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Scavenger receptor B2 is a cellular receptor for enterovirus 71

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Enterovirus 71 (EV71) belongs to human enterovirus species A of the genus *Enterovirus* within the family *Picornaviridae*[1]. EV71, together with coxsackievirus A16 (CVA16), are most frequently associated with hand, foot and mouth disease (HFMD)[1]. Although HFMD is considered a mild exanthematous infection, infections involving EV71, but not CVA16, can progress to severe neurological disease, including fatal encephalitis, aseptic meningitis and acute flaccid paralysis[2]. In recent years, epidemic and sporadic outbreaks of neurovirulent EV71 infections have been reported in Taiwan, Malaysia, Singapore, Japan and China[3–7]. Here, we show that human scavenger receptor class B, member 2 (SCARB2, also known as lysosomal integral membrane protein II or CD36b like-2) is a receptor for EV71. EV71 binds soluble SCARB2 or cells expressing SCARB2, and the binding is inhibited by an antibody to SCARB2. Expression of human SCARB2 enables normally unsusceptible cell lines to support EV71 propagation and develop cytopathic effects. EV71 infection is hampered by the antibody to SCARB2 and soluble SCARB2. SCARB2 also supports the infection of the milder pathogen CVA16. The identification of SCARB2 as an EV71 and CVA16 receptor contributes to a better understanding of the pathogenicity of these viruses.

Virus receptors have essential roles in the early steps of viral infection. They are, therefore, a primary determinant of host range and tissue tropism. EV71 is known as one of the most important pathogens of emerging infectious disease in humans. However, the receptor for EV71 has not been identified yet. Thus, we set out to identify a receptor on human cells for this key pathogen.

RD cells, established from human rhabdomyosarcoma and frequently used to isolate EV71 from clinical specimens, are highly susceptible to EV71. Mouse L929 cells, however, allow only highly inefficient EV71 infection, as we observed only a few viral protein–positive cells (approximately 1 cell in 1×10^5 cells) and no development of cytopathic effects in the cells even when infected at a high multiplicity of infection (MOI) (data not shown). However, we efficiently recovered infectious particles from L929 cells transfected with EV71 genomic RNA (data not shown). These results suggest that the restriction occurs at one of the early steps of infection (attachment, possible internalization or viral uncoating), but not at the replication or assembly steps. Accordingly, we pursued a strategy used in the identification of the poliovirus receptor[8,9]. Briefly, we established L929 transformant (Ltr) cells by transfection of L929 cells with genomic DNA from RD cells. We then detected EV71-susceptible cells via infection with EV71-GFP, which expressed GFP upon infection. Repeated screens led to the generation of two monoclonal cell lines, Ltr051 and Ltr246. We detected a number of GFP-positive cells in Ltr051 and Ltr246 cell cultures upon EV71-GFP infection (**Fig. 1a**). To examine EV71 viral growth kinetics, we infected Ltr051, Ltr246, RD and L929 cells with EV71 strain SK-EV006, isolated from the rectal swab of an individual with encephalitis, at an MOI of 0.01 (**Fig. 1b**). EV71 SK-EV006 grew in Ltr051 cells as efficiently as it did in RD cells (**Fig. 1b**). Ltr246 cells could also support EV71 infection, but the final virus titer was approximately 2.7% of that obtained in Ltr051 cells (**Fig. 1b**). We observed cytopathic effects in Ltr051, Ltr246 and RD cells at 48 h after infection (**Supplementary Fig. 1**). The appearance of cytopathic effects in Ltr246 cells was slower and weaker than in Ltr051 and RD cells (**Supplementary Fig. 1**). Collectively, these results suggest that Ltr051 and Ltr246 cells express a human gene(s) that is essential in the early steps of EV71 infection.

