**Virologica Sinica**

**Supplementary materials**

**NSUN2 mediates distinct pathways to regulate** **enterovirus 71 replication**

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1 Lishi Liu and Zhen Chen contributed equally to this work.

Table S1. qPCR primer and sequence

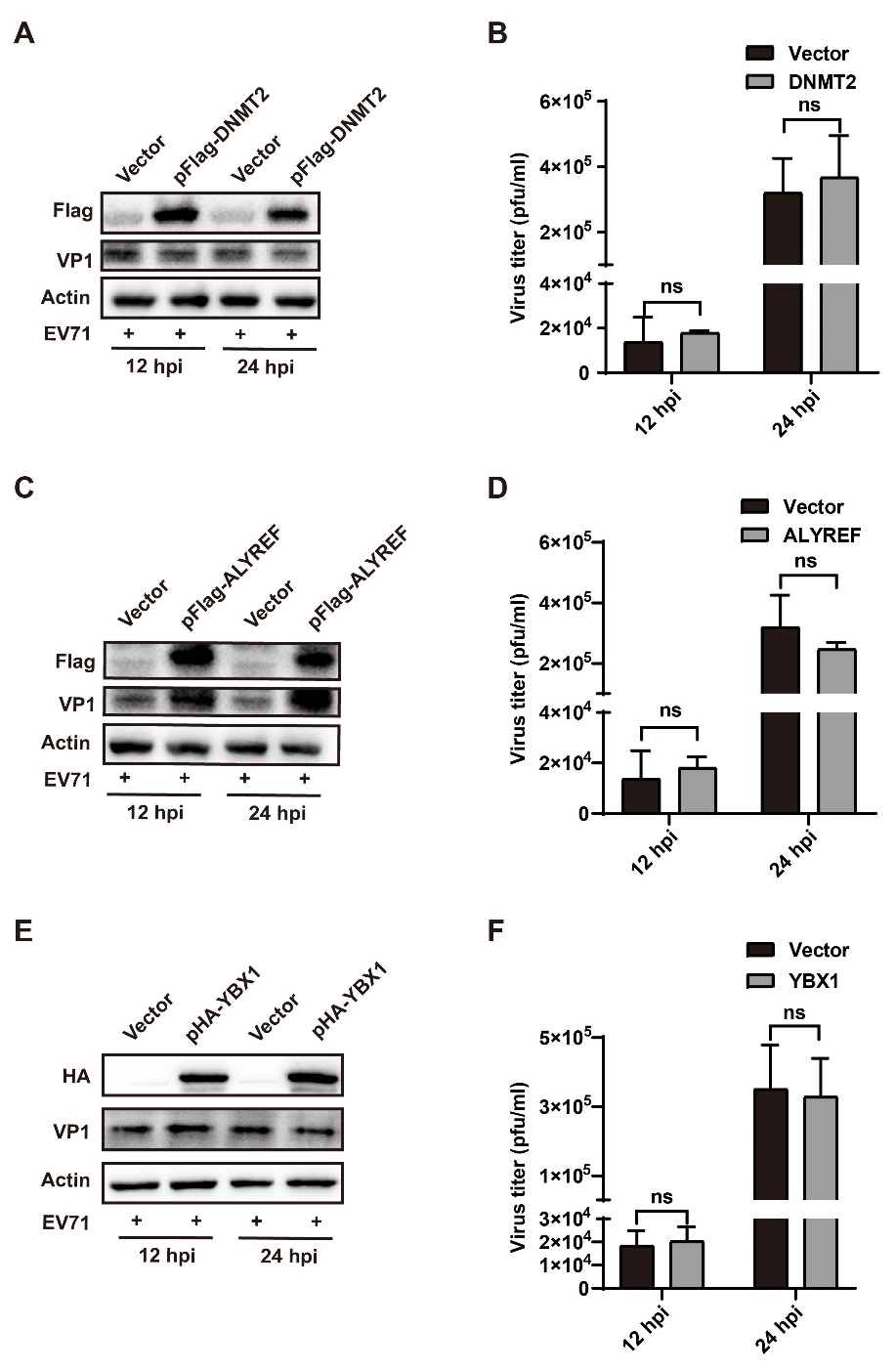
|  |  |
| --- | --- |
| qPCR primer | Sequence |
| GAPDH-F | 5′-GAAGGTGAAGGTCGGAGTC-3′ |
| GAPDH-R | 5′-GAAGATGGTGATGGGATTTC-3′ |
| GAPDH (mice)-F | 5′-CATCACTGCCACCCAGAAGACTG-3′ |
| GAPDH (mice)-R | 5′-ATGCCAGTGAGCTTCCCGTTCAG-3′ |
| EV71-F | 5′-CGAATGCTAGTGATGAGAGTAT-3′ |
| EV71-R | 5′-GAGGAAGATCTATCTCCCCAACT-3′ |
| eGFP-F | 5′-TGAGCAAAGACCCCAACGAG-3′ |
| eGFP-R | 5′-CTTGTACAGCTCGTCCATGC-3′ |
| Luciferase-F | 5′-TCGAAAGAAGTCGGGGAAGC-3′ |
| Luciferase-R | 5′-ATCCCCCTCGGGTGTAATCA-3′ |

