Supporting Information

Suzuki et al. 10.1073/pnas.1311457110

SI Materials and Methods

Cell Culture and Virus Preparation. Human embryonic kidney 293 cells with SV40 T antigen (HEK293T) and human cervical carcinoma cells (HeLa) were obtained from the Health Science Research Resources Bank. SV40-transformed human glial SVG-A cells (kindly provided by W. J. Atwood, Brown University, Providence, RI) (1) were also used. All cells were maintained under 5% (vol/vol) CO_2 at 37 °C in DMEM supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mM L-glutamine, penicillin, and streptomycin (Sigma). Establishment and maintenance of HEK293 cells expressing JCV agnoprotein in an inducible manner (293AG cells) were described previously (2, 3). SVG-A cells stably expressing Myc-tagged AP3D-Y27N or AP3D-Y27C were established using the pIRESneo3-AP3D-Y27N or pIRES-AP3D-Y27C plasmids. The resulting cells were maintained under 5% (vol/vol) CO_2 at 37 °C in DMEM supplemented with 10% (vol/vol) FBS (Clontech), 2 mM L-glutamine, penicillin, streptomycin (Sigma), and 400 μg/mL G418. Establishment of SVG-A cells expressing wild-type agnoprotein (SVG-AG cells) or the RK8AA mutant of agnoprotein (SVG-RK8AA cells) in DOX-inducible manner were performed using Retro-X Tet-On Advanced Inducible Expression System (Clontech). Wild type or the RK8AA mutant of the agnogene followed by the IRES sequence and ERedNLS gene were amplified by PCR with pERedNLS-Agno or pERedNLS-RK8AA and cloned into the pRetroX-Tight-Pur vector (Clontech). The sequence containing the IRES sequence and ERedNLS gene without agnogene was also cloned into the pRetroX-Tight-Pur vector as a mock vector. The resulting plasmids were subjected to retrovirus production with the Retro-X Tet-On Advanced Inducible Expression System according to the manufacturer's instructions. SVG-A cells were inoculated with these recombinant retroviruses and selected in presence of 400 μg/mL G418 and 1 μg/mL puromycin, and the resulting cells were designated as SVG-AG cells expressing wild type of agnoprotein and ERedNLS, SVG-RK8AA cells expressing RK8AA mutant of agnoprotein and ERedNLS, or SVG-Mock cells expressing ERedNLS alone. These cells were maintained under 5% (vol/vol) CO_2 at 37 °C in DMEM supplemented with 10% (vol/vol) Tet system-approved FBS (Clontech), 2 mM L-glutamine, penicillin, streptomycin, 400 μg/mL G418, and 1 μg/mL puromycin. Agnoproteins and ERedNLS were induced with 1 μg/mL DOX. SVG-AG cells stably expressing Myc-tagged AP3D-Y27N or AP3D-Y27C were established using the lentiviral system and maintained under 5% (vol/vol) CO_2 at 37 °C in DMEM supplemented with 10% (vol/vol) Tet system-approved FBS, 2 mM L-glutamine, penicillin, streptomycin (Sigma), 400 μg/mL G418, 1 μg/mL puromycin, and 10 μg/mL blastcidin. For virus preparation, JCV-infected SVG-A cells were harvested and suspended in Tris·HCl (pH 7.5) containing 0.2% BSA (BSA), frozen and thawed three times, and then treated with 0.05 U/mL of neuraminidase type V (Sigma) at 37 °C for 16 h. After incubation at 56 °C for 30 min, cell lysates were centrifuged at 1,000 × *g* for 10 min. The supernatant was quantified by hemagglutination (HA) assays and stored at −80 °C until use.

Construction of Plasmids. For expression of JC polyomavirus (JCV) agnoprotein in mammalian cells, the cDNA of JCV agnoprotein was amplified by PCR using a plasmid encoding the complete JCV genome, pJC1->4pJCV (VG015; Health Science Research Resources Bank), and subcloned into a pCXSN plasmid (4), which was constructed by removing myc tag from pCMV-myc (Clontech) and adding XhoI, SalI, and NotI recognition sites; pCXSN-FlagN (4), which was constructed by adding Flag tag to the pCXSN plasmid at the 5′ region of the XhoI site; pCXSN-MycN (4), which was constructed by adding Myc tag to the pCXSN plasmid at the 5′ region of the XhoI site; pERedNLS (kindly provided by M. Matsuda, Kyoto University, Kyoto, Japan) (4); and pCFPNLS (4), which was constructed by replacing DsRedExpress with sECFP (kindly provided by A. Miyawaki, RIKEN, Wako, Japan). pERedNLS or pCFPNLS contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus between the multiple cloning sites (MCS) and the ERedNLS [DsRed-Express with nuclear localization signal (NLS) of simian virus 40 (SV40) large T antigen fused to its C terminus] or CFPNLS (sECFP with NLS of SV40 large T antigen fused to its C terminus). This plasmid permits both the inserted gene in the MCS and the ERedNLS gene or the CFPNLS gene to be translated from a single bicistronic mRNA. These plasmid-transfected cells were thus labeled by expression of DsRed-Express or sECFP in the nucleus. The gene in MCS is not fused with ERedNLS or CFPNLS but is tagged with a Flag epitope at the NH2 terminus. The substitution mutants of agnoprotein were constructed as described previously (4). The plasmid containing the genome of JCV Mad1-SVEΔ (pUC19-Mad1SVEΔ) was kindly provided by W. J. Atwood (Brown University, Providence, RI) (5, 6). Agnoproteins with mutated viral genomes (ΔAgno and RK8AA) were generated by site-directed mutagenesis as described previously (4). The cDNA of AP3D1 isoform 2 was amplified from the HEK293-derived cDNA library and subcloned into pCMV-myc (Clontech). Successful cloning was confirmed by sequencing. Myc-tagged AP3D-Y27, AP3D-Y27N, and AP3D-Y27C were amplified with pCMV-myc-AP3D1 and subcloned into the pIRESneo3 vector (Clontech). AP3D-Y27, AP3D-Y27N, and AP3D-Y27C were amplified with pIRESneo3-AP3D-Y27 and subcloned into pGEX6P1 vector (GE). Successful cloning was confirmed by sequencing. The plasmid encoding ts045-VSVG-GFP (pVSVG-GFP) was a kind gift from I. Wada (Fukushima Medical University, Fukushima, Japan) (7). The ts045-VSVG protein is a type I transmembrane protein that has been widely used for studying secretory membrane trafficking because it contains a mutation that leads to its reversible misfolding and retention in the ER at 40 °C. The entire coding sequences of VP1 (pET15b-VP1) (8) and RK8AA mutant of agnoprotein (pET15b-His-RK8AA) were amplified by PCR and subcloned into the pET15b expression vector (Novagen) for bacterial expression. The integrities of plasmids were verified by sequencing.

