Supplementary information, Data S1

Materials and Methods

Cell culture, transfection and virus infection

RD and 293T cells were maintained in Dulbecco's modified Eagle's essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C in a 5% CO_2 incubator. Transient transfection was performed using Lipofectamine2000 (Invitrogen), according to the manufacturer's instructions. Enterovirus 71strain SZ98 was obtained from the Chinese Academy of Medical Sciences (Beijing, China). Virus adsorption was performed for 30 min in serum-free medium at 37°C, after which the cells were washed with medium containing 2% FBS and cultured in this medium, with or without DIDS, for 48 h. All virus experiments were performed in a Biosafety Level 2 laboratory.

Plasmids

Viral RNA was extracted from virus infected RD cells using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. cDNA was prepared using M-MLV reverse transcriptase (Invitrogen) with oligo-dT primers. The 2B genes were amplified by PCR using Pyrobest polymerase (Takara). To generate the 2B-coding plasmid, the cDNA was firstly inserted into the pCDNA3'HA vector to add an HA tag at the C-terminus. For cell transfection, the cDNA was cloned into the pCAGGS vector under the control of the chicken β-actin promoter (a kind gift from Dr. Jun-ichi Miyazaki, Osaka University). For oocyte injection, the 2B cDNA was cloned into the pNWP vector, as previously described [1].

Electrophysiology

Complementary RNA of 2B protein with an HA tag was synthesized from pNWP-EV71-2B-HA using the mMESSAGE mMACHINE high-yield capped RNA transcription SP6 Kit (Ambion) and injected into *Xenopus Laevis* oocytes. The cRNA was quantified using denaturing (urea) PAGE. The oocytes were prepared and maintained following standard methods. Twenty-four to 36 hours after an injection of 25ng indicating cRNA, the oocytes were used for electrophysiology or immunofluorescence analysis. A two-electrode voltage clamp instrument (Turbo TEC10, NPI Electronics, Tamm, Germany)

was used to record the currents from the plasma membranes of the injected or uninjected *Xenopus* oocytes. The standard voltage-clamp protocol consisted of rectangular voltage steps from -120 to +20 mV in 10 mV increments applied from a holding voltage of -58 mV. The microelectrodes were filled with 3M KCl and had a resistance of 0.5-1 MΩ. During standard recordings, the oocytes were bathed in standard ORi solution (containing 90 mM NaCl, 2 mM KCl, 2 mM CaCl2 and 5mM MOPS, adjusted to pH7.4 with NaOH) at room temperature (~22°C). In some experiments, equimolar sodium gluconate was substituted for the NaCl. Where indicated, 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS, Sigma) was added directly to the bath solution and the currents were recorded after an incubation period of 3 min.

Antibodies

Monoclonal mouse anti-HA and anti-HA-HRP antibodies were obtained from Sigma. For immunofluorescence analysis, anti-Golgi GM130 (a monoclonal antibody that stains the trans-Golgi complex) was used (BD Biosciences). The secondary antibodies were an Alexa Fluor 488 goat anti-mouse antibody (Invitrogen) and a TRITC conjugated donkey anti-rabbit antibody (BD Biosciences).

Immunoprecipitation and western blot analysis

For immunoprecipitation, 293T cells were collected and lysed with RIPA buffer containing complete protease inhibitor cocktail tablets (Roche). The clarified supernatant was then incubated overnight with an anti-HA monoclonal antibody along with 5% (w/v) BSA and protein A/G beads at 4°C. The beads were washed 5 times with RIPA buffer and lysed in SDS loading buffer for further analysis. In the immunoblotting experiments, the cell lysates and the pellets after immunoprecipitation were boiled for 10 minutes and separated using 15% SDS-PAGE. The proteins were then transferred onto nitrocellulose membranes (Bio-Rad) and detected using anti-HA HRP antibodies. The bands were detected using an enhanced chemiluminescence kit (Thermo).

Immunofluorescence

Oocytes or RD cells were injected with 2B cRNA or transfected with the 2B-HA expression plasmid.

Twenty-four hours after transfection, the cells were fixed with 4% PFA and sequentially incubated with an anti-HA antibody (primary antibody) and anti-mouse IgG Alexa Fluor 488(secondary antibody) and imaged using a confocal fluorescence microscope (Leica).

Plaque Assay

The method was performed as previously described [2]. Briefly, RD cells were cultured to 80% confluence in 6-well plates 24 hours before infection. After a half hour infection, the cells were plated with 0.8% agarose (Lonza Sea Plaque). After 2 days, the cells were fixed in 10% formaldehyde and stained with 0.1% crystal violet.

Real-time RT-PCR

The viral RNA in the supernatant was extracted using the QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer's instructions. The RNA was then reverse-transcribed to generate cDNA using M-MLV reverse transcriptase (Invitrogen) and oligo-dT primers. SYBR Green real-time RT-PCR was performed in a 7900HT Fast Real-Time PCR system (Applied Biosystems). The primers targeting the VP1 of EV71 were as follows: sense, 5'-TGTCCTTAATTCGCACAGCACAGC-3'; antisense, 5'-TTTCTACGCATTTGCGCGTAACCC-3'.

References

1 Lu W, Zheng BJ, Xu K *et al.* Severe acute respiratory syndrome-associated coronavirus 3a protein forms an ion channel and modulates virus release. *Proc Natl Acad Sci USA* 2006; **103**:12540-12545.
2 Schmidt OW, Cooney MK, Kenny GE. Plaque assay and improved yield of human coronaviruses in a human rhabdomyosarcoma cell line. *J Clin Microbiol* 1979; **9**:722-728.