essential for infectious virion production by KSHV (6, 25, 56). Therefore, RTA-independent upregulation of ORF57 expression may further promote the enhancement of viral lytic replication by Tpl2.

The importance of AP-1 transcription factors in viral gene expression and lytic replication has been demonstrated in other members of the gammaherpesvirus family, such as KSHV and EBV (18, 45, 47, 56). Notably, the BZLF1/ZTA gene of EBV encodes a viral transcription factor that is related to Fos and is able to induce productive virus replication (18, 47). Although a previous study has demonstrated that downstream MAPK members such as Erk1/2, p38, and Jnk1/2 play important roles in promoting KSHV lytic gene expression and virus replication through upregulating AP-1 activities (45), which of the several upstream MAP3K members is required for efficient virus replication is not obviously clear. Our study supports a role for Tpl2 as an upstream mediator of the AP-1-dependent activation of RTA, ORF57, and other viral lytic genes during the lytic replication of MHV-68 and possibly other members of the gammaherpesviruses.

One advantage of functional screens with cDNA expression or RNAi gene knockdown is the ability to identify multiple genes that regulate virus replication in a relatively unbiased fashion (3, 10, 26, 34, 43). In addition, multiple components of a known cellular pathway or machinery can be identified through such screens, which help to link a specific pathway or machinery to virus replication (3, 10, 34). Although we employed two similar functional screens in our study to identify cellular pathways regulating virus replication, it is reasonable to believe that a combination of screens of different natures will also be able to identify novel cellular pathways or machineries functioning not only in virus replication but also in other cellular processes. Indeed, a recent study combined a genome-wide RNAi functional screen with interactome data to identify multiple cellular pathways regulating early-stage HIV-1 replication (33). Thus, the use of various combinations of functional screens and other high-throughput approaches will likely help us identify additional cellular pathways and machineries regulating virus replication with unprecedented speed in future studies.

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REFERENCES

1. **Akula, S. M., N. P. Pramod, F. Z. Wang, and B. Chandran.** 2002. Integrin alpha3beta1 (CD 49c/29) is a cellular receptor for Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) entry into the target cells. Cell **108:**407–419.
2. **Bortz, E., L. Wang, Q. Jia, T. T. Wu, J. P. Whitelegge, H. Deng, Z. H. Zhou, and R. Sun.** 2007. Murine gammaherpesvirus 68 ORF52 encodes a tegument protein required for virion morphogenesis in the cytoplasm. J. Virol. **81:** 10137–10150.
3. **Brass, A. L., D. M. Dykxhoorn, Y. Benita, N. Yan, A. Engelman, R. J. Xavier, J. Lieberman, and S. J. Elledge.** 2008. Identification of host proteins required for HIV infection through a functional genomic screen. Science **319:**921–926.
4. **Brown, H. J., M. J. Song, H. Deng, T. T. Wu, G. Cheng, and R. Sun.** 2003. NF-kappaB inhibits gammaherpesvirus lytic replication. J. Virol. **77:**8532–8540.
5. **Brown, P. H., R. Alani, L. H. Preis, E. Szabo, and M. J. Birrer.** 1993. Suppression of oncogene-induced transformation by a deletion mutant of c-jun. Oncogene **8:**877–886.
6. **Byun, H., Y. Gwack, S. Hwang, and J. Choe.** 2002. Kaposi's sarcoma-associated herpesvirus open reading frame (ORF) 50 transactivates K8 and ORF57 promoters via heterogeneous response elements. Mol. Cells. **14:**185–191.
7. **Cesarman, E., Y. Chang, P. S. Moore, J. W. Said, and D. M. Knowles.** 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N Engl. J. Med. **332:**1186–1191.
8. **Chang, Y., E. Cesarman, M. S. Pessin, F. Lee, J. Culpepper, D. M. Knowles, and P. S. Moore.** 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science **266:**1865–1869.
