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Supplemental Information

Entry of Bunyaviruses into Mammalian Cells

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Inventory of Supplemental Information

Supplemental Data Figures

Sup 1 shows the FACS-based infection assay developed for UUKV which has been used to determine the length of the virus cycle and also to assess the effect of all the perturbants tested in this study. Sup 1 is related to Fig 3, 4, 5, 6, 7 and Sup 4, 5, 6 and Sup Table 1.

Sup 2 is related to Fig 1, 2, 3, 4, 5 and 7.

Sup 3 is related to Fig 3.

Sup 4 shows pH dependence of UUKV infectious entry. Sup 4 is related to Fig 3, 5, 6 and Sup 6.

Sup 5 is related to Fig 4, 7 and Sup 4.

Sup 6 is related to Fig 5.

Supplemental Data Table

Sup Table 1 gives a list of cells tested for UUKV infection and shows why authors decided to utilize A549, BHK-21 and BSC40 cells for this study.

Supplemental Data Movies

Sup Movie 1 is related to Fig 4.

Sup Movie 2 is related to Fig 7.

Sup Movie 3 is related to Fig 7.

Supplemental Data Legends

Supplemental Experimental Procedures

Supplemental References

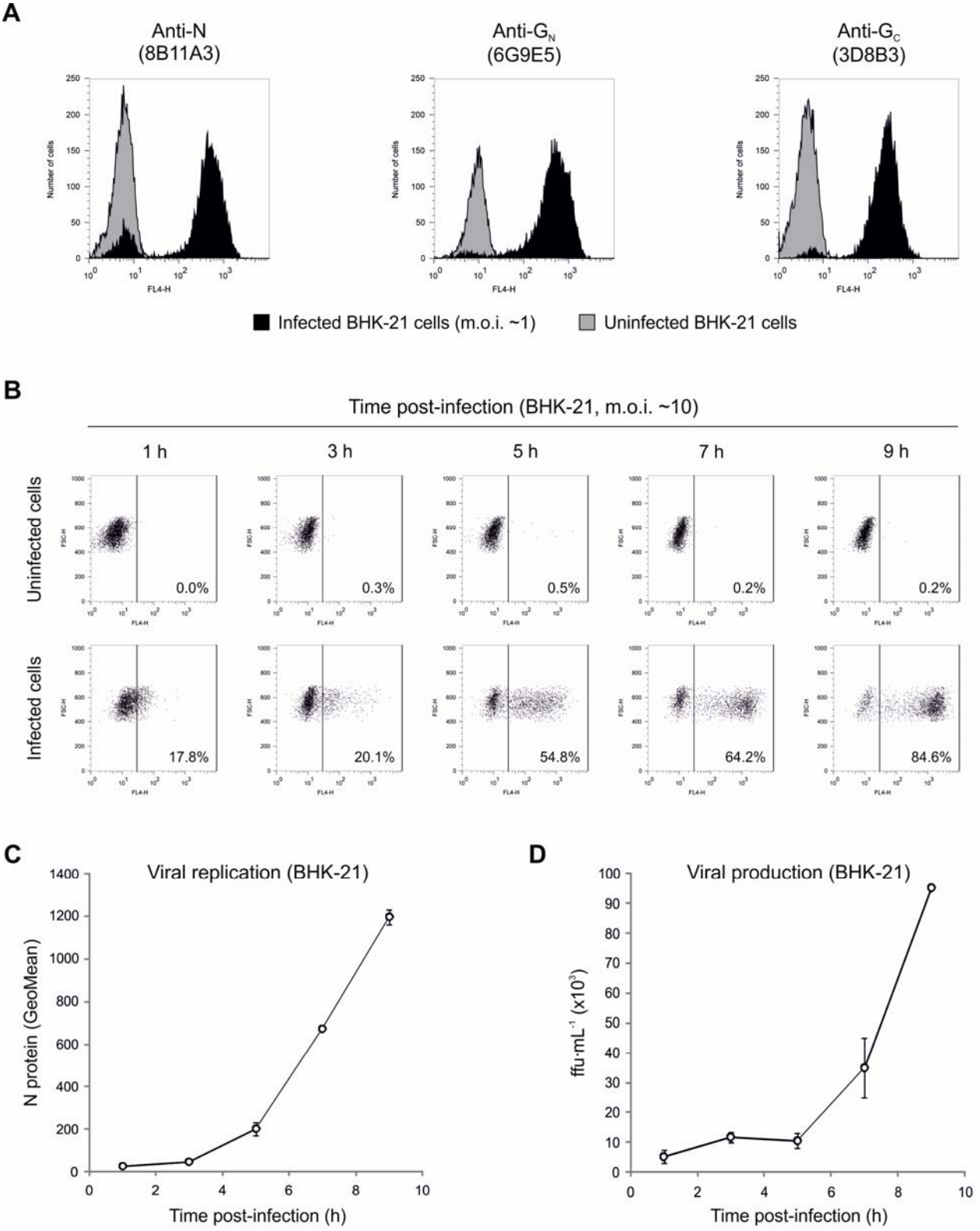
A
Anti-N
(8B11A3)
Anti-G$_N$
(6G9E5)
Anti-G$_C$
(3D8B3)
Number of cells
FL4-H
Infected BHK-21 cells (m.o.i. ~1)
Uninfected BHK-21 cells
B
Time post-infection (BHK-21, m.o.i. ~10)
1 h
3 h
5 h
7 h
9 h
Uninfected cells
Infected cells
0.0%
0.3%
0.5%
0.2%
0.2%
17.8%
20.1%
54.8%
64.2%
84.6%
C
Viral replication (BHK-21)
N protein (GeoMean)
Time post-infection (h)
D
Viral production (BHK-21)
ffu·mL^{-1} (x10^3)
Time post-infection (h)