Primary Antibodies and Reagents. Rabbit anti-JCV agnoprotein, anti-JCV VP1, and anti-JCV Large T polyclonal antibodies were produced as described previously (9–11). Alexa Fluor 488-labeled or Alexa Fluor 647 anti-agnoprotein antibodies were prepared using APEX Antibody Labeling Kits (Invitrogen) according to manufacturer's instructions. Mouse anti-GM130, anti-EEA1, and anti-AP3 δ subunit (AP3D) monoclonal antibodies were purchased from BD Transduction Laboratories. Mouse anti-Lamp1 and anti-CD71 monoclonal antibodies were purchased from BD Pharmingen. Mouse anti-Lamp2 (H4B4), MHC-Class I (HLA-ABC, W6/32), and anti-actin (MAB1501R) monoclonal antibodies were purchased from Santa Cruz, eBioscience, and Chemicon International, respectively. Mouse anti-Flag (M2) monoclonal antibodies were purchased from Sigma. Mouse anti-Myc tag (9E10) monoclonal antibodies were purchased from Calbiochem. Goat anti-GFP polyclonal antibody was purchased from Rockland. Mouse anti-His-tag (OGHis) monoclonal antibodies were purchased from Medical & Biological Laboratories

(MBL). The vATPase inhibitor bafilomycin A1 was obtained from Wako Pure Chemical Industry. Leupeptin, pepstatin A, and E64d were obtained from the Peptide Institute.

In Vitro Synthesized Agnoprotein. In vitro transcription and in vitro translation were performed using the cell-free expression system, the MembraneMax HN Protein Expression Kit (Invitrogen), according to the manufacturer's instructions. The plasmids, such as pURE2-Agnoprotein, pURE2-RK8AA mutant, pURE2-K9Q mutant, and pURE2 plasmids encoding agnoprotein deletion mutants (C6, C18, and C25), which contain T7 promoters, ribosomal binding sites, and terminator sequences, were used as template DNA. The entire coding sequences of wild type, K9Q mutant of JCV agnoprotein, and WT BKV agnoprotein (Dunlop strain) were also subcloned into the pEXP5-CT/TOPO expression vector (Invitrogen) and used as template DNA for expression of the C-terminal hexahistidine (6 × His) tagged agnoprotein. After synthesis of recombinant proteins, the reaction mixture was subjected to SDS/PAGE analysis and immunoblotting using anti-agnoprotein antibody to confirm the expression of proteins.

GST Pull-Down Assay. GST fusion proteins of AP3D-Y27 or its deletion mutants were expressed in *Escherichia coli* (BL21DE3; Novagen) and purified with the use of glutathione Sepharose 4B beads (GE Healthcare). For in vitro GST precipitation assays, GST or GST fusion proteins were mixed with 10 μL of 50% (vol/vol) glutathione Sepharose 4B for 2 h at 4 °C. After addition of cell lysates from 293T cells transfected with pCMV-Agno or in vitro synthesized agnoprotein in TN-TNE buffer [20 mM Tris·HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1% Triton X-100, 10% (vol/vol) glycerol, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and complete protease inhibitor mixture, the mixture was incubated for another 30 min at 4 °C. The beads were separated by centrifugation and washed with TN-TNE buffer, and the bound proteins were subjected to immunoblot analysis.

Preparation and Infection of Recombinant Lentiviruses. The following constructs were kindly provided by H. Miyoshi (RIKEN, Tsukuba, Japan): CSII-CMV-MCS-IRES2-Bsd, a self-inactivating lentiviral construct (12); pCAG-HIVgp, a packaging construct expressing Gag and Pol proteins; and pCMV-VSV-G-RSV-Rev, a construct expressing the vesicular stomatitis virus G glycoprotein (VSV-G) and Rev. This lentiviral system is designed to express a desired gene under the direction of the CMV promoter and the Blastcidin resistance gene as a marker for selection of infected cells. Recombinant lentiviruses constitutively expressing agnoprotein were produced as follows: cDNAs encoding agnoprotein were inserted into the multicloning site of the lentiviral expression vector. The lentiviruses were prepared as previously described (13). Briefly, 293T cells were cotransfected with three plasmids: CSII-CMV-MCS-IRES2-Bsd with or without agnoprotein cDNA insert, pCAG-HIVgp, and pCMV-VSV-G-RSV-Rev using LipofectAmine 2000 (Invitrogen). The culture supernatants containing the recombinant lentiviruses were collected at 48 h after transfection, passed through a 0.45 μm filter, and used for infection experiments. SVG-A cells or 293T cells seeded in the 35-mm dishes were infected at 10% confluence with each recombinant virus. Virus-infected cells were selected by culturing with blastcidin (10 μg/mL) containing media.

Flow Cytometry. Cells were detached using an enzyme-free/PBS-based cell dissociation buffer (Invitrogen) according to the manufacturer's instruction. Aliquots of 10^6 cells were washed in PBS/2% (vol/vol) FBS and suspended in 100 μL of PBS/2% (vol/vol) FBS. Cells were then incubated with 200 ng of primary antibody or control IgG (BD Pharmingen) as a negative control for 30 min at 4 °C. After washing, bound antibodies were visualized by addition of phycoerythrin-conjugated anti-mouse or anti-rabbit Ig antibody (Beckman Coulter). After washing, cells were suspended in 250 μL of PBS/2% (vol/vol) FBS. Cell surface fluorescence was analyzed with a Becton Dickinson FACScanto (BD Bioscience). The data were analyzed by using FlowJo software (Tree Star). For quantification of amount of agnoprotein or MHC-class I, the cells were stained with Alexa Fluor 647 or 488 labeled primary antibodies and subjected to the analysis with FACScanto. Experiments were performed three times independently. The data were analyzed using FlowJo software.

