



LETTER

Inhibition of the Neddylation Pathway Suppresses Enterovirus Replication

Zhe Zhang¹ · Haoran Guo^{1,2} · Jing Wang¹ · Yan Li¹ · Yanhang Gao³ · Quan Liu⁴ · Junqi Niu³ · Wei Wei^{1,2} 

Received: 4 February 2021 / Accepted: 26 May 2021 / Published online: 5 August 2021
© Wuhan Institute of Virology, CAS 2021

Dear Editor,

Human enteroviruses (HEVs) comprise polioviruses, coxsackieviruses, echoviruses, rhinoviruses, and the enterovirus subgroups and belong to the genus *Enterovirus* in the family *Picornaviridae* (Baggen *et al.* 2018). The HEV genome was thought to contain a single open reading frame (ORF) encoding one polyprotein, which was post-translationally processed into structural capsid proteins (VP1–4) and non-structural accessory proteins (2A, 2B, 2C, 3A, 3B, 3C, 3D) (Baggen *et al.* 2018). Recently, we and others, have identified an additional upstream ORF (Guo *et al.* 2019a; Lulla *et al.* 2019) (ORF2/uORF). The encoded protein, ORF2p/UP, facilitates virion release from gut epithelial cells. Given HEVs are globally ubiquitous and cause a diverse array of clinical diseases (Wang *et al.* 2003; Verboon-Macielek *et al.* 2008; Muehlenbachs *et al.* 2015), there is an urgent need to develop broad-spectrum vaccines and therapeutic treatments for enterovirus infections.

Ubiquitin and ubiquitin-like post-translational modifications (PTMs) are essential for determining cell fate and proliferation, embryonic development, and carcinoma progression (Nandi *et al.* 2006). Moreover, accumulating

evidence suggests that different viruses frequently target these PTM signaling pathways (Hofmann *et al.* 2012; Dawson and Mehle 2018; Kumar *et al.* 2020) and modulate the intracellular environment of the host to enhance virus replication. Recently, we found that the PTM neddylation pathway is critical for activating the binding of lentiviral accessory protein Vpx to CRL4 E3 ligase, which induces the degradation of antiviral protein SAMHD1 (Wei *et al.* 2014; Guo *et al.* 2019b). The ubiquitin-like protein NEDD8 has a dedicated E1-activating enzyme (NAE1) and two E2-conjugating enzymes (UBE2M and UBE2F) and is essential for the enzymatic activity of the Cullin-RING E3 ligase family (Rabut and Peter 2008; Enchev *et al.* 2015). The neddylation pathway is considered an optimal target for cancer therapeutics, and the NAE1 inhibitor, MLN4924, has demonstrated remarkable therapeutic benefit in Phase-I clinical trials for cancer therapy (Sarantopoulos *et al.* 2016; Shah *et al.* 2016). Besides, MLN4924 has been found to be an optimal antiviral drug candidate against diverse human viruses including HBV (Xie *et al.* 2021) and influenza A virus (Sun *et al.* 2018).

Herein, we set out to investigate the effects of the neddylation pathway on enterovirus replication and explore the potential use of MLN4924 as an antiviral drug candidate against enterovirus infection. Human rhabdomyosarcoma RD cells were treated with MLN4924 at various concentrations (0.02, 0.05, 0.1, 0.5, 1, and 2 $\mu\text{mol/L}$) and challenged with EV-A71 (multiplicity of infection, MOI of 0.01). Viral titers in the supernatant were determined at 48 h post-infection (hpi), and the cytopathic effect (CPE) was noted. MLN4924 treatment markedly inhibited EV-A71 CPE (Fig. 1A) and significantly reduced the titers of progeny virus in the supernatant (Fig. 1B). In addition, EV-A71 VP1 protein expression decreased in a dose-dependent manner in MLN4924-treated cells (Fig. 1C). MLN4924 inhibited EV-A71 RNA replication in RD cells at an EC₅₀ value of approximately 0.011 $\mu\text{mol/L}$ (Fig. 1D). No significant cytotoxicity or decrease in cell proliferation was observed in MLN4924-treated cells, even at concentrations

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12250-021-00427-2>.