Figure S1. Quantification of UUKV infection by flow cytometry

(A) Cells were exposed to UUKV and harvested 18 h later. The infected cells were fixed, permeabilized, and stained with monoclonal antibodies against the nucleoprotein N or the glycoproteins G_N and G_C before FACS analysis.

(B) UUKV infection was monitored over 9 h with the FACS-based infection assay described in A. The infections are given as a percentage of N protein-positive cells according to the threshold determined for the uninfected cells. The fraction of N protein-positive cells increased over time with the accumulation of newly synthesized N protein. This indicates that the signal detected corresponded to viral replication and not to input particles.

(C) Shows the same data as in B but the infection is given as the total fluorescence associated with the cells. The total fluorescence associated with cells, due to N protein expression, clearly indicated that infected cells expressed a low level of N within the three first hours. Error bars indicate SD.

(D) Supernatants collected during the time course in B were assessed for the production of new infectious viral particles. As UUKV is not lytic, the titers were determined using an infectious focus assay after immunostaining with a polyclonal antibody against all UUKV proteins. Error bars indicate SD.

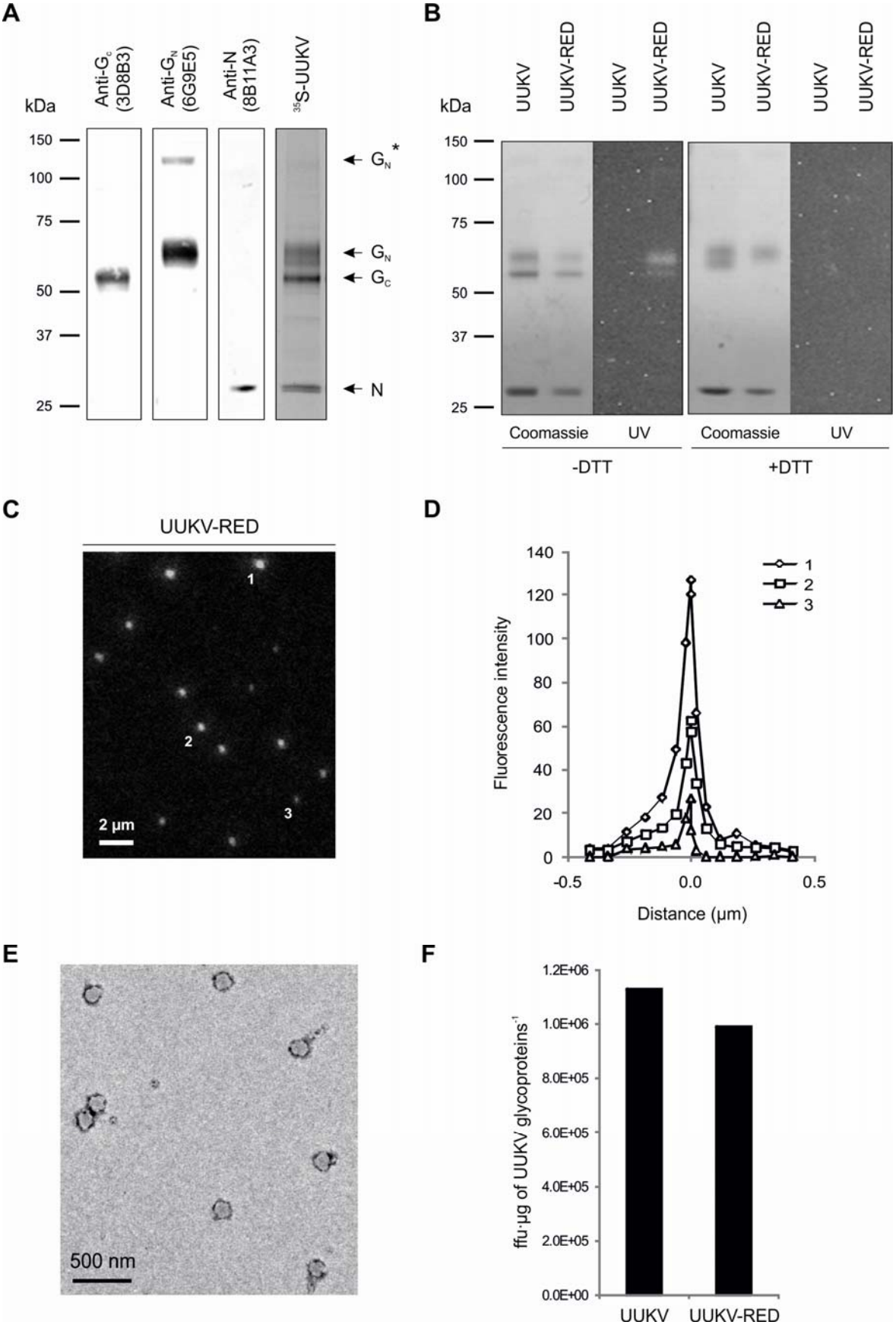
A
kDa
Anti-Gc (3D8B3)
Anti-GN (6G9E5)
Anti-N (8B11A3)
35S-UUKV
150
100
75
50
37
25
GN*
GN
Gc
N
B
kDa
UUKV
UUKV-RED
UUKV
UUKV-RED
UUKV
UUKV-RED
UUKV
UUKV-RED
150
100
75
50
37
25
Coomassie
UV
Coomassie
UV
-DTT
+DTT
C
UUKV-RED
1
2
3
2 µm
D