Transfection, Immunoblot Analysis, and Immunoprecipitation. Cell transfections were performed with LipofectAmine 2000 for 293T cells or Fugene HD for HeLa and SVG-A cells. For immunoblot analysis, cells were harvested at the indicated time points after transfection, lysed in TNE buffer [10 mM Tris·HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10% (vol/vol) glycerol, 1% Triton X-100, and 0.5 mM PMSF], and mixed with Complete protease inhibitor mixture. The cell lysates were fractionated by SDS/PAGE, and the separated proteins were transferred to a polyvinylidene difluoride filter (Millipore). The filter was incubated with primary antibodies, and immune complexes were then detected with horseradish peroxidase-conjugated secondary antibodies and ECL reagents (GE Healthcare). The Flag epitope was detected directly with horseradish peroxidase-conjugated primary antibodies. For detection of the homointeraction of RK8AA mutant of agnoproteins, 293T cells transfected with Myc-tagged RK8AA/Flag-tagged RK8AA or Myc-tagged RK8AA alone were incubated for 72 h and then lysed in TNE buffer and subjected to immunoprecipitation. For detection of the interactions between WT or RK8AA mutant agnoproteins and AP3D, 293AG or 293RK8AA cells were treated for 72 h with or without Doxycycline (DOX) and then lysed in 0.1% Tx lysis buffer [10 mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, and 50 μg/mL DNase I] mixed with Complete protease inhibitor mixture and subjected to immunoprecipitation. Immunoprecipitation was performed by incubation of cell lysates at 4 °C initially for 4 h with antibody-coupled protein G-Sepharose FF beads (GE Healthcare). After washing with cell lysis buffer, the bead-bound proteins were subjected to immunoblot analysis.

siRNA Preparation. The following stealth RNA duplexes were synthesized by Invitrogen: siRNA-AP3-481 sense 5′-AAAUACGUCAGCUUGCAGACCGCGU-3′ and antisense 5′-ACGCGGUCUGCAAGCUGACGUAUUU-3′, siRNA-AP3-564 sense 5′-UUCGCUUGAAGGUGAACUUGGAGGC-3′ and antisense 5′-GCCUCCAAGUUCACCUUCAAGCGAA-3′, siRNA-AP3-1122 sense 5′-AUUCAUAGAGGAGAGACAUGGCAGA-3′ and antisense 5′- UCUGCCAUGUCUCUCCUCUAUGAAU -3′, negative control (siRNA-NC1) sense 5′-GCAUCGUACAGACAAUCUUCAGUUU-3′ and antisense 5′-AAACUGAAGA UUGUCUGUACGAUGC-3′, and negative control (siRNA-NC2) sense 5′-GGAUCUUAUAGGUUAGGAGUCGGUU-3′ and antisense 5′-AACCGACUCCUAACCUAUAAGAUCC-3′. The negative control siRNAs were previously used (14, 3). We also used medium GC duplex siRNA (siRNA-NC3), which does not have homology to any vertebrate transcriptome (Invitrogen). The stealth RNA duplexes against AP2M and AP2M1 Stealth Select 3 RNAi (HSS101953, HSS101954, and HSS101955) were obtained from Invitrogen. The siRNA duplexes against AP1B1, AP2M2, AP3B2, AP3D1, and AP4M1 used in Fig. S5*C* were from a siPerfect Membrane transporter Library (Sigma Genosys).

Yeast Two-Hybrid Screening. The Matchmaker System 3 and HEK293-derived cDNA library were purchased from Clontech. The cDNA of the N-terminal fragment (amino acids 1–24) of wild type (Agno-N24) or RK8AA mutant (RK8AA-N24) of agno-

protein was subcloned into the pGBKT7 yeast shuttle vector (Clontech). AH109 yeast cells were cotransformed with pGBKT7-Agno-N24 (WT) or pGBKT7-RK8AA-N24 and cDNAs from the HEK293 library, and the procedures for yeast growth were according to the manufacturers' protocols. Plasmids were isolated from positive colonies following transformation of *E. coli*, DH5 alpha, and sequenced. The sequence data were analyzed by BLAST in NCBI.

Immunofluorescence. For analysis of the colocalization of agnoprotein with AP3D, EEA-1, or Lamp-2, JCV-infected SVG-A cells or DOX-treated SVG-AG/SVG-RK8AA cells were fixed for 3 min in 100% methanol at −80 °C. After blocking with 1% BSA, cells were incubated with anti-agnoprotein/anti-AP3D, anti-agnoprotein/anti-EEA-1, or anti-agnoprotein/anti–Lamp-2 antibodies overnight at 4 °C and stained with Alexa Fluor 488-conjugated anti-mouse Ig antibody and Alexa Fluor 594-conjugated anti-rabbit Ig antibodies (Molecular Probes) for 1 h at room temperature. Nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI) simultaneously with secondary antibody. The cells were observed with a confocal laser-scanning microscope (Olympus). For analysis of colocalization of VSVG-GFP and GM130, 293T cells were transfected with pVSVG-GFP and pERedNLS-Agno for evaluation of VSVG-GFP transport to the plasma membrane. The cells were cultured first at nonpermissive temperature (39.5 °C) for the ts045-VSVG-GFP transport for 20 h, followed by a permissive temperature (32 °C). After 3 h incubation, the cells were fixed for 20 min in 4% (wt/vol) paraformaldehyde (PFA)/PBS, permeabilized with 0.5% Triton X-100 in PBS, and incubated at room temperature with 1% BSA in PBS. The cells were then incubated with anti-GM130 antibody followed by staining with Alexa Fluor 647-conjugated anti-mouse Ig antibody and were analyzed by confocal microscopy. For analysis of the localization of agnoprotein, SVG-AG or SVG-RK8AA cells were fixed for 20 min in 4% (wt/vol) PFA/PBS, permeabilized with 0.5% Triton X-100 in PBS, and incubated at room temperature with 1% BSA in PBS. The cells were then incubated with anti-agnoprotein antibody followed by staining with Alexa Fluor 488-conjugated anti-rabbit Ig antibody. Actin filaments and nuclei were labeled with Alexa Fluor 647-conjugated phallodin and DAPI simultaneously with secondary antibody. The cells were imaged with an inverted fluorescence/phase-contrast microscopy (Olympus) equipped with cooled CCD camera (Olympus). The levels of protein expression in single cells were analyzed by Multi Wavelength Cell Scoring Application Modules of MetaMorph software. The number of agnoprotein-positive punctuate objects was analyzed by Granularity Application Module of MetaMorph software (Molecular Devices).