Notes: The table shows the sequences of the qPCR primers used in this study.

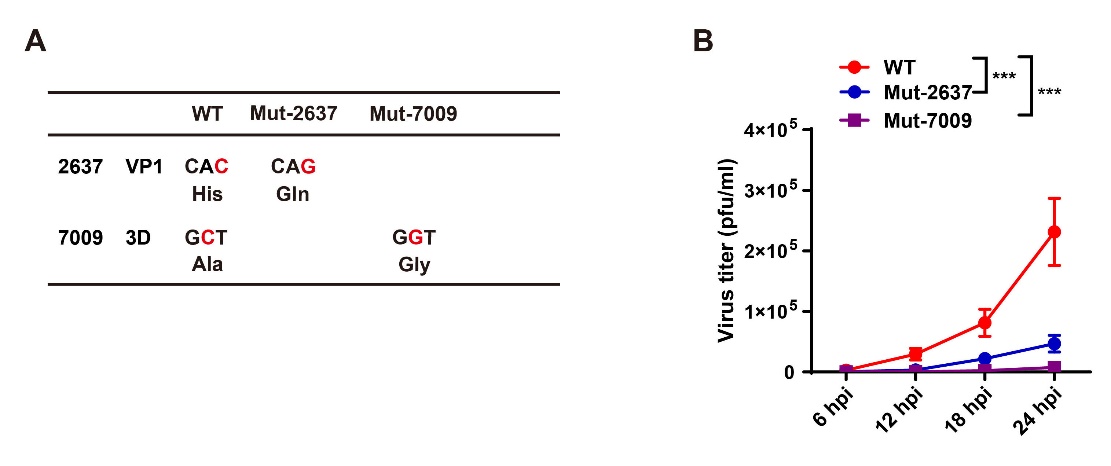
Table S2. The m5C sites of EV71 RNA detected by Direct RNA sequencing

|  |  |  |  |
| --- | --- | --- | --- |
| Vero | | RD | |
| location | score | location | score |
| 584 | 0.9929 | 584 | 0.9934 |
| 1460 | 0.9736 | 1460 | 0.9605 |
| 2637 | 0.9468 | 2637 | 0.9492 |
| 7009 | 0.9546 | 7009 | 0.9293 |
| 108 | 0.9265 | 108 | 0.9669 |
| 207 | 0.9535 | 207 | 0.94 |
| 231 | 0.9243 | 287 | 0.9237 |
| 287 | 0.9106 | 364 | 0.9636 |
| 364 | 0.913 | 473 | 0.9585 |
| 473 | 0.9486 | 495 | 0.9371 |
| 480 | 0.9375 | 587 | 0.996 |
| 495 | 0.9429 | 753 | 0.9156 |
| 587 | 0.9881 | 772 | 0.9589 |
| 735 | 0.9101 | 961 | 0.9935 |
| 772 | 0.963 | 984 | 0.9421 |
| 811 | 0.9515 | 1000 | 0.9074 |
| 961 | 0.9735 | 1024 | 0.9244 |
| 984 | 0.9306 | 1108 | 0.9131 |
| 1024 | 0.9402 | 1133 | 0.9182 |
| 1108 | 0.9111 | 1271 | 0.9354 |
| 1133 | 0.9225 | 1277 | 0.9163 |
| 1271 | 0.9522 | 1394 | 0.9445 |
| 1277 | 0.9324 | 1434 | 0.917 |
| 1286 | 0.9315 | 1568 | 0.904 |
| 1301 | 0.9078 | 1639 | 0.9048 |
| 1394 | 0.9554 | 1685 | 0.9207 |
| 1568 | 0.9121 | 1708 | 0.9138 |
| 1639 | 0.914 | 1741 | 0.9413 |
| 1708 | 0.9176 | 1853 | 0.9192 |
| 1741 | 0.954 | 1854 | 0.9134 |
| 1853 | 0.9314 | 1904 | 0.9043 |
| 1854 | 0.9486 | 1926 | 0.9918 |
| 1904 | 0.9277 | 1938 | 0.9156 |
| 1926 | 0.9887 | 2119 | 0.922 |
| 1938 | 0.9169 | 2121 | 0.9574 |
| 2119 | 0.9427 | 2639 | 0.9142 |
| 2121 | 0.9441 | 2665 | 0.9568 |
| 2531 | 0.9189 | 2811 | 0.9509 |
| 2639 | 0.9198 | 2941 | 0.9086 |
| 2665 | 0.97 | 2942 | 0.9859 |
| 2811 | 0.9303 | 3078 | 0.9508 |
| 2917 | 0.9053 | 3096 | 0.9387 |
| 2941 | 0.9017 | 3114 | 0.929 |
| 2942 | 0.9899 | 3115 | 0.9346 |
| 3078 | 0.959 | 5677 | 0.9148 |
| 3096 | 0.9339 | 5704 | 0.9003 |
| 3114 | 0.9431 | 7187 | 0.9107 |
| 3115 | 0.9535 |  |  |
| 4242 | 0.9135 |  |  |
| 5704 | 0.9109 |  |  |