✉ Wei Wei
wwei6@jlu.edu.cn

- ¹ Institute of Virology and AIDS Research, First Hospital, Jilin University, Changchun 130021, China
- ² Key Laboratory of Organ Regeneration and Transplantation of Ministry of Education, Institute of Translational Medicine, First Hospital, Jilin University, Changchun 130021, China
- ³ Department of Hepatology, First Hospital, Jilin University, Changchun 130021, China
- ⁴ Department of Emerging Infectious Diseases, Institute of Translational Medicine, First Hospital, Jilin University, Changchun 130021, China

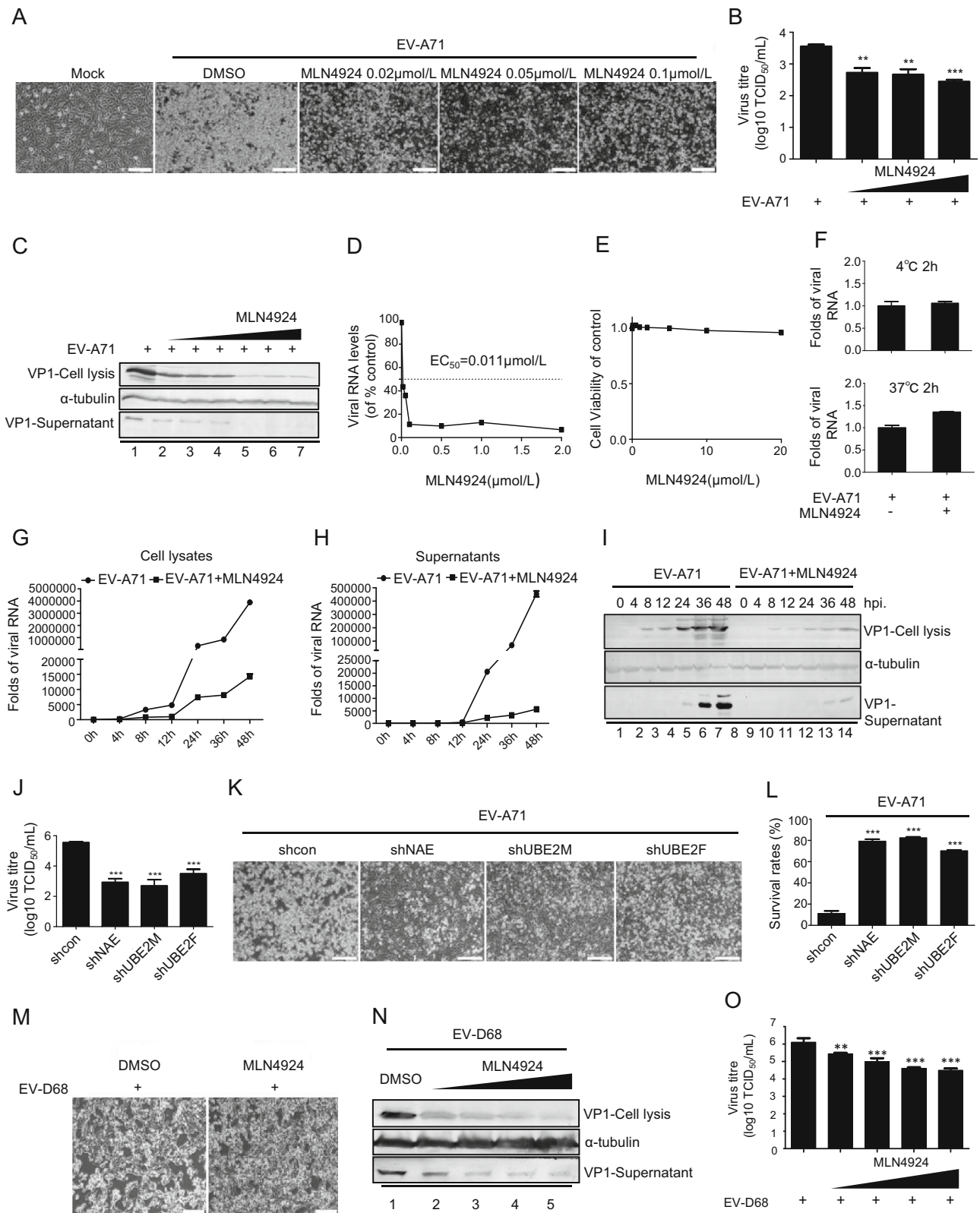


Fig. 1 Neddylation blockade inhibits viral replication of enteroviruses. **A–D** MLN4924- or DMSO-treated RD cells were infected with EV-A71. **A** Cytopathic effect (CPE) was observed at 48 h post-infection (hpi). Scale bar = 200 μ m. **B** Quantification of infectious virions. Supernatants were collected 48 hpi, and viral titers were calculated as TCID₅₀. The concentration of MLN4924 used ranged from 0.02–0.1 μ mol/L. **C** The protein expression levels of enterovirus VP1 protein were detected using immunoblotting. A range of MLN4924 concentrations were used (0.02, 0.05, 0.1, 0.5, 1 and 2 μ mol/L). **D** Effect of MLN4924 on EV-A71 RNA replication (EC₅₀ = 0.011 μ mol/L). The concentration of MLN4924 used ranged from 0.02–2 μ mol/L. **E** Cell viability assay. The cytotoxicity of MLN4924 was determined in mock-infected RD cells. Cells were cultured in media containing a range of MLN4924 concentrations (0–20 μ mol/L), and cell proliferation and cytotoxicity were evaluated using the MTS assay. **F** Virus attachment and entry assays. RD cells were incubated at 4 °C for 30 min before EV-A71 infection and then incubated at 4 °C or 37 °C for 2 h to facilitate virus attachment or entry, respectively. qRT-PCR was performed to quantify viral RNA. The concentration of MLN4924 was 0.5 μ mol/L. (**G** and **H**) the concentrations of intracellular and supernatant viral RNA at specific time points post-infection were determined using qRT-PCR. The concentration of MLN4924 was 0.5 μ mol/L. (**I**) The effect of MLN4924 treatment on EV-A71 VP1 protein expression at specific time points post-infection. The concentration of