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Supplemental Information

The Intracellular Cargo Receptor ERGIC-53 Is Required for the Production of Infectious Arenavirus, Coronavirus, and Filovirus Particles

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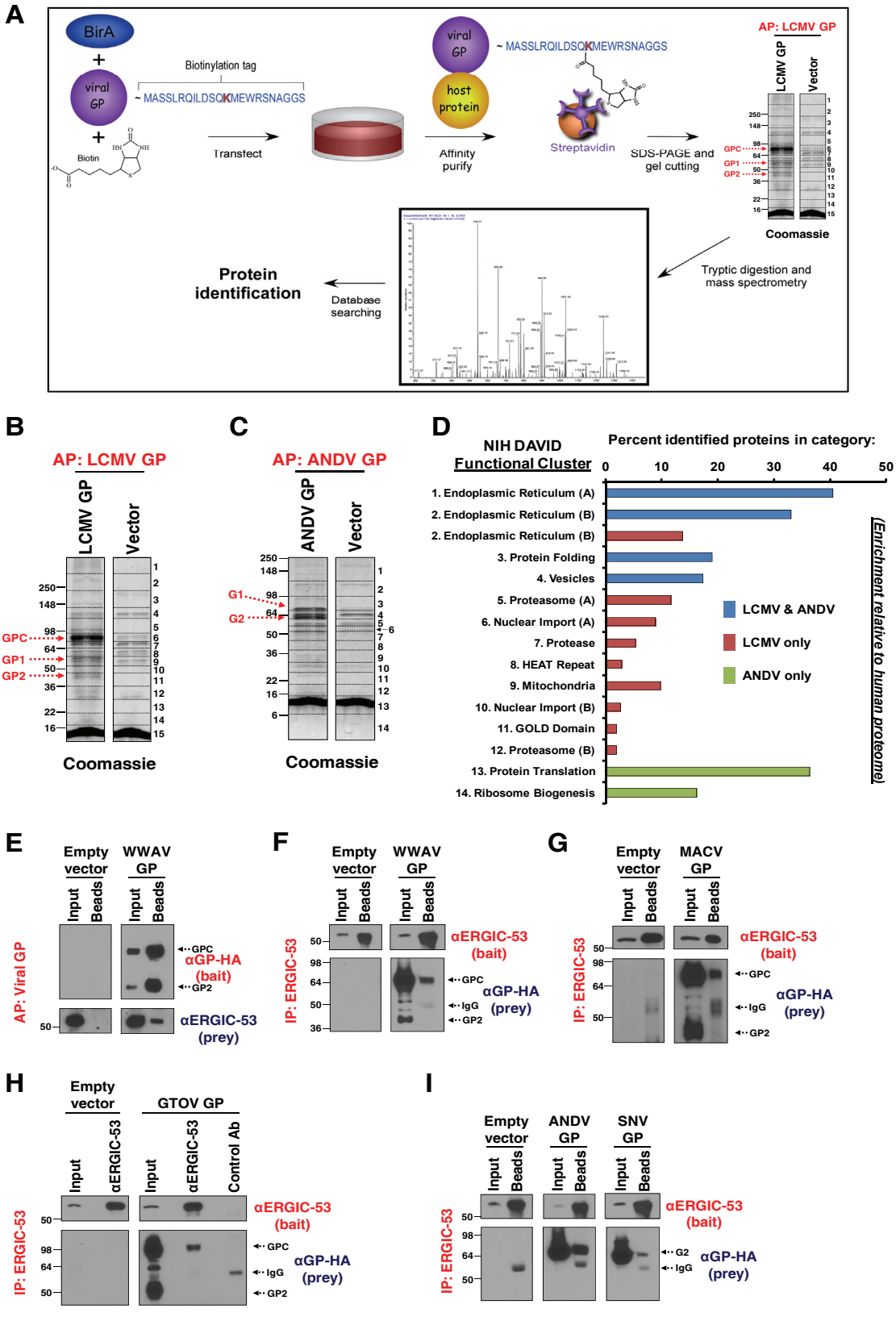
A
BirA
viral GP
Biotin
Biotinylation tag
~ MASSLRQILDSQKMEWRSNAGGS
Transfect
viral GP
host protein
Affinity purify
Streptavidin
SDS-PAGE and gel cutting
AP: LCMV GP
LCMV GP
Vector
GPC
GP1
GP2
Coomassie
Tryptic digestion and mass spectrometry
Database searching
Protein identification
B
AP: LCMV GP
LCMV GP
Vector
GPC
GP1
GP2
Coomassie
C
AP: ANDV GP
ANDV GP
Vector
G1
G2
Coomassie
D
NIH DAVID Functional Cluster
Percent identified proteins in category:
1. Endoplasmic Reticulum (A)
2. Endoplasmic Reticulum (B)
2. Endoplasmic Reticulum (B)
3. Protein Folding
4. Vesicles
5. Proteasome (A)
6. Nuclear Import (A)
7. Protease
8. HEAT Repeat
9. Mitochondria
10. Nuclear Import (B)
11. GOLD Domain
12. Proteasome (B)
13. Protein Translation
14. Ribosome Biogenesis
LCMV & ANDV
LCMV only
ANDV only
(Enrichment relative to human proteome)
E
Empty vector
WWAV GP
Input
Beads
AP: Viral GP
GPC
αGP-HA (bait)
GP2
αERGIC-53 (prey)
F
Empty vector
WWAV GP
IP: ERGIC-53
αERGIC-53 (bait)
GPC
αGP-HA (prey)
IgG
GP2
G
Empty vector
MACV GP
IP: ERGIC-53
αERGIC-53 (bait)
GPC
αGP-HA (prey)
IgG
GP2
H
Empty vector
GTOV GP
Input
αERGIC-53
Control Ab
IP: ERGIC-53
αERGIC-53 (bait)
GPC
αGP-HA (prey)
IgG
GP2
I
Empty vector
ANDV GP
SNV GP
Input
Beads
IP: ERGIC-53
αERGIC-53 (bait)
G2
IgG
αGP-HA (prey)