To identify the putative human gene(s) in Ltr051 and Ltr246 cells, we performed a transcriptome analysis comparing the two transformants and L929 cells via human microarray analysis. A total of 14 genes in Ltr051 cells and 33 genes in Ltr246 cells showed a more than twofold increase in expression in Ltr051 or Ltr246 cells compared with L929 cells (**Supplementary Tables 1** and **2**). We selected protein-coding genes with more than fourfold increased signals or putative transmembrane protein-coding genes with more than twofold increased signals for further analysis. To determine whether these 22 selected genes were integrated into the genomes of Ltr051 or Ltr246 cells, we performed PCR with genomic DNA from Ltr051 and Ltr246 cells as the template. We amplified the *SCARB2* gene from the genomic DNA of Ltr051 cells, whereas we amplified the *CCL2*

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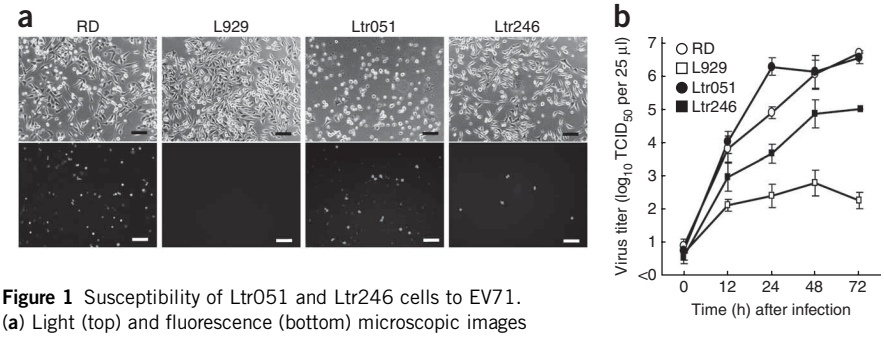

Figure 1 Susceptibility of Ltr051 and Ltr246 cells to EV71. (**a**) Light (top) and fluorescence (bottom) microscopic images of Ltr051, Ltr246, RD and L929 cells infected with EV71-GFP at 24 h after infection. Scale bar, 200 μm. (**b**) Virus titers in Ltr051 cells, Ltr246 cells, RD cells and L929 cells infected with EV71 SK-EV006 at an MOI of 0.01. Virus titers were determined at 0, 12, 24, 48 and 72 h after infection. The data are shown as mean virus titers ± s.d. ($n = 3$). $TCID_{50}$, 50% tissue culture infective dose.

(encoding CC chemokine ligand-2) and *MEPCE* (methylphosphate capping enzyme) genes from Ltr246 cells (**Supplementary Tables 1** and **2**). We did not amplify the other 19 genes from either genomic DNA.

To determine whether expression of SCARB2, CCL2 or BCDIN3 makes L929 cells susceptible to EV71, we transfected L929 cells with a plasmid encoding SCARB2, CCL2 or BCDIN3 (pCA-SCARB2, pCA-CCL2 or pCA-BCDIN3) and then infected the cells with EV71-GFP. We detected GFP-positive cells in L929 cell cultures transfected with pCA-SCARB2 alone (**Supplementary Fig. 2**). We obtained similar results when we used BHK-21 and CHO cells as recipient cells (**Supplementary Fig. 2**). In contrast, L929 cells did not become susceptible to EV71-GFP by individual transfection of pCA-CCL2 and pCA-BCDIN3 or by transfection of both plasmids together (data not shown). These results suggest that human SCARB2 is sufficient to permit EV71 infection of Ltr051 cells. We presume that the susceptibility of Ltr246 cells to EV71 may be due to an unlisted human protein-coding gene. Further studies are required to identify the gene.

SCARB2 belongs to the scavenger receptor class B subfamily, which also includes *SCARB1* (*Cla-1*) and *CD36*[10,11]. SCARB2 is a type III double-transmembrane protein with N- as well as C-terminal cytoplasmic tails and is located primarily in lysosomes and endosomes[11]. SCARB2 participates in membrane transportation and the reorganization of the endosomal-lysosomal compartment[12]. Deficiency of SCARB2 in mice causes ureteric pelvic junction obstruction, deafness and peripheral neuropathy[13].