Statistical Analysis. All data were expressed as mean ± SD. Student *t* test was used to analyze differences between two groups. A value of $P < 0.05$ was considered as statistically significant.

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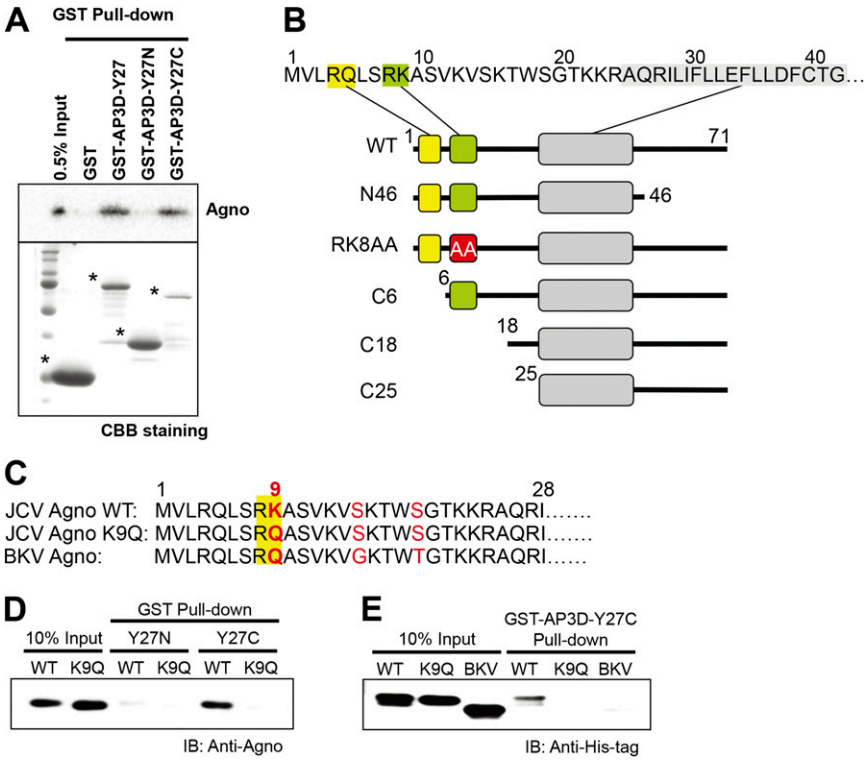

Fig. S1. Identification of AP3D as an agnoprotein interacting protein. (*A*) GST pull-down assay with the GST-AP3D-Y27, GST-AP3D-Y27N, or GST-AP3D-Y27C. After incubation with cellular lysates transfected with pCMV-Agno, the GST fusion proteins were precipitated with glutathione-Sepharose, and bound proteins were subjected to immunoblot analysis with anti-agnoprotein Ab (*Upper*). GST fusion proteins of AP3D-Y27, AP3D-Y27N, and AP3D-Y27C were subjected to coomassie brilliant blue (CBB) staining (*Lower*). Asterisks indicate the recombinant proteins. (*B*) Schematic representation of the constructs of WT and mutants of agnoprotein. The yellow boxes indicate the amino acids, which are necessary for targeting the endoplasmic reticulum (ER) (*4*). The green boxes indicate the basic amino acid cluster, which is important for viroporin activity. The gray boxes indicate a hydrophobic amino acid stretch. Arg-8 and Lys-9 of agnoprotein were substituted by Ala in the RK8AA mutant, indicated as a red box. (*C*) Comparison of the N-terminal regions of agnoproteins of JCV and BKV. The differences of amino acids are indicated with red colored characters. Lys-9 of JCV agnoprotein was substituted by Gln in the K9Q mutant (JCV Agno K9Q). (*D*) GST pull-down assay with the GST-fused proteins (GST-AP3D-Y27N or GST-AP3D-Y27C) and in vitro synthesized JCV agnoproteins (WT and K9Q). After incubation with in vitro synthesized JCV agnoproteins, the GST fusion proteins were precipitated with glutathione-Sepharose, and beads-bound proteins were subjected to immunoblot analysis with anti-agnoprotein Ab. The aliquots from mixtures before a pull-down experiment were also subjected to immunoblot analysis as 10% input. (*E*) GST pull-down assay with the GST-AP3D-Y27C and in vitro synthesized the C-terminal hexahistidine (6 × His) tagged agnoproteins of JCV or BKV. After incubation with in vitro synthesized agnoproteins, the GST fusion proteins were precipitated with glutathione-Sepharose, and beads-bound proteins were subjected to immunoblot analysis with anti-His tag Ab. The aliquots from mixtures before pull-down experiment were also subjected to immunoblot analysis as 10% input.