9. **Chen, R. H., C. Abate, and J. Blenis.** 1993. Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. Proc. Natl. Acad. Sci. U. S. A. **90:**10952–10956.
10. **Cherry, S., T. Doukas, S. Armknecht, S. Whelan, H. Wang, P. Sarnow, and N. Perrimon.** 2005. Genome-wide RNAi screen reveals a specific sensitivity of IRES-containing RNA viruses to host translation inhibition. Genes Dev. **19:**445–452.
11. **Chiariello, M., M. J. Marinissen, and J. S. Gutkind.** 2000. Multiple mitogen-activated protein kinase signaling pathways connect the cot oncoprotein to the c-jun promoter and to cellular transformation. Mol. Cell. Biol. **20:**1747–1758.
12. **Choi, H. S., B. S. Kang, J. H. Shim, Y. Y. Cho, B. Y. Choi, A. M. Bode, and Z. Dong.** 2008. Cot, a novel kinase of histone H3, induces cellular transformation through up-regulation of c-fos transcriptional activity. FASEB J. **22:**113–126.
13. **Countryman, J., and G. Miller.** 1985. Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. Proc. Natl. Acad. Sci. U. S. A. **82:**4085–4089.
14. **Delecluse, H. J., R. Feederle, B. O'Sullivan, and P. Taniere.** 2007. Epstein Barr virus-associated tumours: an update for the attention of the working pathologist. J. Clin. Pathol. **60:**1358–1364.
15. **Dourmishev, L. A., A. L. Dourmishev, D. Palmeri, R. A. Schwartz, and D. M. Lukac.** 2003. Molecular genetics of Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) epidemiology and pathogenesis. Microbiol. Mol. Biol. Rev. **67:**175–212.
16. **Dumitru, C. D., J. D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J. H. Lin, C. Patriotis, N. A. Jenkins, N. G. Copeland, G. Kollias, and P. N. Tsichlis.** 2000. TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell **103:**1071–1083.
17. **Dupin, N., T. L. Diss, P. Kellam, M. Tulliez, M. Q. Du, D. Sicard, R. A. Weiss, P. G. Isaacson, and C. Boshoff.** 2000. HHV-8 is associated with a plasmablastic variant of Castleman disease that is linked to HHV-8-positive plasmablastic lymphoma. Blood **95:**1406–1412.
18. **Farrell, P. J., D. T. Rowe, C. M. Rooney, and T. Kouzarides.** 1989. Epstein-Barr virus BZLF1 trans-activator specifically binds to a consensus AP-1 site and is related to c-fos. EMBO J. **8:**127–132.
19. **Feederle, R., M. Kost, M. Baumann, A. Janz, E. Drouet, W. Hammerschmidt, and H. J. Delecluse.** 2000. The Epstein-Barr virus lytic program is controlled by the co-operative functions of two transactivators. EMBO J. **19:**3080–3089.
20. **Flemington, E., and S. H. Speck.** 1990. Identification of phorbol ester response elements in the promoter of Epstein-Barr virus putative lytic switch gene BZLF1. J. Virol. **64:**1217–1226.
21. **Gavrin, L. K., N. Green, Y. Hu, K. Janz, N. Kaila, H. Q. Li, S. Y. Tam, J. R. Thomason, A. Gopalsamy, G. Ciszewski, J. W. Cuozzo, J. P. Hall, S. Hsu, J. B. Telliez, and L. L. Lin.** 2005. Inhibition of Tpl2 kinase and TNF-alpha production with 1,7-naphthyridine-3-carbonitriles: synthesis and structure-activity relationships. Bioorg. Med. Chem. Lett. **15:**5288–5292.
22. **Gwack, Y., H. J. Baek, H. Nakamura, S. H. Lee, M. Meisterernst, R. G. Roeder, and J. U. Jung.** 2003. Principal role of TRAP/mediator and SWI/SNF complexes in Kaposi's sarcoma-associated herpesvirus RTA-mediated lytic reactivation. Mol. Cell. Biol. **23:**2055–2067.