Fluorescence intensity
140
120
100
80
60
40
20
0
-0.5
0.0
0.5
Distance (µm)
1
2
3
E
500 nm
F
ffu·µg of UUKV glycoproteins-1
1.2E+06
1.0E+06
8.0E+05
6.0E+05
4.0E+05
2.0E+05
0.0E+00
UUKV
UUKV-RED

Figure S2. Labeling of UUKV with [^{35}S] or fluorescent dye

(A) ^{35}S-labeled particles (^{35}S-UUKV) were analyzed by non-reducing SDS-PAGE and the purity assessed by autoradiography (fourth panel). The specificity was controlled by western blotting using monoclonal antibodies against the nucleoprotein N and the glycoproteins G_N and G_C. G_N^* indicates oligomers of G_N.

(B) UUKV was labeled with a thiosulfate-activated fluorescent dye at a 1:5 molar ratio between UUKV glycoproteins and dye. The unlabeled (UUKV) and fluorescent particles (UUKV-RED) were analyzed by non-reducing (-dithiothreitol [DTT]) and reducing (+DTT) SDS-PAGE with blue staining (Coomassie) or fluorography (UV). The glycoproteins of UUKV-RED were the only fluorescently labeled proteins. In reducing conditions (DTT or TCEP), the fluorescent dye was completely removed. Note that contrary to non-reduced samples, G_N and G_C co-migrated in SDS-PAGE after reduction.

(C) Confocal microscopy pictures of UUKV-RED show individual fluorescent spots of different intensities (1 to 3) with a diameter of 0.3 to 0.5 µm.

(D) The distribution of the relative fluorescence intensity of one spot fits a Gaussian function (fit is calculated using the central limit theorem equation). The relative fluorescence intensity distributions for three different spots are shown (1 to 3). The distributions of the signal suggest that the light was emitted from single particles.

