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Electronic Supplementary Material

Inhibition of the Neddylation Pathway Suppresses Enterovirus Replication

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Materials and Methods

Cells

Human rhabdomyosarcoma RD cells (ATCC), human embryonic kidney 293T cells (ATCC) and human lung carcinoma A549 cells (ATCC) were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution. Human colon carcinoma HT-29 cells (Cell Bank of the Chinese Academy of Sciences) were cultured in McCoy's 5a Medium supplemented with 10% foetal bovine serum (FBS) and penicillin/streptomycin solution. All cells were cultured under standard incubator conditions (37°C and 5% CO_2).

To generate NAE, UBE2M or UBE2F stable knock-down cell lines, we used shRNA plasmids targeting NAE, UBE2M or UBE2F co-transfected with pRSV-Rev(Addgene, 12253), pMDLg/pRRE (Addgene, 12251), and pCMV-VSV-G (Addgene, 8454) into HEK293T cells. Two days after transfection, the cell culture medium were collected and incubated with RD cells for 48 h, were then selected in 10% FBS-DMEM supplemented with puromycin (1 μg/mL; Sigma).

Viruses

EV-A71 prototype Anhui2007 provided by Professor Cheng Tong and EV-D68 prototype Fermon purchased from ATCC, were both propagated in RD cells. The viruses in the supernatants of infected cells were repeatedly freeze-thawed for 3 times 72 h post transfection and harvested by low-speed centrifugation, then passed through a 0.22-mm filter. The viral particles were finally pelleted through a 20% sucrose cushion in a SW28 rotor by centrifugation at 28,000 rpm for 120 min. Purified virions

were stored at –80°C. MLN4924 (MedChemExpress) was dissolved in Dimethyl sulfoxide (DMSO) and kept at –20 °C.

Cell Viability Assay

Nearly 3000 cells were seeded per well in 96-well plates with complete medium and incubated at 37°C in a 5% CO_2 incubator. Then, cell culture medium was removed and replaced with complete medium containing various concentrations of MLN4924. Negative control (only media) were included on each plate. The cells were incubated for 48 h and then 20 μL MTS was added to each well. Three hours later, Absorbance was detected using a BioTek ELISA reader (BioTek Instruments, Inc.) at a wavelength of 490 nm. Each data point represents the average of three replicates in cell culture.

Viral Attachment Assays

For virus attachment experiments, RD cells were first washed with cold DMEM, and then EV-A71 viruses were added to RD cells. After incubation at 4°C or 37 °C for 2 h, RD cells were washed with cold DMEM to remove unbound viruses. Total RNAs were extracted using Trizol (Life Technologies) and the bound virus RNA was determined by using qRT-PCR.

Virus Titer Assay

RD cells were cultured in 96-well plates at a density of 13,000 cells per well. EV-A71 was serially diluted (10-fold) with DMEM containing 1% FBS and added to cells. Virus titers were determined by the appearance of cytopathic effects (CPEs) in RD cells using a microtitration analysis in accordance with the Reed-Muench method. Viral titres were expressed as the 50% tissue culture infectious dose ($TCID_{50}$).

RNA Quantitation by qRT-PCR

RNA isolated from cells or supernatant was converted to cDNA using a reverse transcription kit (Transgen Biotech, Beijing, China). PCR amplification was performed using a Roche Z480 instrument (Roche, Basel, Switzerland) with SYBR Green supermix (Monad, Wuhan, China).The reactions were performed under the following conditions: 95°C for 5 min, 40 cycles of 95°C for 30 s and 60°C for 1 min, followed by a dissociation protocol. Single peaks in the melting curve analysis indicated specific amplicons. The relative levels of EV-A71 RNA and EV-D68 RNA in different

samples were determined using a comparative $2^{-\Delta\Delta CT}$ method with normalization against the *GAPDH* gene.

Immunoblotting

Cell lysates and supernatants treated under different conditions were separated via SDS-PAGE, and were transferred to nitrocellulose membranes using a semidry apparatus(Bio-Rad).The membranes were probed with various primary antibodies against the proteins of interest, secondary antibodies were alkaline phosphatase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). The membranes were stained with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma-Aldrich) and visualized for band quantification.