MLN4924 was 0.5 μ mol/L. **J–L** Stable RD-shNAE1, RD-shUBE2M, and RD-shUBE2F cells were challenged with EV-A71 (MOI of 0.01). **J** Quantification of progeny virions. **K** CPE observed in RD cells at 48 hpi. Scale bar = 200 μ m. **L** The survival rates of infected cells were determined using trypan blue staining. **M–O** MLN4924- or DMSO-treated HEK293T cells were infected with EV-D68. **M** CPE observed in HEK293T cells 48 hpi. The concentration of MLN4924 was 0.5 μ mol/L. Scale bar = 200 μ m. **N** Protein expression levels of EV-D68 VP1 protein were detected using immunoblotting. The concentration of MLN4924 used ranged from 0.02–0.5 μ mol/L. **O** Quantification of progeny virions. Supernatants were collected 48 hpi and viral titers calculated using TCID₅₀ assay. The concentration of MLN4924 used ranged from 0.02–0.5 μ mol/L. **P* < 0.05, ****P* < 0.01, *****P* < 0.001.

as high as 20 μ mol/L, as determined using the MTS assay (Fig. 1E). MLN4924 treatment conferred resistance to EV-A71 infection in human colon carcinoma HT-29 cells (Supplementary Fig. S1).

Furthermore, we determined the effects of MLN4924 on EV-A71 entry into host cells. RD cells were treated with either MLN4924 or DMSO and then incubated with EV-A71 for 2 h at 4 °C or 37 °C to facilitate virus attachment or entry, respectively. qRT-PCR was used to detect viral RNA (vRNA). We found that MLN4924 treatment did not significantly affect EV-A71 virus entry (Fig. 1F). Time-dose analysis of EV-A71 RNA replication indicated a strong decrease in the accumulation of viral RNA in the cell lysate (Fig. 1G) and in the supernatant (Fig. 1H) of MLN4924-treated RD cells, when compared to that in DMSO-treated cells. We next analyzed VP1 protein expression in RD cells infected with EV-A71 either in the presence of 0.5 μ mol/L MLN4924 or DMSO over a time course. VP1 protein expression was detectable at 8 hpi in

both MLN4924- and DMSO-treated cells. At later time points, a dramatic reduction in VP1 protein expression was observed in both the cell-associated and supernatant-associated fractions of MLN4924-treated cells as compared to the DMSO-treated controls (Fig. 1I). Hence, MLN4924 suppresses EV-A71 replication at a post-entry step.