We established L-SCARB2 and L-Empty cells, which stably retained pCA-SCARB2 and pCAGGS-PUR, respectively. Because SCARB2 is known to localize mainly to the lysosomal membrane, we evaluated whether SCARB2 is also expressed in the plasma membrane. L-SCARB2 and L-Empty cells were stained with an antibody to human SCARB2 without permeabilization (**Fig. 2a**). We also stained RD and L929 cells as a positive and a negative control, respectively. We clearly detected cell surface expression of human SCARB2 on L-SCARB2 and RD cells (**Fig. 2a**). Next, to evaluate the growth kinetics of EV71, we infected these cells with EV71 SK-EV006. The growth kinetics of EV71 SK-EV006 in L-SCARB2 cells was similar to those in RD cells (**Fig. 2b**). In accordance with viral growth, we found cytopathic effects at 48 h after infection in both L-SCARB2 and RD cells (**Fig. 2c**). These data indicate that EV71 enters and propagates in L-SCARB2 cells and spreads efficiently.

To determine whether L-SCARB2 cells are susceptible to other EV71 strains, we infected L-SCARB2 cells with EV71 strains belonging to genogroups A, B and C (**Supplementary Fig. 3**). At 48 h after infection, all tested strains induced obvious cytopathic effects in both L-SCARB2 and RD cells (**Supplementary Fig. 3**). To evaluate the viral growth kinetics of representative strains from each genogroup, we infected these cells with strain BrCr (genogroup A), strain Nagoya (genogroup B) and strain Isehara (genogroup C). BrCr, Nagoya and Isehara grew in L-SCARB2 cells with a similar efficiency to that observed in RD cells (**Fig. 2d**). These results suggest that SCARB2 is able to serve as an efficient cellular receptor for several, and possibly all, EV71 strains. Strains BrCr and Isehara hardly propagated in L929 cells, whereas strains SK-EV006 and Nagoya inefficiently

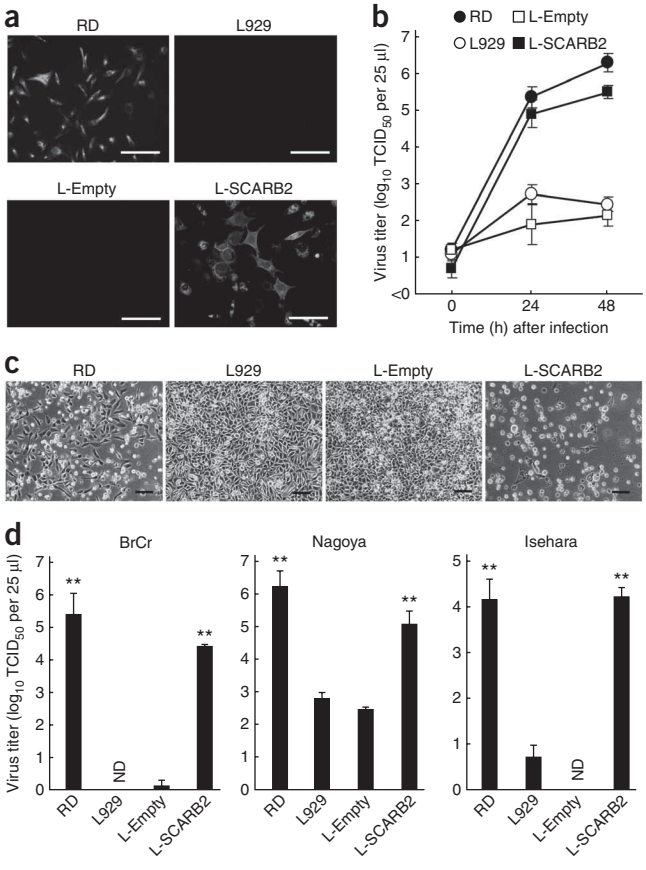

Figure 2 SCARB2 confers susceptibility to EV71 to mouse L929 cells. (**a**) Cell surface expression of SCARB2 on L-SCARB2, L-Empty, RD and L929 cells stained with antibody to SCARB2 without permeabilization. Scale bar, 200 μm. (**b**) Virus titers in L-SCARB2 cells, L-Empty cells, RD cells and L929 cells infected with SK-EV006 at an MOI of 0.01. Virus titration was performed at 0, 24 and 48 h after infection. The data are shown as mean virus titers ± s.d. ($n = 3$). (**c**) L-SCARB2, L-Empty, RD and L929 cells were infected with SK-EV006 at an MOI of 0.01. These cells were imaged via light microscopy at 48 h after infection. Scale bar, 200 μm. (**d**) Virus titers in L-SCARB2, L-Empty, RD and L929 cells infected with BrCr, Nagoya or Isehara at an MOI of 0.01. Virus titers were determined at 48 h after infection. The data are shown as mean virus titers ± s.d. ($n = 3$). $^{**}P < 0.01$ by Student's t test compared with the virus titer in L929 cells. ND, not detected.