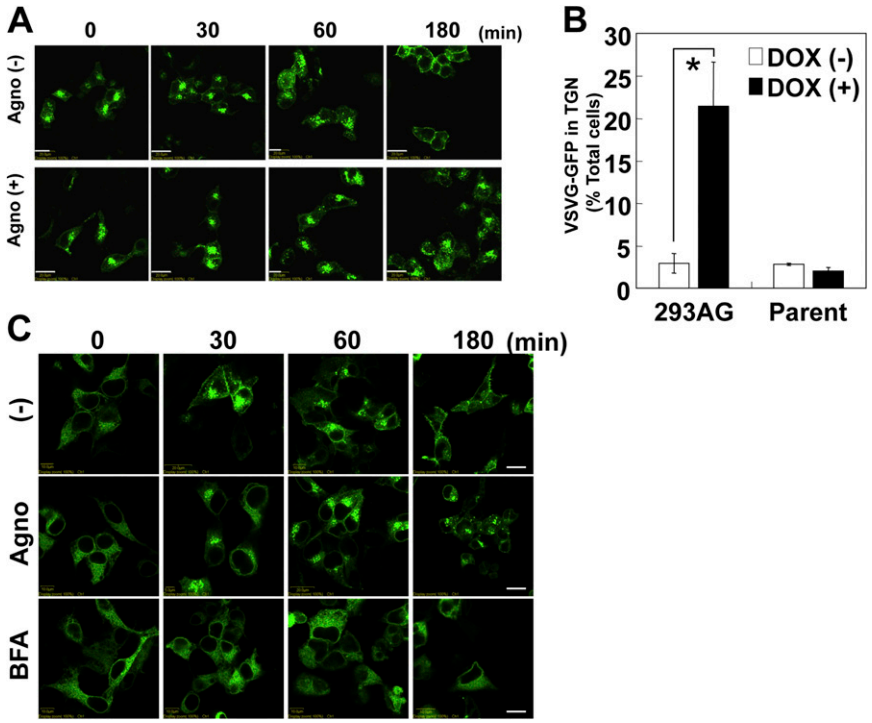

Fig. S2. Agnoprotein blocks intracellular trafficking of vesicular stomatitis virus G protein (VSVG). (*A* and *B*) The 293AG cells in the absence or presence of DOX were transfected with pVSVG-GFP. The cells were incubated for 20 h at 39.5 °C. The temperature was then shifted to 20 °C for 3 h to accumulate VSVG-GFP in the perinuclear region and then to 32 °C in the presence of cycloheximide to permit VSVG-GFP transport from the perinuclear region. The cells were fixed at the indicated time points. The localization of VSVG-GFP protein was analyzed by confocal microscopy. (Scale bars, 20 μm.) (*B*) The graph indicates the average proportion of cells with the trans-Golgi network (TGN) of VSVG-GFP in 200 cells. Results are the average of three independent experiments, and the significance of changes was analyzed by Student *t* test (*$P < 0.05$). (*C*) The 293AG cells in the absence (–) or presence (Agno) of DOX were transfected with pVSVG-GFP. The cells were incubated for 20 h at 39.5 °C to accumulate VSVG-GFP in the ER. The temperature was then shifted to 32 °C in the presence of cycloheximide to permit VSVG-GFP transport from the ER. Brefeldin A (BFA) (5 μg/mL) was added to the cells in the absence of DOX as a control VSVG-GFP transport inhibition from the ER to the Golgi. The cells were fixed at the indicated time points. The localization of VSVG-GFP protein was analyzed by confocal microscopy. In the presence of BFA the VSVG-GFP signal was still clearly present in the ER at 180 min. In contrast, almost all of the VSVG-GFP was transported to the perinuclear region in the presence of agnoprotein, suggesting that the transport of VSVG-GFP between the ER and Golgi apparatus, which is independent of AP3D, was disrupted by BFA but not by agnoprotein. Results are representative of three independent experiments. (Scale bars, 20 μm.)

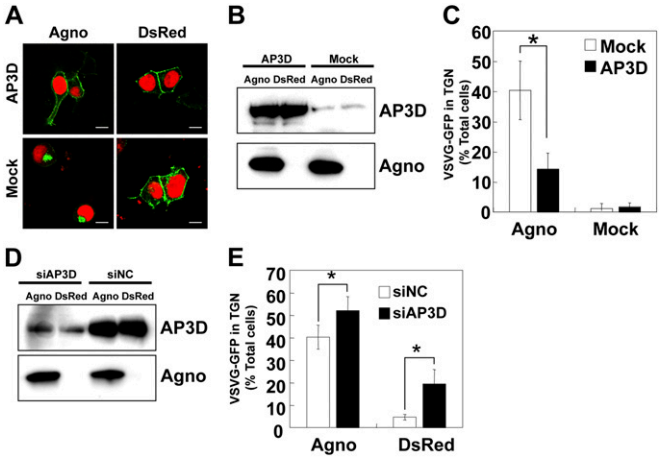

Fig. S3. Agnoprotein impairs AP-3–mediated vesicular trafficking. (*A–C*) The 293T cells were transfected with pVSVG-GFP, pERedNLS-Agno (Agno), or pERedNLS (DsRed) and pCMV-Myc-AP3D (AP3D) or pCMV-Myc (Mock) for measurement of VSVG-GFP transport to the plasma membrane. The cells were cultured first at nonpermissive temperature (39.5 °C) for 20 h, followed by 20 °C for 3 h to accumulate VSVG-GFP in the TGN, and were subsequently shifted to 32 °C to permit VSVG-GFP transport from the TGN. Thereafter, the cells were fixed and analyzed by confocal microscopy. The cell lysates corresponding to the imaging analysis were subjected to immunoblot analysis with anti-AP3D (AP3D) and anti-agnoprotein (Agno) antibodies. The levels of AP3D expression in cells transfected with pCMV-Myc-AP3D (AP3D) were significantly higher than that with pCMV-Myc empty vector (Mock). The graph indicates the average proportion of cells with the TGN of VSVG-GFP in 200 cells. Results are the average of three independent experiments, and the significance of changes was analyzed by Student *t* test (**P* < 0.05). Green represents VSVG-GFP signal, and red represents DSRed expression in nuclei as a marker of transfection. (*D*) The 293T cells were transfected with pERedNLS-Agno (Agno) or pERedNLS (DsRed) and siAP3D or siNC. The cells were lysed and subjected to immunoblotting. The levels of AP3D expression in cells transfected with siAP3D were significantly lower than that with siNC. (*E*) The 293T cells were transfected with siAP3D or siNC and incubated for 48 h. Then, the cells were transfected with pVSVG-GFP and pERedNLS-Agno (Agno) or pERedNLS (DsRed) for measurement of VSVG-GFP transport to the plasma membrane. The cells were cultured at 39.5 °C for 20 h, followed by incubation at 32 °C. Thereafter, the cells were fixed at indicated time points and analyzed by confocal microscopy. The bar graph indicates the average proportion of cells with TGN localization of VSVG-GFP in 200 cells. Results are representative of three independent experiments, and the significance of changes was analyzed by Student *t* test (**P* < 0.05).