23. **Gwack, Y., H. Byun, S. Hwang, C. Lim, and J. Choe.** 2001. CREB-binding protein and histone deacetylase regulate the transcriptional activity of Kaposi's sarcoma-associated herpesvirus open reading frame 50. J. Virol. **75:** 1909–1917.
24. **Gwack, Y., H. Nakamura, S. H. Lee, J. Souvlis, J. T. Yustein, S. Gygi, H. J. Kung, and J. U. Jung.** 2003. Poly(ADP-ribose) polymerase 1 and Ste20-like kinase hKFC act as transcriptional repressors for gamma-2 herpesvirus lytic replication. Mol. Cell. Biol. **23:**8282–8294.
25. **Han, Z., and S. Swaminathan.** 2006. Kaposi's sarcoma-associated herpesvirus lytic gene ORF57 is essential for infectious virion production. J. Virol. **80:**5251–5260.
26. **Hao, L., A. Sakurai, T. Watanabe, E. Sorensen, C. A. Nidom, M. A. Newton, P. Ahlquist, and Y. Kawaoka.** 2008. Drosophila RNAi screen identifies host genes important for influenza virus replication. Nature **454:**890–893.
27. **Holland, L. E., K. P. Anderson, C. Shipman, Jr., and E. K. Wagner.** 1980. Viral DNA synthesis is required for the efficient expression of specific herpes simplex virus type 1 mRNA species. Virology **101:**10–24.
28. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14:**8–19.
29. **Hwang, S., T. T. Wu, L. M. Tong, K. S. Kim, D. Martinez-Guzman, A. D. Colantonio, C. H. Uittenbogaart, and R. Sun.** 2008. Persistent gammaherpesvirus replication and dynamic interaction with the host in vivo. J. Virol. **82:**12498–12509.
30. **Jia, Q., V. Chernishof, E. Bortz, I. McHardy, T. T. Wu, H. I. Liao, and R. Sun.** 2005. Murine gammaherpesvirus 68 open reading frame 45 plays an essential role during the immediate-early phase of viral replication. J. Virol. **79:**5129–5141.
31. **Kaleeba, J. A., and E. A. Berger.** 2006. Kaposi's sarcoma-associated herpesvirus fusion-entry receptor: cystine transporter xCT. Science **311:**1921–1924.
32. **Khanal, P., K. Y. Lee, K. W. Kang, B. S. Kang, and H. S. Choi.** 2009. Tpl-2 kinase downregulates the activity of p53 and enhances signaling pathways leading to activation of activator protein 1 induced by EGF. Carcinogenesis **30:**682–689.
33. **Konig, R., Y. Zhou, D. Elleder, T. L. Diamond, G. M. Bonamy, J. T. Irelan, C. Y. Chiang, B. P. Tu, P. D. De Jesus, C. E. Lilley, S. Seidel, A. M. Opaluch, J. S. Caldwell, M. D. Weitzman, K. L. Kuhen, S. Bandyopadhyay, T. Ideker, A. P. Orth, L. J. Miraglia, F. D. Bushman, J. A. Young, and S. K. Chanda.** 2008. Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. Cell **135:**49–60.
34. **Krishnan, M. N., A. Ng, B. Sukumaran, F. D. Gilfoy, P. D. Uchil, H. Sultana, A. L. Brass, R. Adametz, M. Tsui, F. Qian, R. R. Montgomery, S. Lev, P. W. Mason, R. A. Koski, S. J. Elledge, R. J. Xavier, H. Agaisse, and E. Fikrig.** 2008. RNA interference screen for human genes associated with West Nile virus infection. Nature **455:**242–245.