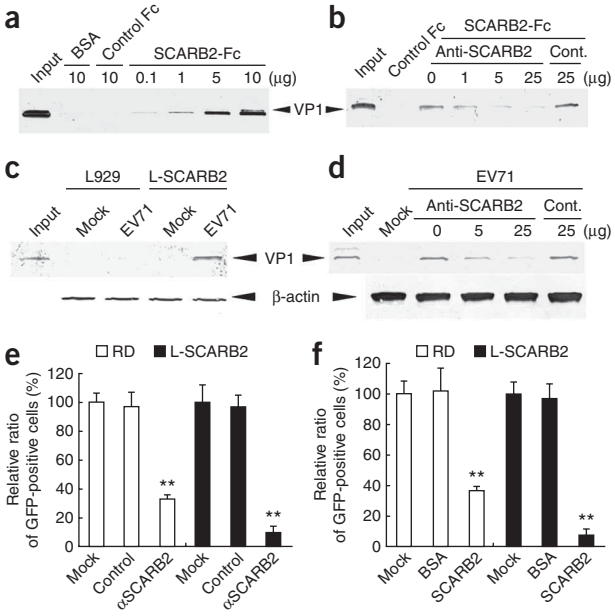

Figure 3 Interaction of SCARB2 with EV71. (**a**) Western blot analysis of EV71 incubated with BSA, control Fc or SCARB2-Fc bound to protein G–agarose. The precipitated proteins were analyzed with antibody to EV71 (recognizing mainly VP1). (**b**) Western blot analysis of SCARB2-Fc (2 μg) bound to protein G–agarose pretreated with indicated amount of antibody to SCARB2 (anti-SCARB2) or a normal goat IgG (Cont.) before incubation with EV1. The precipitated proteins were analyzed with antibody to EV71. (**c**) Western blot analysis of L-SCARB2 or L929 cells incubated with EV71 strain SK-EV006 (EV71) or 5% FCS-DMEM (Mock). After washing steps, these cells were lysed and then analyzed with antibody to EV71 or to β-actin. (**d**) Western blot analysis of L-SCARB2 cells treated with the indicated amount of antibody to SCARB2 or a normal goat IgG before incubation with EV71. Total cell lysates were analyzed with antibody to EV71 or to β-actin. (**e**) GFP-positive cell counts among L-SCARB2 cells and RD cells pretreated with 50 μg ml^{-1} of antibody to SCARB2 or a normal goat IgG before EV71-GFP infection. Mock treatment is set at 100%. The data are shown as mean percentages ± s.d. ($n = 5$). $^{**}P < 0.01$ by Student's t test when compared with the mock infection. (**f**) GFP-positive cell counts among L-SCARB2 cells and RD cells infected with EV71-GFP that had been mixed with 12 μg ml^{-1} of SCARB2-Fc or BSA. Mock treatment is set at 100%. The data are shown as mean percentages ± s.d. ($n = 5$). $^{**}P < 0.01$ by Student's t test when compared with the mock infection.

propagated in these cells (**Fig. 2b**,**d**). Some strains may be able to infect L929 cells via mouse Scarb2, as overexpression of mouse Scarb2, which has an 85.8% amino-acid sequence identity to human SCARB2, makes mouse cells susceptible to EV71 although not as efficiently as human SCARB2 (data not shown).