(E) Unfixed UUKV-RED particles negatively stained with phosphotungstic acid under neutral pH conditions viewed by EM. Pictures show isolated particles. As already described for some other bunyaviruses (Schmaljohn and Nichol, 2007), the viruses appeared pleiomorphic in shape and homogenous in terms of size (132 +/- 8 nm, n=8).

(F) The fluorescent particles were as infectious as unlabeled particles. The titers of UUKV and UUKV-RED were normalized according to the amount of glycoproteins G_N and G_C (foci forming units [ffu] per µg of glycoproteins). Furthermore, we noticed no impact of ^{35}S-labeling on UUKV

infectivity; the titers were similar to those of non-radioactive particles (about 10^9 to 10^{10} ffu·mL^{-1} after concentration and purification) (data not shown).

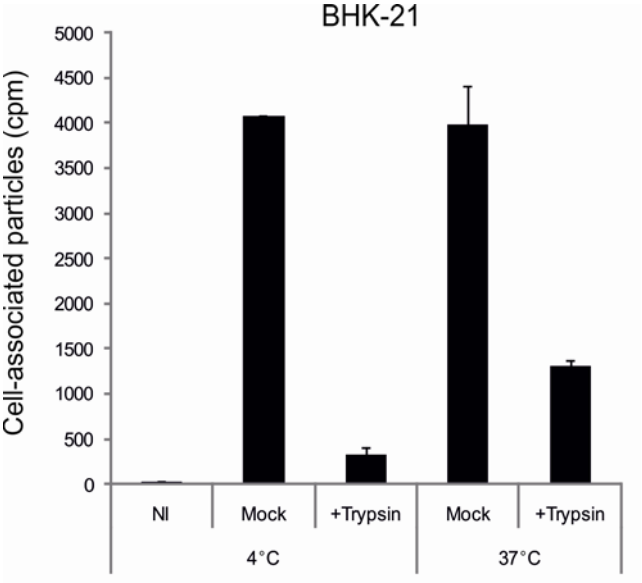

Figure S3. ^{35}S-virus-based internalization assay

^{35}S-labeled UUKV (m.o.i. ~3) was bound to cells on ice. Cells were then washed to remove unbound material and maintained on ice or shifted to 37°C before treatment with trypsin for 1 h at 4°C to remove cell-surface bound viruses. NI for non-infected cells. Error bars indicate SD.