35. **Liang, Y., J. Chang, S. J. Lynch, D. M. Lukac, and D. Ganem.** 2002. The lytic switch protein of KSHV activates gene expression via functional interaction with RBP-Jkappa (CSL), the target of the Notch signaling pathway. Genes Dev. **16:**1977–1989.
36. **Liang, Y., and D. Ganem.** 2003. Lytic but not latent infection by Kaposi's sarcoma-associated herpesvirus requires host CSL protein, the mediator of Notch signaling. Proc. Natl. Acad. Sci. U. S. A. **100:**8490–8495.
37. **Liu, S., I. V. Pavlova, H. W. Virgin IV, and S. H. Speck.** 2000. Characterization of gammaherpesvirus 68 gene 50 transcription. J. Virol. **74:**2029–2037.
38. **Lukac, D. M., J. R. Kirshner, and D. Ganem.** 1999. Transcriptional activation by the product of open reading frame 50 of Kaposi's sarcoma-associated herpesvirus is required for lytic viral reactivation in B cells. J. Virol. **73:**9348–9361.
39. **Martinez-Guzman, D., T. Rickabaugh, T. T. Wu, H. Brown, S. Cole, M. J. Song, L. Tong, and R. Sun.** 2003. Transcription program of murine gammaherpesvirus 68. J. Virol. **77:**10488–10503.
40. **Mavromara-Nazos, P., and B. Roizman.** 1987. Activation of herpes simplex virus 1 gamma 2 genes by viral DNA replication. Virology **161:**593–598.
41. **Monje, P., M. J. Marinissen, and J. S. Gutkind.** 2003. Phosphorylation of the carboxyl-terminal transactivation domain of c-Fos by extracellular signal-regulated kinase mediates the transcriptional activation of AP-1 and cellular transformation induced by platelet-derived growth factor. Mol. Cell. Biol. **23:**7030–7043.
42. **Murphy, L. O., S. Smith, R. H. Chen, D. C. Fingar, and J. Blenis.** 2002. Molecular interpretation of ERK signal duration by immediate early gene products. Nat. Cell Biol. **4:**556–564.
43. **Naghavi, M. H., S. Valente, T. Hatziioannou, K. de Los Santos, Y. Wen, C. Mott, G. G. Gundersen, and S. P. Goff.** 2007. Moesin regulates stable microtubule formation and limits retroviral infection in cultured cells. EMBO J. **26:**41–52.
44. **Nash, A. A., and N. P. Sunil-Chandra.** 1994. Interactions of the murine gammaherpesvirus with the immune system. Curr. Opin. Immunol. **6:**560–563.
45. **Pan, H., J. Xie, F. Ye, and S. J. Gao.** 2006. Modulation of Kaposi's sarcoma-associated herpesvirus infection and replication by MEK/ERK, JNK, and p38 multiple mitogen-activated protein kinase pathways during primary infection. J. Virol. **80:**5371–5382.

46. **Rappocciolo, G., F. J. Jenkins, H. R. Hensler, P. Piazza, M. Jais, L. Borowski, S. C. Watkins, and C. R. Rinaldo, Jr.** 2006. DC-SIGN is a receptor for human herpesvirus 8 on dendritic cells and macrophages. J. Immunol. **176:**1741–1749.
47. **Rooney, C. M., D. T. Rowe, T. Ragot, and P. J. Farrell.** 1989. The spliced BZLF1 gene of Epstein-Barr virus (EBV) transactivates an early EBV promoter and induces the virus productive cycle. J. Virol. **63:**3109–3116.
48. **Salmeron, A., T. B. Ahmad, G. W. Carlile, D. Pappin, R. P. Narsimhan, and S. C. Ley.** 1996. Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase. EMBO J. **15:**817–826.
49. **Simas, J. P., and S. Efstathiou.** 1998. Murine gammaherpesvirus 68: a model for the study of gammaherpesvirus pathogenesis. Trends Microbiol. **6:**276–282.