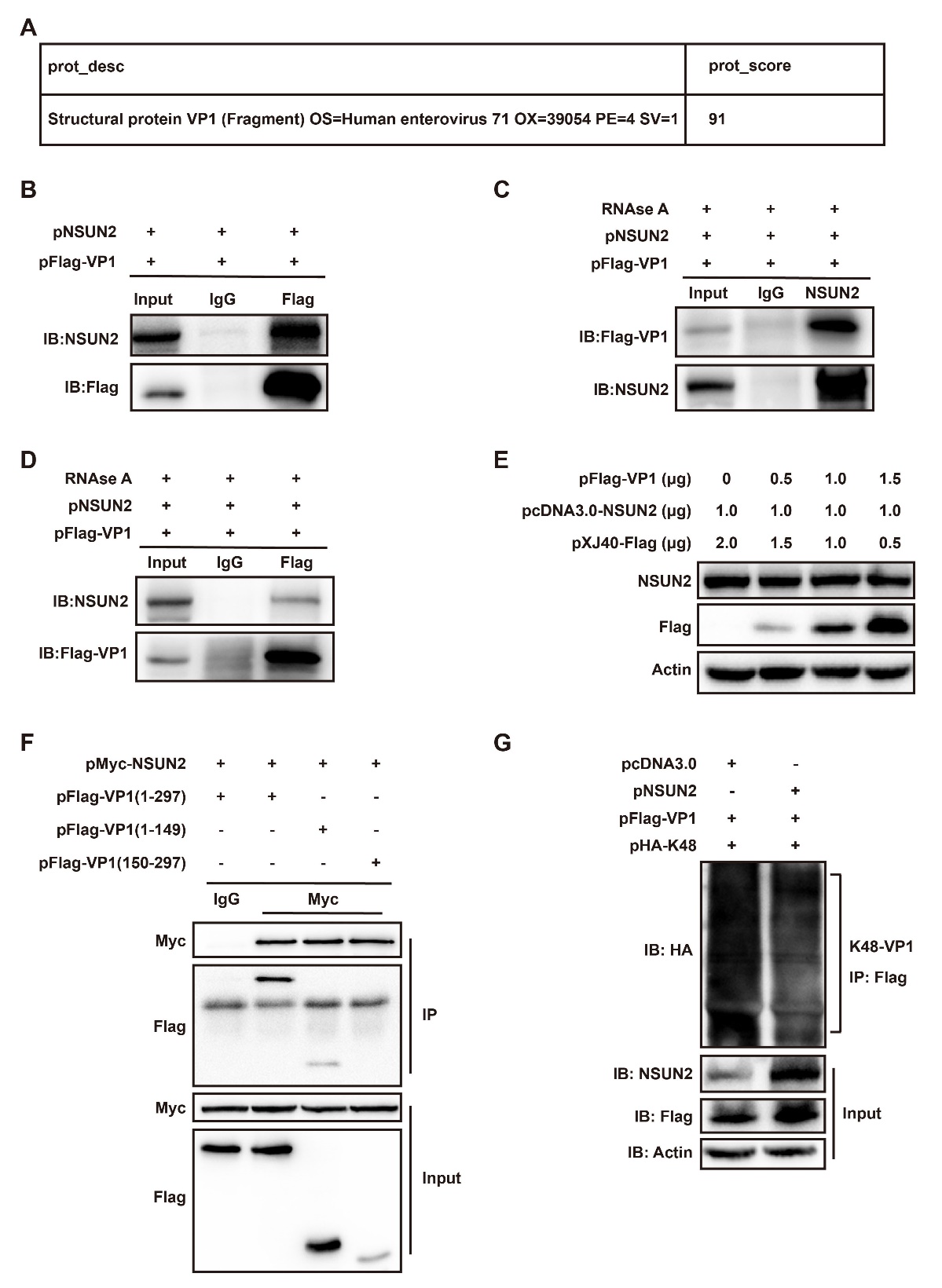
Notes: Direct RNA sequencing was conducted using the poly A-purified RNAs from EV71-infected Vero cells and RD cells. The m5C sites shown in Figure 1D were clearly presented as data in the supplemental table 2.