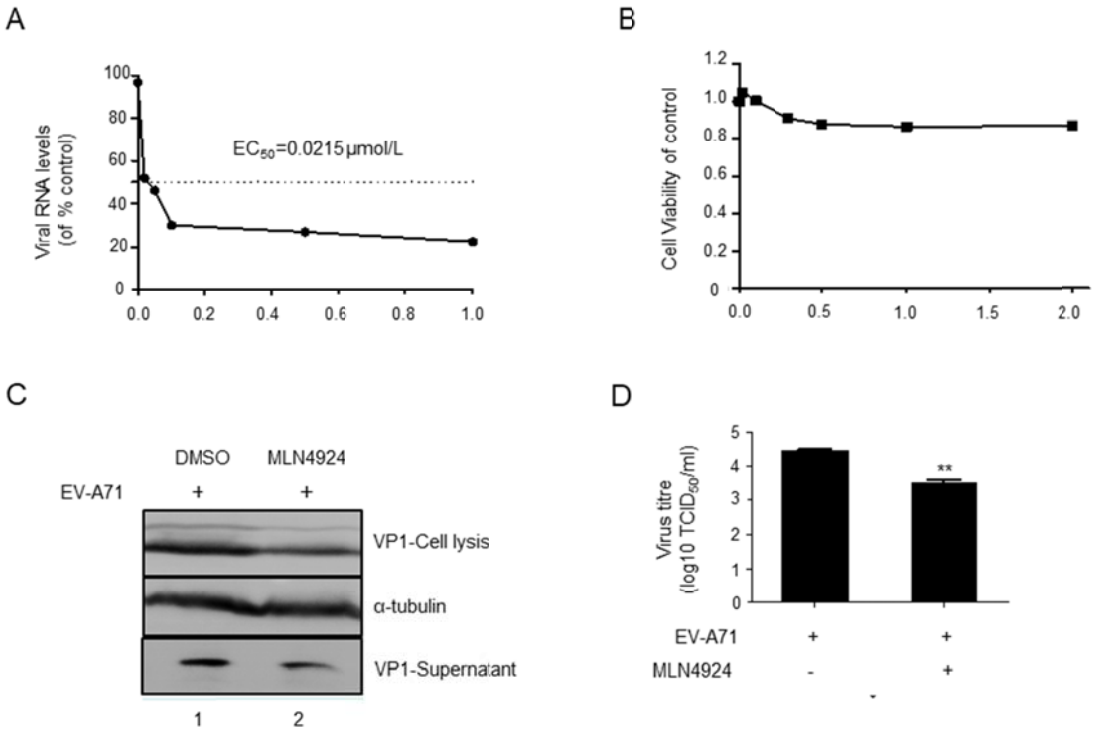

Figure S1. MLN4924 inhibits viral replication of EV-A71 in HT-29 cells. **A** Effect of MLN4924 on EV-A71 RNA replication (EC_{50} = 0.0215 μmol/L). **B** Cell viability assay. Cells were cultured in media containing a range of MLN4924 concentrations (0–2 μmol/L), and cell proliferation and cytotoxicity were evaluated using the MTS assay. **C** The effect of MLN4924 treatment on VP1 protein expression in EV-A71-infected HT-29 cells at 48 h post-infection. **D** Supernatants were collected 48 hpi, and viral titers were calculated as $TCID_{50}$.