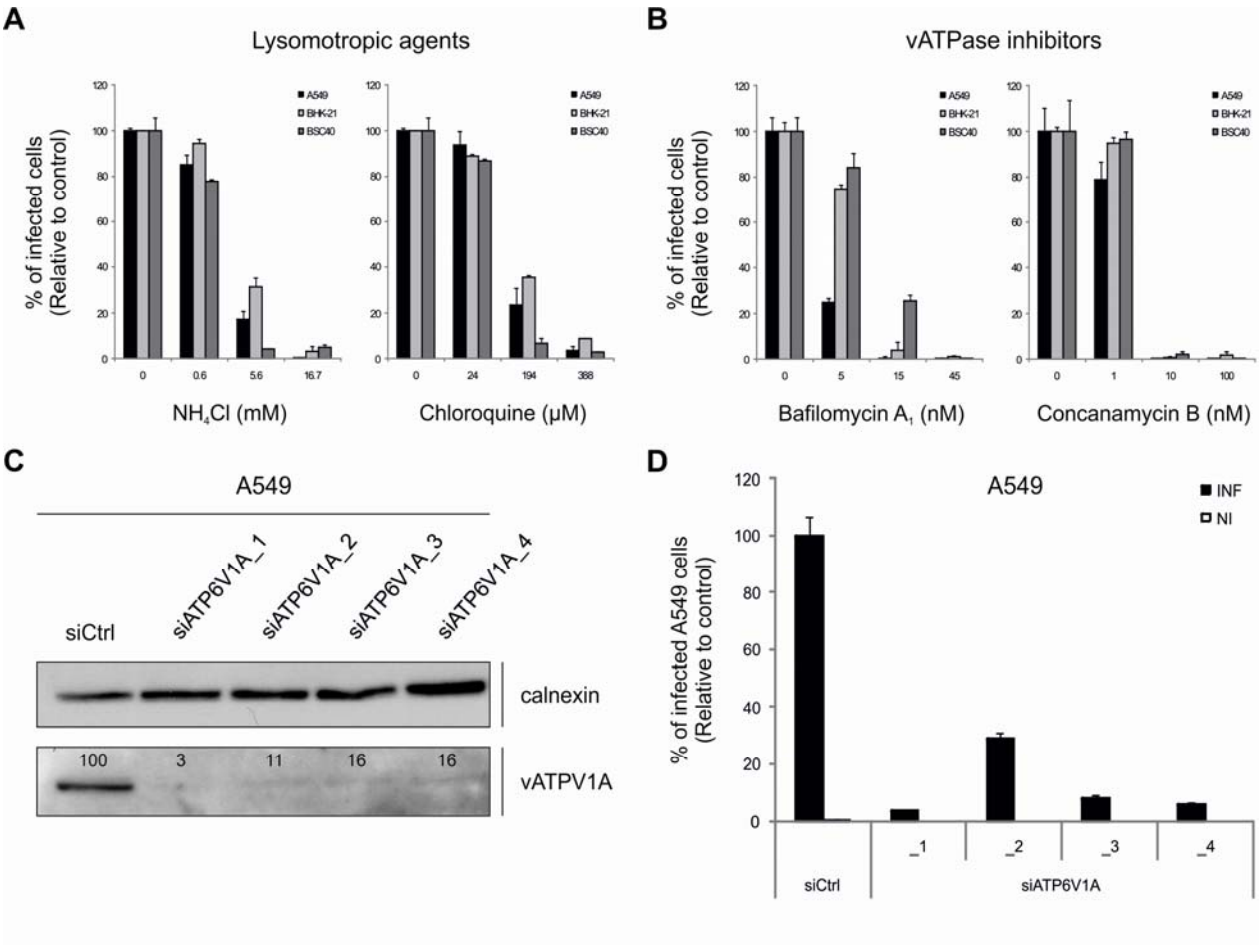

Figure S4. UUKV infects cells in a pH-dependent manner

(A and B) Cells were pre-treated with pH interfering drugs at varying concentrations and then infected with UUKV in the presence of the drugs (NH_4Cl and chloroquine in panel A, and bafilomycin A1 and concanamycin B in panel B). Data are normalized to samples where the inhibitors had been omitted. Error bars indicate SD.

(C) Efficiency of vATPV1A knockdown assayed by western blotting. vATPV1A protein levels are expressed as percentages of vATPV1A levels in cells treated with vATPV1A siRNAs (siATP6V1A_1 to _4) normalized to level of calnexin and vATPV1A level in control cells treated with negative-control siRNA (siCtrl).

(D) Cells treated with vATPV1A siRNAs were infected with UUKV (m.o.i. ~2). The quantification of infection was performed as described in Figure 3E. NI and INF for non- and infected cells respectively. Error bars indicate SD.