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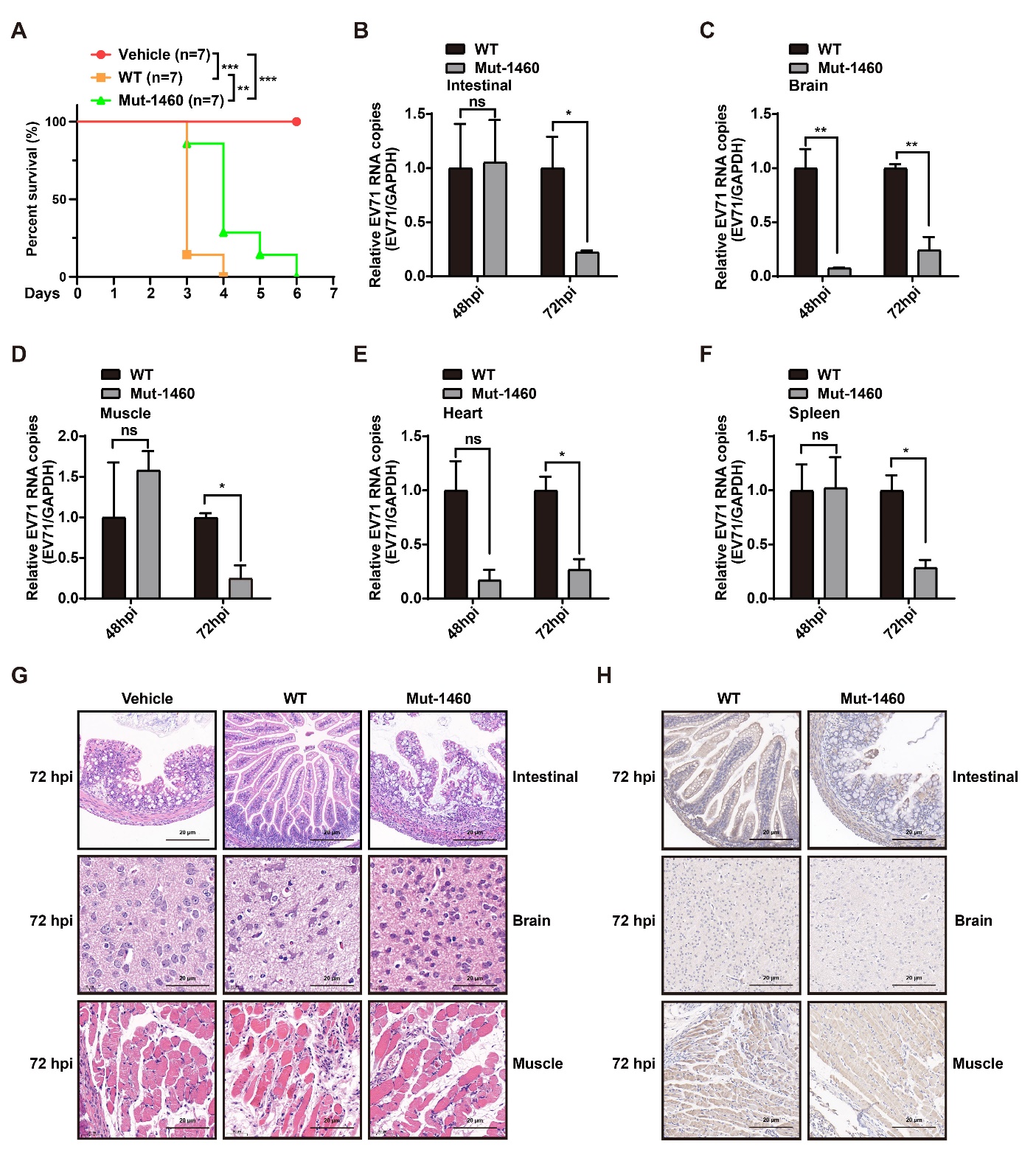
**Figure S1.** DNMT2, ALYREF and YBX1 do not affect enterovirus 71 (EV71) replication. **A, C, E** EV71-infected RD cells were transfected with an empty vector or indicated plasmids. At 12 h (left two lanes) and 24 h (right two lanes) post-transfection, the expression levels of DNMT2, ALYREF, YBX1, VP1 and Actin proteins were detected using the indicated Abs. **B, D, F** Numbers of progeny EV71 viruses in RD cells were measured at 12 h (left two bars) and 24 h (right two bars) and presented as a bar graph.Unpaired Student’s *t*-tests were performed for all bar graphs, and data are presented as the means ± SEMs (n = 3). \**P* ≤ 0.05, \*\**P* ≤ 0.01. ns: not significant, two-way ANOVA.