To determine whether EV71 directly binds SCARB2, we conducted pull-down assays with soluble SCARB2-Fc (extracellular region of SCARB2 fused to the Fc region of human IgG) (**Fig. 3a**). We incubated EV71 SK-EV006 with SCARB2-Fc and protein G–agarose beads, and we analyzed the precipitated proteins by western blotting with an antibody to EV71. The antibody to EV71 predominantly detected VP1 (**Fig. 3a**). We detected an EV71-VP1 band in a SCARB2-Fc concentration–dependent manner (**Fig. 3a**). The binding of EV71 to SCARB2-Fc was inhibited by the antibody to SCARB2 in a dose-dependent manner (**Fig. 3b**). Binding of SCARB2-Fc to poliovirus, whose receptor is CD155, was not observed under similar conditions (data not shown). These data indicate that SCARB2 directly and specifically binds EV71.

To assess whether EV71 binds SCARB2 in the plasma membrane, we conducted a virus attachment assay with L-SCARB2 or L929 cells. We incubated these cells with EV71 SK-EV006 and detected attached virus by western blotting (**Fig. 3c**). EV71 SK-EV006 was attached to L-SCARB2 cells (**Fig. 3c**), and the attachment was inhibited by the antibody to SCARB2 in a dose-dependent manner (**Fig. 3d**). These data indicate that SCARB2 mediates the attachment of EV71 to the cells.

To ascertain whether the binding of SCARB2 to EV71 mediates EV71 infection, we next performed an infection inhibition assay with either the antibody to SCARB2 or the SCARB2-Fc. We preincubated L-SCARB2 and RD cells with the antibody and then infected them with EV71-GFP. EV71 infection was blocked by pretreatment of L-SCARB2 and RD cells with the antibody in a dose-dependent manner (**Supplementary Fig. 4a**). The inhibition efficiency for L-SCARB2 cells was higher than that for RD cells, in which inhibition was not complete even at the highest antibody concentration (**Fig. 3e**). We next tested the infection inhibition assay with SCARB2-Fc. We infected L-SCARB2 and RD cells with EV71-GFP after we incubated the virus with SCARB2-Fc. We found that the inhibition effect was dose dependent (**Supplementary Fig. 4b**). Again, the inhibition efficiency for L-SCARB2 cells was higher than that for RD cells at the highest SCARB2-Fc concentration (**Fig. 3f**). These data indicate that EV71 enters cells through a SCARB2-dependent pathway. In addition, the difference in the extent of infection inhibition by the antibody and SCARB2-Fc between the L-SCARB2 and RD cells suggests that RD cells might have a separate, minor path for the early steps of EV71 infection.

To confirm the importance of SCARB2 in EV71 infection, we examined whether EV71 also uses SCARB2 in infecting other human cell lines. We detected SCARB2 on the surface of HeLa, HEp-2, 293T and HepG2 cells, and the infection efficiency of EV71-GFP was well correlated with the level of cell surface SCARB2 expression (**Supplementary Fig. 5a**,**b**). By infection inhibition assay, the infection of these cells by EV71-GFP was clearly inhibited by pretreatment with the antibody at a similar degree to RD cells (**Supplementary Fig. 5c**). These data indicate that SCARB2 has a dominant role in the early steps of EV71 infection of the tested human cell lines.

We next tested whether SCARB2 mediates infection of other human enterovirus species A viruses. For this purpose, we infected L-SCARB2 cells and L-Empty cells with CVA2, CVA3, CVA4, CVA5, CVA6, CVA7, CVA8, CVA10, CVA12, CVA14 and CVA16 (**Table 1** and **Supplementary Fig. 6**). At 48 h after infection, CVA16 induced cytopathic effects in both L-SCARB2 and RD cells (**Supplementary Fig. 6**). CVA2, CVA3, CVA4, CVA5, CVA6, CVA8 and CVA12 induced