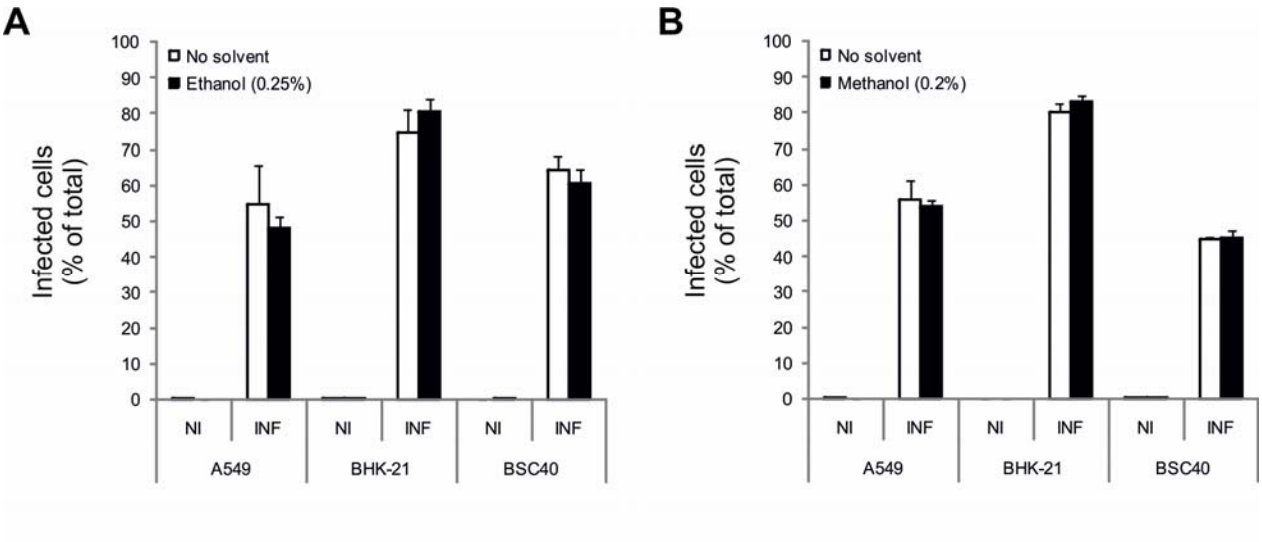

Figure S5. Low concentration of solvents has no effect on UUKV infection

(A and B) Cells were pre-treated with ethanol (A) or methanol (B), at the highest final concentrations used for drug assays in this study, and then infected with UUKV in the presence of these solvents. NI and INF for non- and infected cells respectively. Error bars indicate SD.

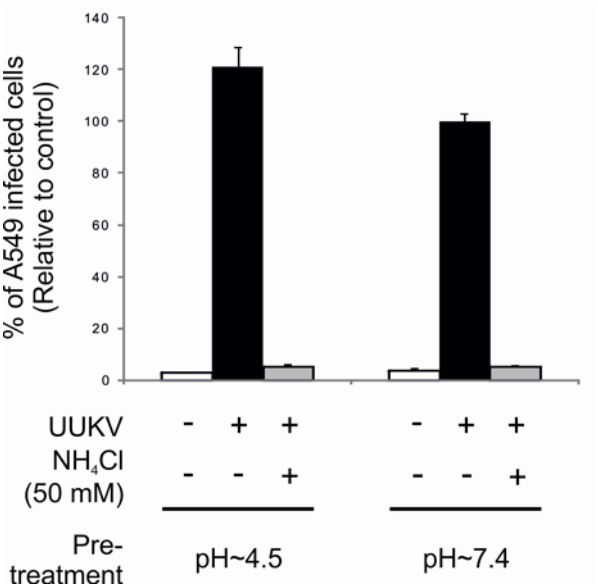

Figure S6. NH_4Cl efficiently blocks UUKV infection in low-pH treated cells

To assess the capacity of cells to replicate virus and of NH_4Cl to block penetration after very low pH treatment, cells were incubated in buffer at low pH for 1.5 min and then exposed to UUKV (m.o.i. ~1) in the presence or absence of NH_4Cl (50 mM) for 1 h at 37°C. The values were normalized to samples treated at pH ~7.4 where NH_4Cl had been omitted. Error bars indicate SD.

Table S1. Cells infected by UUKV

Cell line	Specie	Tissue	Sensitivity to UUKV infection[a]	Production of new viral particles[b]
3T3	Mouse	Fibroblast	-	-
A431	Human	Skin	+	ND
A549	Human	Lung	++	++
AP61	Mosquito	Larva (whole)	-	-
BHK-21	Hamster	Kidney	+++	+++
BSC40	Monkey	Kidney	++	+
C6/36	Mosquito	Larva (whole)	-	-
CHO	Hamster	Ovary	++	++
CV1	Monkey	Kidney	+	ND
HEK-293T	Human	Kidney	++	ND
HeLa	Human	Cervical carcinoma	+	-
Huh-7	Human	Hepatocarcinoma	++	ND
raji	Human	B lymphocyte	-	-*
THP1	Human	Monocyte	-	-*
Vero	Monkey	Kidney	++	+

[a] The sensitivity of cells to UUKV infection is based on the infection index at 18 hours post-infection (m.o.i. ~1) as follow: +++ superior to 30%, ++ from 10% to 30%, + from 1% to 10%, - inferior to 1%. The infection index was determined by quantification of N-positive cells using the FACS-based analysis described in Supplement 1B.

[b] The production of new viral particles was determined by foci assay and is given according to the size of foci 72 h post-infection as follow: +++ superior to 1 mm, ++ from 0.5 to 1 mm, + inferior to 0.5 mm, - no foci. ND for not determined.