Then, we evaluated the role of neddylation in EV-A71 replication. There are two well-known pathways involved in neddylation: Rbx1-UBE2M and Rbx2-UBE2F (Huang *et al.* 2009). We generated stable knockdown RD cell lines using shRNA targeting endogenous E1 (NAE1) and E2 (UBE2M or UBE2F) enzymes (Supplementary Fig. S2). The cells were then challenged with EV-A71. These results showed that the downregulation of NAE1, UBE2M, and UBE2F dramatically reduced the production of infectious virions (Fig. 1J) and enterovirus CPE (Fig. 1K and 1L), as compared with those in control cells. Thus, the PTM neddylation pathway is a determinant factor for supporting EV-A71 replication.

These results led us to examine the antiviral activity of MLN4924 against other enteroviruses. Unlike EV-A71, which is transmitted via the fecal–oral route, EV-D68 is transmitted through coughing and sneezing. We treated HEK293T cells with MLN4924 concentrations ranging from 0.02 to 0.5 μ mol/L, and infected the cells with EV-D68 (MOI 0.1) for 48 h. MLN4924 treatment significantly blocked EV-D68 CPE (Fig. 1M), VP1 protein expression (Fig. 1N), and infectious virion production (Fig. 1O). MLN4924 treatment potently inhibited EV-D68 replication in human lung carcinoma A549 cells (Supplementary Fig. S3).

Protein neddylation is the process of transferring NEDD8 to a lysine residue on the substrate; it modulates the activity of its substrates. UBE2M and UBE2F are the two NEDD8-conjugating E2 enzymes, and they exhibit distinct biological functions. UBE2M is responsible for the neddylation of Cullin1, Cullin2, Cullin3, Cullin4A, and Cullin4B, whereas UBE2F facilitates the neddylation of Cullin5. Moreover, UBE2M and UBE2F have different non-Cullin substrates. Both UBE2M and UBE2F are required for enterovirus replication, implying that enteroviruses utilized a complicated neddylation-ubiquitination network for modulating the intracellular microenvironment conducive for viral infection. Identification of NEDD8-conjugated substrates, which are critical for viral replication, is essential to extend our understanding of the life cycle of enteroviruses.

Considering the remarkable therapeutic benefit of MLN4924 treatment in patients with diverse cancers, we explored its potential as an inhibitor of enterovirus replication. Here we provide evidence that MLN4924 is a potent inhibitor of enterovirus replication and suggest that neddylation is crucial for the life cycle of enteroviruses.

Furthermore, we show that the components of the neddylation pathway can serve as potential targets for therapeutic intervention.

Acknowledgements We thank Y. Li and G. Zhou for technical assistance and Drs. T. Cheng, and XF. Yu for key reagents. This work was supported in part by funding from the National Natural Science Foundation of China (81772183 and 31800150), the Department of Science and Technology of Jilin Province (No. 20190304033YY and 20180101127JC), the open project of Key Laboratory of Organ Regeneration and Transplantation, Ministry of Education, the Program for JLU Science and Technology Innovative Research Team (2017TD-08), and Fundamental Research Funds for the Central Universities.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement This article does not contain any studies with human or animal subjects performed by any of the authors.