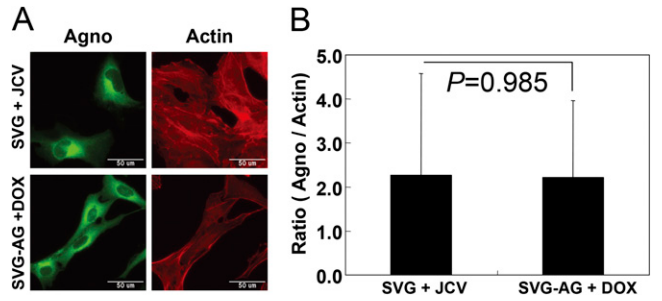

Fig. S4. Establishment of DOX-inducible agnoprotein-expressing cells, SVG-AG cells. (*A* and *B*) The levels of agnoprotein expression in SVG-AG cells were similar to those in JCV-infected parent SVG-A cells. SVG-AG cells were incubated with 1 μg/mL of DOX for 72 h, and SVG-A cells were infected with JCV and incubated for 96 h. These cells were fixed and subjected to immunofluorescence analysis using anti-agnoprotein and anti-actin Abs followed by Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 647-conjugated anti-mouse IgG Abs, respectively. The cell images were acquired by fluorescence microscopy and analyzed using MetaMorph software. (Scale bars, 50 μm.) (*B*) The bar graph indicates the ratio of total fluorescence intensity of agnoprotein in a single cell which is normalized by total fluorescence intensity of actin. No significant differences between SVG-AG cells and JCV-infected SVG-A cells were observed (*P* = 0.985).

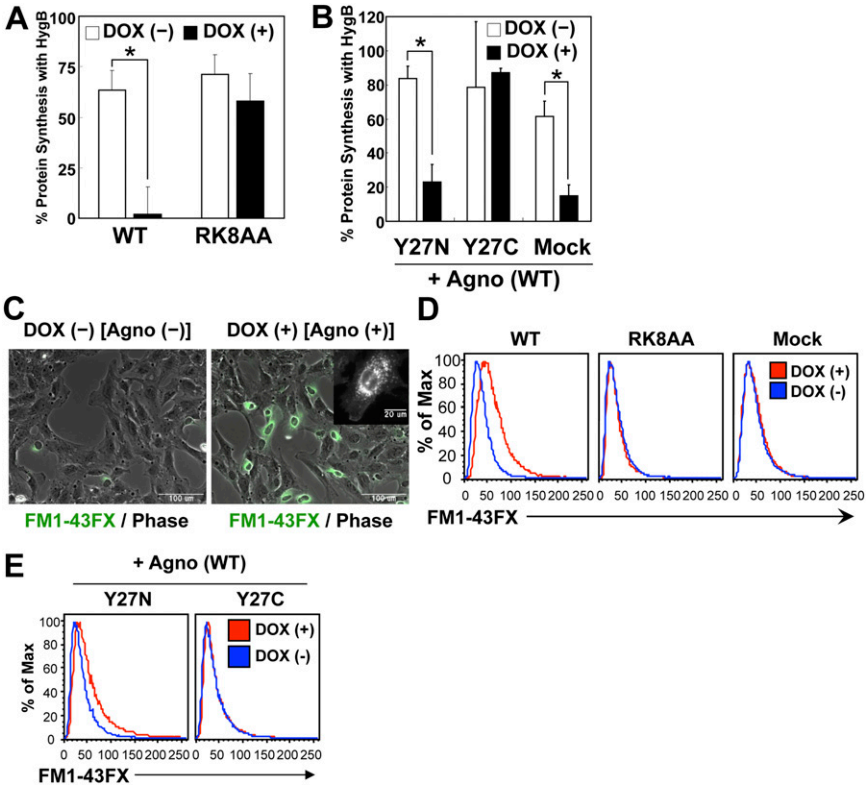

Fig. S5. Association of agnoprotein with AP3D is fundamental to membrane permeabilization. (*A*) DOX-inducible agnoprotein-expressing cells, SVG-AG cells (WT) or SVG-RK8AA cells (RK8AA), were incubated with or without 1 μg/mL DOX for 72 h. Nascent protein syntheses in these cells with or without 500 μg/mL HygB were labeled using the Click-iT AHA Alexa Fluor 488 Protein Synthesis Kit and observed by fluorescence microscopy. The cell images were analyzed using MetaMorph software. The bar graph indicates the proportion of mean signal intensity of Alexa Fluor 488 of the cells in the presence of HygB compared with those in the absence of HygB. Results are average of three independent experiments, and the significance of changes was analyzed by Student *t* test (**P* < 0.05). (*B*) SVG-AG cells stably expressing partial fragments of AP3D (Y27N or Y27C) or empty vector (Mock) were incubated with or without 1 μg/mL DOX for 72 h. Nascent protein syntheses in these cells were similarly observed. The cell images were analyzed using MetaMorph software. The bar graph indicates the proportion of mean signal intensity of Alexa Fluor 488 of the cells in the presence of HygB against without those in the absence of HygB. Results are average of three independent experiments, and the significance of changes was analyzed by Student *t* test (**P* < 0.05). (*C*) SVG-AG cells were incubated with or without 1 μg/mL DOX for 72 h. Then the cells were stained with FM1-43FX dye in HBSS without magnesium or calcium on ice for 1 min and fixed with 4% PFA. The cells were analyzed by fluorescence microscopy. Phase, phase contrast images. (Scale bars, 100 μm.) (*Inset*) High magnification of cells stained by FM1-43FX. (Scale bar, 20 μm.) (*D*) SVG-AG cells (WT), SVG-RK8AA (RK8AA) cells, or SVG-Mock cells, in which DsRed as induction marker proteins are expressed with DOX treatment and were incubated with or without 1 μg/mL DOX for 72 h. Thereafter, the cells were stained with FM1-43FX dye in HBSS without magnesium or calcium on ice for 1 min and fixed with 4% PFA. The intensities of FM1-43FX were analyzed by flow cytometry. (*E*) SVG-AG cells stably expressing partial fragments of AP3D (Y27N or Y27C) were incubated with or without 1 μg/mL DOX for 72 h, stained with FM1-43FX, fixed with 4% PFA, and analyzed by flow cytometry.