* raji and THP1 are non adherent cells. Consequently, production of new infectious particles was assessed by titration of infected-cell supernatant.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Viruses, siRNA, antibodies, plasmids and inhibitors

GFP-recombinant VSV and the prototype strains of influenza A X-31 and Semliki forest viruses have been described previously (Helenius et al., 1980; Kilbourne, 1969; Schnell et al., 1996). SFV and VSV were kindly provided by T. Heger and H.K. Johannsdottir. CHC siRNAs (siCHC_1, SI02651747; siCHC_2, SI00299873) and vATPV1A siRNAs (siATP6V1A_1, SI00307489; siATP6V1A_2, SI00307496; siATP6V1A_3, SI00307503; siATP6V1A_4, SI00307510) were all from Qiagen. AllStars negative-control siRNA (Qiagen) was used as control siRNA (siCtrl). Mouse antibodies 6G9E5 and 3D8B3 are directed against conformation-dependent epitopes in the glycoproteins G_N and G_C, respectively (Persson and Pettersson, 1991). Mouse antibody 8B11A3 is directed against a linear epitope in the N protein (Persson and Pettersson, 1991). The polyclonal rabbit antibody U5 is directed against all viral proteins (Kuismanen et al., 1982). A polyclonal rabbit antibody against all viral proteins of SFV and a monoclonal mouse antibody against the influenza A virus nucleoprotein were used to detect viral replication. Mouse antibody against CHC (clone 23) and chicken antibody against vATPV1A (ATP6V1A) were purchased from Thermo Scientific and Becton Dickinson, respectively. Mouse antibodies against β-actin and α-tubulin were purchased from Sigma. The polyclonal rabbit anti-calnexin antibody was described previously (Hammond et al., 1994). Plasmids encoding EGFP-LAMP-1, EGFP-Rab5a, EGFP-Rab5a S34N, EGFP-Rab5a Q79L, EGFP-Rab7a, EGFP-Rab7a T22N and EGFP-Rab7a Q67L have all been described previously (Pelkmans et al., 2004; Falcon-Perez et al., 2005). Plasmid coding for PH-PLCΔ1EGFP was kindly providing by J. Mercer. NH_4Cl and chloroquine (Sigma) stocks were dissolved in water, colcemid and MG132 (Sigma) in methanol, and bafilomycin A1 (Sigma), wortmannin (Sigma) and concanamycin B (Alexis) in absolute ethanol.

Note that dimethyl sulfoxide (DMSO) was not used as solvent for drugs in this study because by itself, it reduced UUKV infection (data not shown).

Production, purification and labeling of UUKV

Sub-confluent monolayers of BHK-21 cells were exposed to UUKV for 1 h in FCS-free medium at a multiplicity of 0.1 ffu·cell^{-1}, washed and then incubated at 37°C in FCS-free medium for 48 h. For the production of ^{35}S-labeled UUKV, DMEM (without methionine and cysteine) was used as culture medium and supplemented with 100 µCi·mL^{-1} of [^{35}S] cysteine and methionine (Pro-Mix ^{35}S, Amersham Biosciences). Supernatants containing virus were clarified and subsequently centrifuged for 2 h at 100,000 g through a 30% sucrose cushion made in TNE buffer (10 mM Tris-HCl, pH~7.4 containing 100 mM NaCl and 2 mM EDTA). The virus pellet was resuspended in TNE buffer. For the production of fluorescent particles, the glycoproteins concentration was measured and C5-thiosulfate-activated fluorescent dye (TS-link Bodipy TR, Invitrogen) was added in a 1:5 molar ratio while vortexing. Both ^{35}S- and fluorescent-labeled particles were banded in a 5% to 45% Optiprep iodixanol density gradient (Sigma) made in TNE buffer and complemented with 8.6% sucrose.

Virus titration

Virus was titered on cells by focus immuno-detection assay as described elsewhere for other viruses (Lozach et al., 2005). Briefly, following infection of confluent monolayers with 10-fold dilutions of UUKV in FCS-free medium, cells were grown in presence of medium containing 2% FCS and supplemented with 0.6% carboxymethyl-cellulose to abolish virus spread. Foci were detected by immunostaining with the antibody U5.