References

- Baggen J, Thibaut HJ, Strating J, van Kuppeveld FJM (2018) The life cycle of non-polio enteroviruses and how to target it. *Nat Rev Microbiol* 16:368–381
- Dawson AR, Mehle A (2018) Flu's cues: exploiting host post-translational modifications to direct the influenza virus replication cycle. *PLoS Pathog* 14:e1007205
- Enchev RI, Schulman BA, Peter M (2015) Protein neddylation: beyond cullin-RING ligases. *Nat Rev Mol Cell Biol* 16:30–44
- Guo H, Li Y, Liu G, Jiang Y, Shen S, Bi R, Huang H, Cheng T, Wang C, Wei W (2019a) A second open reading frame in human enterovirus determines viral replication in intestinal epithelial cells. *Nat Commun* 10:4066
- Guo H, Zhang N, Shen S, Yu XF, Wei W (2019b) Determinants of lentiviral Vpx-CRL4 E3 ligase-mediated SAMHD1 degradation in the substrate adaptor protein DCAF1. *Biochem Biophys Res Commun* 513:933–939
- Hofmann H, Logue EC, Bloch N, Daddacha W, Polsky SB, Schultz ML, Kim B, Landau NR (2012) The Vpx lentiviral accessory protein targets SAMHD1 for degradation in the nucleus. *J Virol* 86:12552–12560
- Huang DT, Ayrault O, Hunt HW, Taherbhoy AM, Duda DM, Scott DC, Borg LA, Neale G, Murray PJ, Roussel MF, Schulman BA (2009) E2-RING expansion of the NEDD8 cascade confers specificity to cullin modification. *Mol Cell* 33:483–495
- Kumar R, Mehta D, Mishra N, Nayak D, Sunil S (2020) Role of host-mediated post-translational modifications (PTMs) in RNA virus pathogenesis. *Int J Mol Sci* 22:323
- Lulla V, Dinan AM, Hosmillo M, Chaudhry Y, Sherry L, Irigoyen N, Nayak KM, Stonehouse NJ, Zilbauer M, Goodfellow I, Firth AE (2019) An upstream protein-coding region in enteroviruses modulates virus infection in gut epithelial cells. *Nat Microbiol* 4:280–292
- Muehlenbachs A, Bhatnagar J, Zaki SR (2015) Tissue tropism, pathology and pathogenesis of enterovirus infection. *J Pathol* 235:217–228
- Nandi D, Tahiliani P, Kumar A, Chandu D (2006) The ubiquitin-proteasome system. *J Biosci* 31:137–155
- Rabut G, Peter M (2008) Function and regulation of protein neddylation. "Protein modifications: beyond the usual suspects" review series. *EMBO Rep* 9:969–976
- Sarantopoulos J, Shapiro GI, Cohen RB, Clark JW, Kauh JS, Weiss GJ, Cleary JM, Mahalingam D, Pickard MD, Faessel HM, Berger AJ, Burke K, Mulligan G, Dezube BJ, Harvey RD (2016) Phase I study of the investigational NEDD8-activating enzyme inhibitor pevonedistat (TAK-924/MLN4924) in patients with advanced solid tumors. *Clin Cancer Res* 22:847–857
- Shah JJ, Jakubowiak AJ, O'Connor OA, Orłowski RZ, Harvey RD, Smith MR, Lebovic D, Diefenbach C, Kelly K, Hua Z, Berger AJ, Mulligan G, Faessel HM, Tirrell S, Dezube BJ, Lonial S (2016) Phase I study of the novel investigational NEDD8-activating enzyme inhibitor pevonedistat (MLN4924) in patients with relapsed/refractory multiple myeloma or lymphoma. *Clin Cancer Res* 22:34–43
- Sun H, Yao W, Wang K, Qian Y, Chen H, Jung YS (2018) Inhibition of neddylation pathway represses influenza virus replication and pro-inflammatory responses. *Virology* 514:230–239
- Verboon-Macielek MA, Krediet TG, Gerards LJ, de Vries LS, Groenendaal F, van Loon AM (2008) Severe neonatal parechovirus infection and similarity with enterovirus infection. *Pediatr Infect Dis J* 27:241–245
- Wang SM, Lei HY, Huang KJ, Wu JM, Wang JR, Yu CK, Su IJ, Liu CC (2003) Pathogenesis of enterovirus 71 brainstem encephalitis in pediatric patients: roles of cytokines and cellular immune activation in patients with pulmonary edema. *J Infect Dis* 188:564–570
- Wei W, Guo H, Liu X, Zhang H, Qian L, Luo K, Markham RB, Yu XF (2014) A first-in-class NAE inhibitor, MLN4924, blocks lentiviral infection in myeloid cells by disrupting neddylation-dependent Vpx-mediated SAMHD1 degradation. *J Virol* 88:745–751
- Xie M, Guo H, Lou G, Yao J, Liu Y, Sun Y, Yang Z, Zheng M (2021) Neddylation inhibitor MLN4924 has anti-HBV activity via modulating the ERK-HNF1 α -C/EBP α -HNF4 α axis. *J Cell Mol Med* 25:840–854