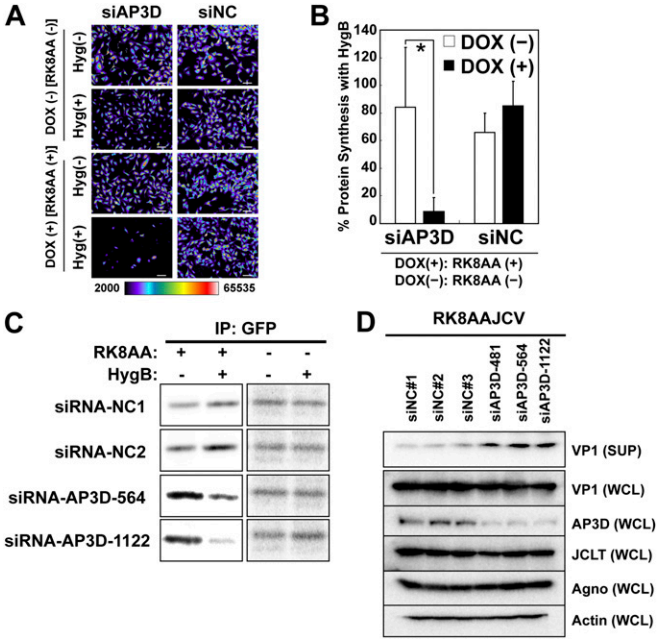

Fig. S6. Disruption of AP-3–mediated vesicular trafficking is necessary for the viroporin activity of agnoprotein. (*A* and *B*) SVG-RK8AA (RK8AA) cells were transfected with siAP3D or siNC and incubated with or without 1 μg/mL DOX for 72 h. Nascent protein syntheses in these cells in the presence or absence of 500 μg/mL of HygB was labeled using Click-iT AHA Alexa Fluor 488 and analyzed by fluorescence microscopy. The cell images were analyzed using MetaMorph software. (Scale bars, 100 μm.) (*B*) The bar graph indicates the average proportion of total fluorescence intensity of the cells in the presence of HygB against those in the absence of HygB. Results are average of three independent experiments, and the significance of changes was analyzed by Student *t* test (*$P < 0.05$). (*C*) HeLa cells, which were transfected with siAP3Ds or siNCs and incubated for 24 h, were transfected with pCFPNLS-RK8AA (RK8AA) or control vector and incubated for another 72 h. The cells were pretreated with or without HygB (400 μg/mL), radiolabeled with [^{35}S] Met-Cys, and incubated in the presence (+) or absence (–) of HygB for 2 h. The cell extracts were harvested, and the CFPNLS protein as a transfection marker protein was immunoprecipitated with an anti-GFP antibody and analyzed by SDS/PAGE. The membrane permeability for HygB by suppression of AP3D was confirmed by the ^{35}S metabolic assay. (*D*) SVG-A cells were transfected with RK8AAJCV mutant genome. After 24 h, cells were transfected with siRNAs as indicated. Cells were incubated another 96 h, and then whole-cell lysates (WCL) and supernatants (SUP) were harvested simultaneously. The collected samples were applied to SDS/PAGE and immunoblotting with the indicated antibodies. Culture supernatants were concentrated 25 times by centrifugation.