Table 1 Induction of cytopathic effects by coxsackieviruses

Virus	RD cells	L929 cells	L-Empty cells	L-SCARB2 cells
CVA2	+	–	–	–
CVA3	+	–	–	–
CVA4	+	–	–	–
CVA5	+	–	–	–
CVA6	+	–	–	–
CVA7	+	+	+	+
CVA8	+	–	–	–
CVA10	+	+	+	+
CVA12	+	–	–	–
CVA14	+	+	+	+
CVA16	+	–	–	+

cytopathic effects only in RD cells (**Supplementary Fig. 6**). CVA7, CVA10 and CVA14 induced cytopathic effects in all four cell lines (**Supplementary Fig. 6**); thus, it was impossible to determine whether the cytopathic effects induced by these viruses were due to human SCARB2–mediated infection. We examined the viral growth kinetics of these viruses in L-SCARB2 cells. CVA16 grew efficiently in L-SCARB2 and RD cells, whereas CVA2, CVA3, CVA4, CVA5, CVA6, CVA8 and CVA12 grew at a high efficiency only in RD cells (**Supplementary Fig. 7**). These data indicate that CVA16 is also able to infect cells through a SCARB2-dependent pathway and that infection with most other human enterovirus species A is not dependent upon SCARB2.

In this study, we obtained two L929 cell transformants susceptible to EV71 infection and revealed that Ltr051 cells express human SCARB2. EV71 propagation in both L-SCARB2 and Ltr051 cells was as efficient as it is in RD cells. EV71 infection of RD cells was mostly blocked by antibody to SCARB2 and by SCARB2-Fc. Ltr246 cells were also susceptible to EV71, but their infection efficiency was lower than that of Ltr051 cells and RD cells. These results indicate that SCARB2 has a dominant role in EV71 infection of RD cells, although another minor receptor presents in Ltr246 cells might also contribute to EV71 infection.

During fatal cases of human EV71 infection, EV71 has been recovered from the central nervous system, lymph nodes, skin, intestinal mucosa and infectious lesions found in nearly all organs[14]. Because SCARB2 is expressed ubiquitously[11], it is possible that SCARB2 may be involved in systemic EV71 infections. SCARB2 serves as a receptor for EV71 strains isolated from individuals with HFMD as well as from individuals with encephalitis. SCARB2 also serves as a receptor for CVA16. It is noteworthy that although SCARB2 may be involved in the pathogenesis of HFMD caused by these viruses, EV71 occasionally induces neurologic symptoms, and CVA16 rarely does. Further study should clarify the involvement of SCARB2 in EV71 and CVA16 infection *in vivo* and may elucidate the mechanism behind the different levels of pathogenicity observed for these similar viruses.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Accession codes. Microarray data have been deposited in the Gene Expression Omnibus database with accession code GSE16358.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

S.Y. designed and performed the majority of the experiments, analyzed the data and wrote the manuscript; Y.Y. constructed pSVA-EV71-GFP and established Ltr051 and Ltr246 cells; J.L. constructed pSVA-EV71; N.H., T.M. and T.T. performed microarray analysis; S.K. designed the project, assisted with the experiments, analyzed the data and wrote the manuscript.

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ONLINE METHODS

Viruses. We propagated EV71 (strains BrCr/USA/70 (BrCr; genogroup A), SK-EV006/Malaysia/97 (SK-EV006; genogroup B), Nagoya/Japan/73 (Nagoya; genogroup B), C7/Japan/97 (C7; genogroup B), Hungary/78 (Hungary; genogroup B), 258/Bulgaria/75 (258; genogroup B), 1095/Japan/97 (1095l; genogroup C) and Isehara/Japan/99 (Isehara; genogroup C)) in Vero cells for use in this study[15]. BrCr was isolated from an individual with meningitis; SK-EV006, C7, Hungary and 258 were from individuals with encephalitis; and Nagoya, 1095 and Isehara were from individuals with HFMD. All strains were provided by H. Shimizu.

Cloning of Ltr051 and Ltr246 cells. We transfected L929 cells with genomic DNA from RD cells and BamHI-digested pcDNA3.1.After 24 h, we divided the cells into 100–200 pools and selected with DMEM containing 5% FCS and 1 mg ml^{-1} G418. After 7 d, we split each pool, which contained approximately 100 G418-resistant clones, in two and cultured until the cells formed colonies. We infected one of the pools with EV71-GFP to test for EV71-susceptible cells and subjected the other, containing EV71-susceptible cells, to repeated cloning procedures and established it as Ltr051 cells. We established Ltr246 cells from an independent transfection.