**Figure S2.** 5-Methylcytosine (m5C) modifications promote enterovirus 71 (EV71) replication. **A** The diagram of Mut-2637 and Mut-7009 EV71 strains relative with WT EV71. **B** The TCID50 virus titers of progeny EV71 viruses in Vero cells infected with WT or the other three mutant EV71 strains from (**A**) were measured at multiple time points (0, 6, 12, 18, and 24 h) and presented as a line chart. Unpaired Student’s *t*-tests were performed for the line chart, and data are presented as the means ± SEMs (n = 3). \*\*\**P* ≤ 0.001.



**Figure S3.** NSUN2 targets VP1 for binding in an RNA-independent manner and inhibits its K48 ubiquitination. **A** The table presents one of the proteins pulled down with NSUN2, specifically VP1, and the indicated scores according to the mass spectrum (MS) analysis. **B** VP1 targets NSUN2 for binding. Co-IP samples using anti-Flag Abs (right 2 lanes) and input samples (left lane) obtained from RD cells expressing exogenous NSUN2 and VP1 were examined using the indicated Abs. **C, D** NSUN2 targets VP1 for binding in an RNA-independent manner. Co-IP samples obtained from the RNase A-treated RD cells expressing NSUN2 and VP1 using anti-NSUN2 (**C**) or anti-Flag (**D**) and input samples were separately examined using the indicated Abs. **E** Exogenous VP1 did not affect NSUN2 expression. Levels of NSUN2, VP1 and Actin were measured in RD cells co-transfected with a constant amount of NSUN2 plasmid and an increasing amount of VP1 plasmid using the indicated Abs. Empty vectors were supplemented for equal amounts of transfected plasmids. **F** VP1 interacts with NSUN2 via its N-terminus. Co-IP samples (top two panels) obtained from HEK293T cells expressing exogenous NSUN2 and VP1 (left two lanes) or two truncated mutants [VP1(1–149) and VP1(150–297)] using anti-Myc Abs and input samples (bottom 2 panels) were examined using the indicated Abs. **G** IP samples obtained from HEK293T cells expressing exogenous VP1 and mutant ubiquitin (only contain K48) with (left lane) or without (right lane) exogenous NSUN2 using anti-Flag Abs. Levels of VP1 ubiquitination were measured using anti-HA Abs (top panel), and input samples were measured using the indicated Abs (bottom three panels).



**Figure S4.** A mutant enterovirus 71 (EV71) strain (Mut-1460) lacking 5-methylcytosine (m5C)-modified residues exhibits significantly attenuated pathogenicity in mice. **A** Mice were separately challenged with the same numbers (105 pfu) of wild-type (WT, yellow) or m5C-mutant (Mut-1460) (green) EV71 strains, and DMEM was set as control (vehicle) (red). Each group consisted of seven mice, and the indicated survival time was measured and presented as a line chart. A log-rank (Mantel-Cox) test was performed (\**P* ≤ 0.05, \*\**P* ≤ 0.01). **B–F** After euthanizing AG6 mice challenged with WT or Mut-1460 EV71 strains for 48 h or 72 h, levels of viral RNAs in intestinal (**B**), brain (**C**), muscle (**D**), heart (**E**) and spleen (**F**), samples were quantified and presented as bar graphs. Unpaired Student’s *t*-tests were performed for the line chart and all bar graphs, and data are presented as the means ± SEMs (n = 3). \**P* ≤ 0.05, \*\**P* ≤ 0.01. ns: not significant. **G, H** After challenging mice with DMEM (vehicle) or WT or Mut-1460 EV71 viruses for 72 h, hematoxylin and eosin staining (**G**) or immunohistochemistry (**H**) to detect EV71 particles using anti-VP1 Abs was performed on the intestine (top panel), brain (middle panel), and limb muscle (bottom panel). The scale bar is 20 μm.