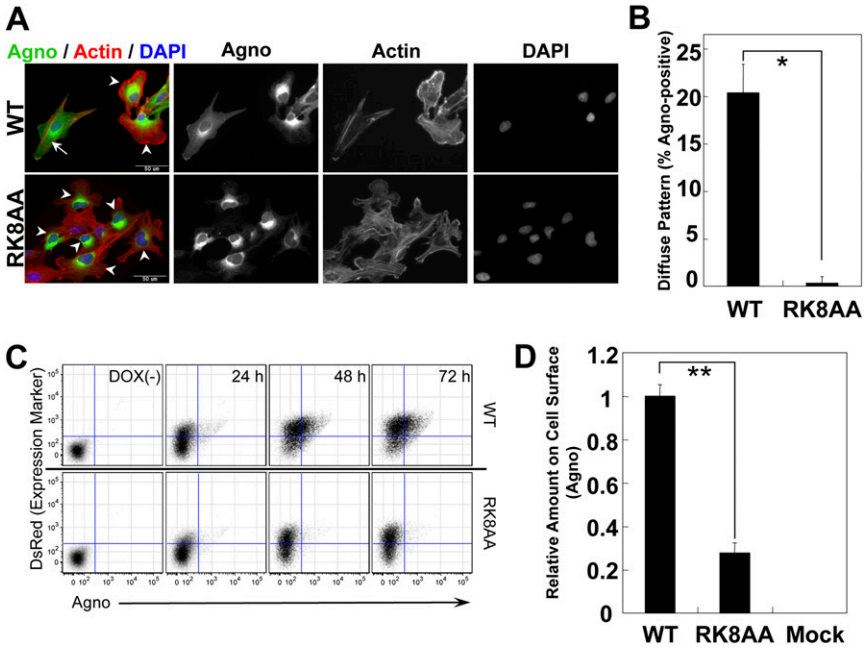

Fig. S7. RK8AA mutant is poorly expressed on the cellular surface. (*A* and *B*) SVG-AG cells (WT) or SVG-RK8AA cells (RK8AA) were incubated with 1 μg/mL DOX for 72 h and subjected to immunofluorescence analysis using anti-agnoprotein antibody followed by Alexa Fluor 488-conjugated anti-rabbit IgG antibody (green). Actin filaments and nuclei were labeled with Alexa Fluor 647 phalloidin (red) and DAPI (blue) simultaneously. The cells in which agnoprotein was predominantly localized in the perinuclear region (perinuclear pattern) are indicated by arrowheads. An arrow indicates the cells in which agnoprotein localized diffusely (diffuse pattern) in the cytoplasm. (Scale bars, 50 μm.) (*B*) The bar graph indicates the average proportion of cells with the diffuse pattern of localization compared with total agnoprotein-expressing cells (*$P < 0.05$). (*C*) SVG-AG cells (WT) or SVG-RK8AA cells (RK8AA) were incubated with or without 1 μg/mL DOX for indicated time points. The cells were subjected to cell surface staining using anti-agnoprotein antibody followed by Alexa Fluor 488-conjugated anti-rabbit IgG antibody and then analyzed by flow cytometry. The fluorescence of DsRed was used as a marker for induction of proteins by DOX treatment. (*D*) SVG-AG cells (WT), SVG-RK8AA cells (RK8AA), or SVG-Mock cells (Mock) were incubated with 1 μg/mL DOX for 72 h. The levels of agnoprotein expression on cell surface were measured by flow cytometry using Alexa Fluor 647 labeled anti-agnoprotein antibody. The bar graph indicates the relative mean fluorescence intensity of agnoprotein (**$P < 0.01$).

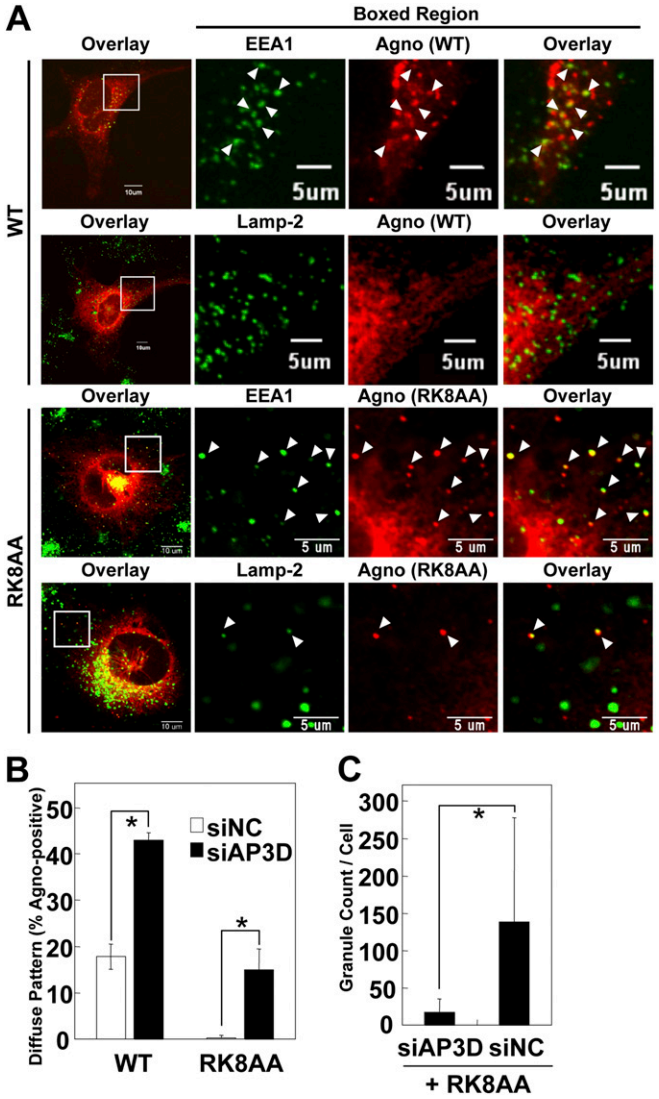

Fig. S8. RK8AA mutant of agnoprotein localized in the lysosome. (*A*) Confocal microscopy analysis of SVG-A cells transfected with WTJCV genome showed the colocalization of WT agnoprotein (WT) with EEA1, an early endosome marker, but not with Lamp-2, the lysosome marker. In contrast, SVG-A cells transfected with RK8AAJCV genome showed the colocalization of RK8AA mutant of agnoprotein with both EEA1 and Lamp-2. The boxed areas in the first column are shown at higher magnification in the second through fourth columns. Arrowheads indicate the colocalization of agnoprotein with marker proteins. [Scales bars, 10 μm (first column) and 5 μm (second through fourth columns).] (*B*) SVG-AG cells (WT) or SVG-RK8AA cells (RK8AA) transfected with siAP3D or siNC were incubated with 1 μg/mL DOX for 72 h and thereafter subjected to immunofluorescence analysis. The bar graph indicates the average proportion of cells with the diffuse pattern of agnoprotein expression. Results are average of three independent experiments, and the significance of changes was analyzed by Student *t* test (**P* < 0.05). (*C*) SVG-RK8AA cells (RK8AA) were transfected with siAP3D or siNC and incubated with 1 μg/mL DOX for 72 h. The cells were subjected to immunofluorescence analysis using anti-agnoprotein antibody followed by Alexa Fluor 488-conjugated anti-rabbit IgG antibody and observed by fluorescence microscopy. The granule localizations of agnoprotein were quantified by MetaMorph software. The bar graph indicates the average number of granular localization of agnoprotein in a single cell. Results are average of three independent experiments, and the significance of changes was analyzed by Student *t* test (**P* < 0.05).

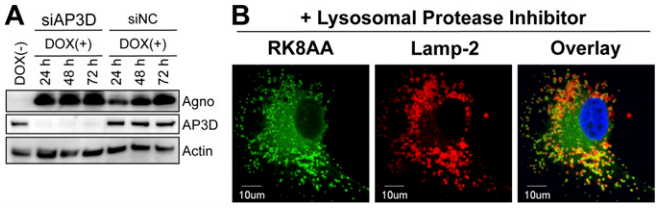

Fig. S9. Agnoprotein was degraded by AP-3–dependent pathway. (*A*) SVG-AG cells were transfected with siAP3D or siNC and incubated with or without 1 μg/mL DOX for the indicated time and subjected to immunoblotting using anti-AP3D, anti-actin, or anti-agnoprotein antibodies. (*B*) DOX-treated SVG-RK8AA cells were cultured in the presence of lysosomal protease inhibitor for 48 h and processed for immunofluorescence staining. Confocal microscopy analysis of the cells showed prominent colocalization of RK8AA mutant with Lamp-2. (Scale bars, 10 μm.)