Microarray analysis and genomic PCR. We extracted total RNA from Ltr051, Ltr246 and L929 cells with an RNeasy Mini Kit (Qiagen). We used each total RNA sample as a template to obtain amplified RNA (aRNA) using an Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion). We labeled each aRNA with Cy5 or Cy3 and competitively hybridized for 17 h at 65 °C with a Whole Human Genome Microarray kit 4x44K (Agilent) ($n = 3$). We analyzed the microarray images, obtained with a GenePix 4000B (Axon), by GenePix Pro (Axon). We calculated human gene expression levels in Ltr051 or Ltr246 cells as relative values to those in L929 cells. Increased signals would have been induced by the integration and expression of human genes. They would also have been elicited by the upregulation of mouse messenger RNAs, cross-hybridized with microarray probes target to human mRNA, owing to an incidental integration of human promoters, human enhancers or human signal transduction genes. We focused on the former cases. To examine whether increased signals were due to integrated human genes, we extracted genomic DNA from Ltr051 and Ltr246 cells and subjected it to PCR using AmpliTaq Gold (Applied Biosystems) and appropriate primers (**Supplementary Table 3**). We confirmed that all primer sets could amplify the target sequences from the genomic DNA of RD cells but not L929 cells. We analyzed the hydrophobicity of the candidate proteins with the SOSUI program[16].

Pull-down assay. We incubated EV71 strain SK-EV006 (8.55×10^6 $TICD_{50}$) or poliovirus (6×10^6 plaque-forming units) with BSA (10 μg), human IgG Fc (10 μg, R&D Systems) or human SCARB2-Fc (0.1 to 10 μg, R&D Systems) and protein G–agarose (25 μl, Sigma) in 1 ml of DMEM for 2 h at 4 °C. We then washed the beads twice with DMEM, suspended them in SDS sample buffer and incubated them for 5 min at 95 °C. After the beads were removed, we subjected the samples to 18% SDS-PAGE followed by western blotting with a rabbit antibody to EV71 (ref. 15) or a rabbit antibody to poliovirus[17]. In the binding inhibition test, we treated beads coated with SCARB2-Fc (2 μg) with antibody to SCARB2 (1–25 μg ml^{-1}, R&D Systems) for 1 h at 4 °C before the virus was mixed.

Virus attachment assay. We detached L-SCARB2 or L929 cells in PBS containing 0.05% EDTA and mixed them with EV71 strain SK-EV006 (8.55×10^6 $TCID_{50}$) for 1 h at 4 °C. We washed these cells twice with PBS, suspended them in SDS sample buffer and incubated them for 5 min at 95 °C. We then subjected them to 18% SDS-PAGE followed by western blotting with the antibody to EV71 or to β-actin (clone AC-74, Sigma). In the attachment inhibition test, we pretreated these cells with the antibody to SCARB2 (5 or 25 μg ml^{-1}) for 1 h at 4 °C before the virus was mixed.

Infection inhibition assays. To examine the effect of the antibody to SCARB2, we preincubated RD and L-SCARB2 cells with the antibody to SCARB2 (0.5, 1, 5, 10, 25 or 50 μg ml^{-1}) for 30 min at 37 °C. We subsequently added EV71-GFP to the cells. After 18 h, we counted GFP-positive cells under the microscope. To examine the effect of soluble SCARB2, we mixed EV71-GFP with the SCARB2-Fc (0.125, 0.25, 0.75, 1.5, 3, 6 or 12 μg ml^{-1}) and incubated for 60 min at 37 °C. We then added the mixture to L-SCARB2 and RD cells. After 18 h, we counted GFP-positive cells under the microscope. The experiments were repeated five times.

Flow cytometry. We performed flow cytometry as previously reported[18] with some modifications. Briefly, we detached cells in PBS containing 0.02% EDTA, stained them with the antibody to SCARB2 followed by the Alexa Fluor 488–conjugated donkey antibody to goat IgG (Invitrogen), and then analyzed the cells by using a FACSCalibur with Cell Quest Pro software (Becton Dickinson).

Statistical analyses. We analyzed data obtained from three to five independent experiments by Student's t test. We expressed results as means ± s.d. and considered them significant when $P < 0.05$.

Additional Methods. Detailed methodology is described in the **Supplementary Methods**.

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