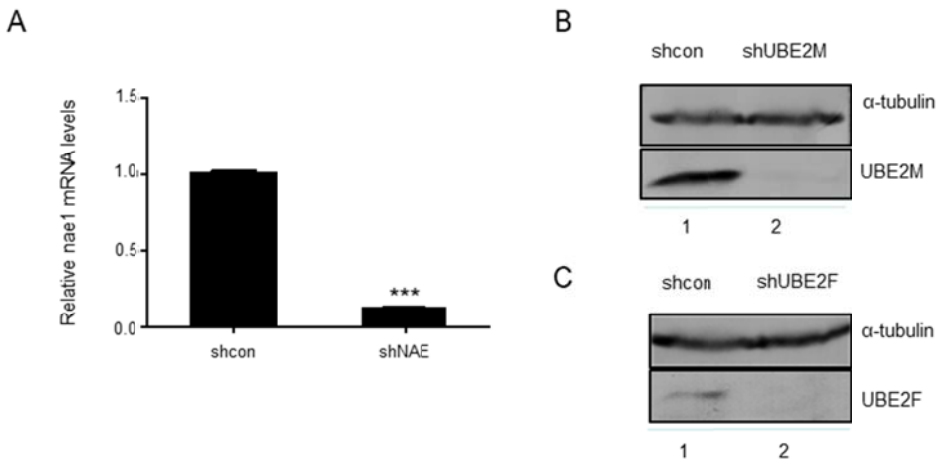

Figure S2. Validation of endogenous protein expression in stable knocked down RD cells. **A** NAE mRNA levels were determined using quantitative PCR. **B** and **C** The expression levels of endogenous UBE2M and UBE2F were determined using immunoblotting.

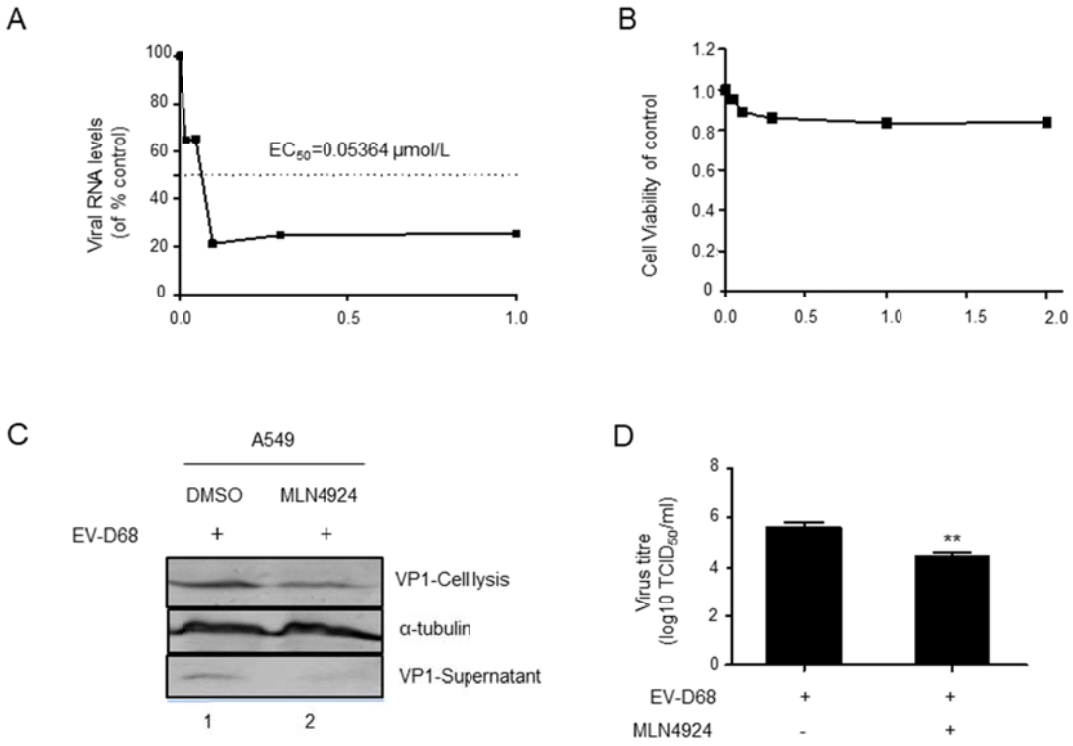

Figure S3. MLN4924 blocks viral replication of EV-D68 in A549 cells. A Effect of MLN4924 on EV-D68 RNA replication (EC_{50} = 0.05364 μmol/L). **B** Cell viability assay. Cells were cultured in media containing a range of MLN4924 concentrations (0–2 μmol/L), and cell proliferation and cytotoxicity were evaluated using the MTS assay. **C** The effect of MLN4924 treatment on VP1 protein expression in EV-D68-infected A549 cells at 48 h post-infection. **D** Supernatants were collected 48 hpi, and viral titers were calculated as $TCID_{50}$.