50. **Smith, G. A., and L. W. Enquist.** 1999. Construction and transposon mutagenesis in Escherichia coli of a full-length infectious clone of pseudorabies virus, an alphaherpesvirus. J. Virol. **73:**6405–6414.
51. **Stevenson, P. G., and P. C. Doherty.** 1998. Kinetic analysis of the specific host response to a murine gammaherpesvirus. J. Virol. **72:**943–949.
52. **Sun, R., S. F. Lin, L. Gradoville, Y. Yuan, F. Zhu, and G. Miller.** 1998. A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus. Proc. Natl. Acad. Sci. U. S. A. **95:**10866–10871.
53. **Sunil-Chandra, N. P., S. Efstathiou, J. Arno, and A. A. Nash.** 1992. Virological and pathological features of mice infected with murine gamma-herpesvirus 68. J. Gen. Virol. **73:**2347–2356.
54. **Treisman, R.** 1994. Ternary complex factors: growth factor regulated transcriptional activators. Curr. Opin. Genet. Dev. **4:**96–101.
55. **Virgin, H. W., IV, P. Latreille, P. Wamsley, K. Hallsworth, K. E. Weck, A. J. Dal Canto, and S. H. Speck.** 1997. Complete sequence and genomic analysis of murine gammaherpesvirus 68. J. Virol. **71:**5894–5904.
56. **Wang, S. E., F. Y. Wu, H. Chen, M. Shamay, Q. Zheng, and G. S. Hayward.** 2004. Early activation of the Kaposi's sarcoma-associated herpesvirus RTA, RAP, and MTA promoters by the tetradecanoyl phorbol acetate-induced AP1 pathway. J. Virol. **78:**4248–4267.
57. **Wang, S. E., F. Y. Wu, Y. Yu, and G. S. Hayward.** 2003. CCAAT/enhancer-binding protein-alpha is induced during the early stages of Kaposi's sarcoma-associated herpesvirus (KSHV) lytic cycle reactivation and together with the KSHV replication and transcription activator (RTA) cooperatively stimulates the viral RTA, MTA, and PAN promoters. J. Virol. **77:**9590–9612.
58. **Wang, Y., H. Li, Q. Tang, G. G. Maul, and Y. Yuan.** 2008. Kaposi's sarcoma-associated herpesvirus ori-Lyt-dependent DNA replication: involvement of host cellular factors. J. Virol. **82:**2867–2882.
59. **Whitmarsh, A. J., P. Shore, A. D. Sharrocks, and R. J. Davis.** 1995. Integration of MAP kinase signal transduction pathways at the serum response element. Science **269:**403–407.
60. **Wu, T. T., T. Park, H. Kim, T. Tran, L. Tong, D. Martinez-Guzman, N. Reyes, H. Deng, and R. Sun.** 2009. ORF30 and ORF34 are essential for expression of late genes in murine gammaherpesvirus 68. J. Virol. **83:**2265–2273.
61. **Wu, T. T., L. Tong, T. Rickabaugh, S. Speck, and R. Sun.** 2001. Function of Rta is essential for lytic replication of murine gammaherpesvirus 68. J. Virol. **75:**9262–9273.
62. **Wu, T. T., E. J. Usherwood, J. P. Stewart, A. A. Nash, and R. Sun.** 2000. Rta of murine gammaherpesvirus 68 reactivates the complete lytic cycle from latency. J. Virol. **74:**3659–3667.
63. **Xie, J., H. Pan, S. Yoo, and S. J. Gao.** 2005. Kaposi's sarcoma-associated herpesvirus induction of AP-1 and interleukin 6 during primary infection mediated by multiple mitogen-activated protein kinase pathways. J. Virol. **79:**15027–15037.
64. **Yang, Z., and C. Wood.** 2007. The transcriptional repressor K-RBP modulates RTA-mediated transactivation and lytic replication of Kaposi's sarcoma-associated herpesvirus. J. Virol. **81:**6294–6306.