Protein analysis

For western blotting, viral protein extracts from purified-virus stock, and protein extracts from siRNA-treated cells, were analyzed by SDS-PAGE (NuPAGE Novex Bis-Tris gels, Invitrogen) and transferred to Immobilon P membranes (Millipore). Incubation with primary antibodies 8B11A3, 6G9E5, 3D8B3 (1 $\mu g \cdot mL^{-1}$) or against CHC (1:100) and β-actin (1:1,000) was followed by incubation with an anti-mouse horseradish peroxidase-conjugated secondary antibody (1:2,000). Incubation with primary antibodies against vATPV1A (1:2,500) and calnexin (1:20,000) was followed by incubation with an anti-chicken and anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:2,500 and 1:10,000), respectively. Bound antibodies were detected by exposure to enhanced chemiluminescence reagents (ECL+, Amersham Biosciences). For ^{35}S-UUKV proteins analysis, samples were separated by SDS-PAGE and gels were fixed, dried, and exposed to SuperRX film (Fujifilm). For fluorescent-labeled UUKV, proteins were separated by SDS-PAGE and subsequently were either exposed to UV (fluorography) or fixed and stained with Coomassie blue solution (50% methanol 10% acetic acid 0.25% Serva Blue G [Serva]).

Cell-cell fusion assay

Virus binding (m.o.i. ~1,000) was synchronized on ice. Virus-bound cells were subsequently incubated for 5 min at 37°C in RPMI1640 0.2% BSA buffered at varying pH with either 30 mM HEPES (pH~7.0 to 7.5), 30 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH~5.5 to 6.5) or 30 mM citric acid (pH~4.5 to 5.25). Subsequently, cells were extensively washed and then incubated in complete medium (pH~7.4) at 37°C for 3 h before fixation and permeabilization with 0.1% Triton X-100 (Sigma). Nuclei and cytoplasms were stained for 30 min at room temperature with bisbenzimide (0.2 $\mu g.mL^{-1}$, Invitrogen) and CellMask Deep Red (1:5,000, Invitrogen), respectively. Syncitia were monitored by fluorescence microscopy as described above. Fusion was quantified by counting the number of cells and nuclei present in a microscope field after

fusion. A fusion index (*f*) was computed according to the equation $f = (1 - [c/n])$, where *c* is the number of cells in a field after fusion and *n* the number of nuclei. An average field contained 250 to 350 nuclei.

Plasma membrane-virus fusion assay

UUKV was fused at the plasma membrane as previously described (Helenius et al., 1980). Briefly, virus binding (m.o.i. ~5 for A549 cells, 30 for BHK-21 and 10 for BSC40) was synchronized on ice, and then, virus-bound cells were treated with buffers at different pH for 1.5 min as described for the cell-cell fusion assay. Following pH treatment, cells were extensively washed on ice and then incubated in complete medium (pH~7.4) supplemented with NH_4Cl (50 mM) at indicated temperatures for either 7 h (A549 and BHK-21 cells) or 18 h (BSC40 cells).

Immunofluorescence staining

For assessing microtubule disorganization, cells treated 3 h on ice with colcemid (10 µM) were fixed using medium containing 4% formaldehyde, permeabilized with 0.05% saponin, and then incubated with a primary mouse antibody against α-tubulin (1:1,000) for 1 h. Cells were then incubated with an anti-mouse Alexa Fluor 488-conjugated secondary antibody (1:500, Molecular Probes) for 45 min. Cover slips were mounted with ImmuMount from Thermo Shandon (Pittsburgh, Pennsylvania, United States) and analyzed by confocal microscopy as described in the experimental procedures part.

DNA transfection

Cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions either in 24w-plates or in 8w-chamber Lab-Tek II (Nalge Nunc).

siRNA-mediated knockdown

Transfections for siRNA silencing were performed using Interferin transfection reagent (Polyplus) according to the manufacturer's forward protocol. Briefly, A549 cells in 24w-plates were seeded one day prior to transfection. The siRNAs (20 nM) were diluted in FCS-free medium and then, together with Interferin (3 µL), added to the cells. For siATP6V1A_1 to _4, cells were infected 72 h post-transfection. For siCHC_1 and _2, 48 h post-transfection, cells were re-transfected, and incubated for an additional 48 h before infection. Knockdown was verified by Western blotting using β-actin and calnexin for normalization.

Flow Cytometry assays

As previously described (Quirin et al., 2008), infected cells were fixed and then permeabilized with a saponin-based buffer to stain intracellular viral antigens with antibodies. Briefly, permeabilized cells were incubated with primary antibody (1 µg.mL^{-1}) for 1 h, washed, and subsequently exposed to an anti-mouse or an anti-rabbit Alexa Fluor 647-conjugated secondary antibody (1:500, Molecular Probes) for 45 min. The infection index was determined by FACS analysis with a FACS Calibur cytometer (Becton Dickinson) and using Flowjo software (Treestar).

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