Figure S1. Proteomics Workflow, Cut Map of Coomassie-Stained Gels Containing Human Proteins Purified in Complex with LCMV GP or ANDV GP, Functional Clustering Analysis of Host Protein Partners, and Additional Viral GPs that Associate with ERGIC-53, Related to Figure 1

(A) Workflow for identification of human proteins that associate with the GPs encoded by LCMV and ANDV. HEK 293T cells were co-transfected with a plasmid encoding each respective viral GP with a C-terminal HA epitope tag and a biotin acceptor peptide (BAP), along with a second plasmid that encodes BirA, a bacterial biotin ligase, to ensure biotinylation of the viral GPs. As a control, cells were co-transfected with the BirA plasmid and an empty vector. Two days later, biotinylated GPs and associated host proteins were affinity purified (AP) from whole cell lysates using magnetic streptavidin beads and separated on 4-20% polyacrylamide gels for Coomassie staining. To determine the identity of cellular proteins captured, each Coomassie-stained gel lane was cut into sections (see dashed lines) for in-gel, tryptic digestion and mass spectrometry analysis as described in the Extended Experimental Procedures.

(B and C) Cut maps of the affinity purified LCMV GP (B) and ANDV GP (C) samples following SDS-PAGE and Coomassie staining.

(D) NIH DAVID functional clustering identifies enriched protein functional categories from proteomic datasets representing proteins identified binding to both LCMV and ANDV GP proteins, LCMV GP alone, or ANDV GP alone. Official gene symbols (see Tables S1A-S1C) of these data sets were entered into NIH DAVID, searched under medium stringency choosing *Homo sapiens* as background. Functional clusters showing four-fold or more increases were chosen for display here. Functional clusters were simplified by providing labels 1-14 above (see

Table S1D for details). The percent of proteins (average of functional cluster subsets) in each data set in each of these categories is provided as well as the relative enrichment of a given cluster relative to the human proteome. Note that one protein may be found in more than one category. Also note that because the size of each dataset is different a category may show a higher percentage, but a lower enrichment when compared to the same category in a different data set.

(E) HEK 293T cells were transfected with the BirA plasmid and a modified pCAGGS plasmid encoding WWAV GP with a C-terminal HA epitope tag and BAP or, as a control, an empty pCAGGS plasmid. Whole cell lysates (input) were collected 2 days later and incubated with streptavidin beads to isolate each biotinylated GP species (GPC and GP2). Input lysates and captured bead fractions were screened for GP species (GPC and GP2) (bait) and ERGIC-53 (prey) via Western blot.

(F - I) HEK 293T cells were transfected with a plasmid encoding the indicated viral GPs with a C-terminal HA epitope tag or an empty plasmid. Two days later ERGIC-53 was immunoprecipitated from whole cell lysates (input). Inputs and immunoprecipitated bead fractions were screened for ERGIC-53 (bait) and the various GP species (GPC and GP2 for arenaviruses; G2 for hantaviruses) (prey) via Western blot.

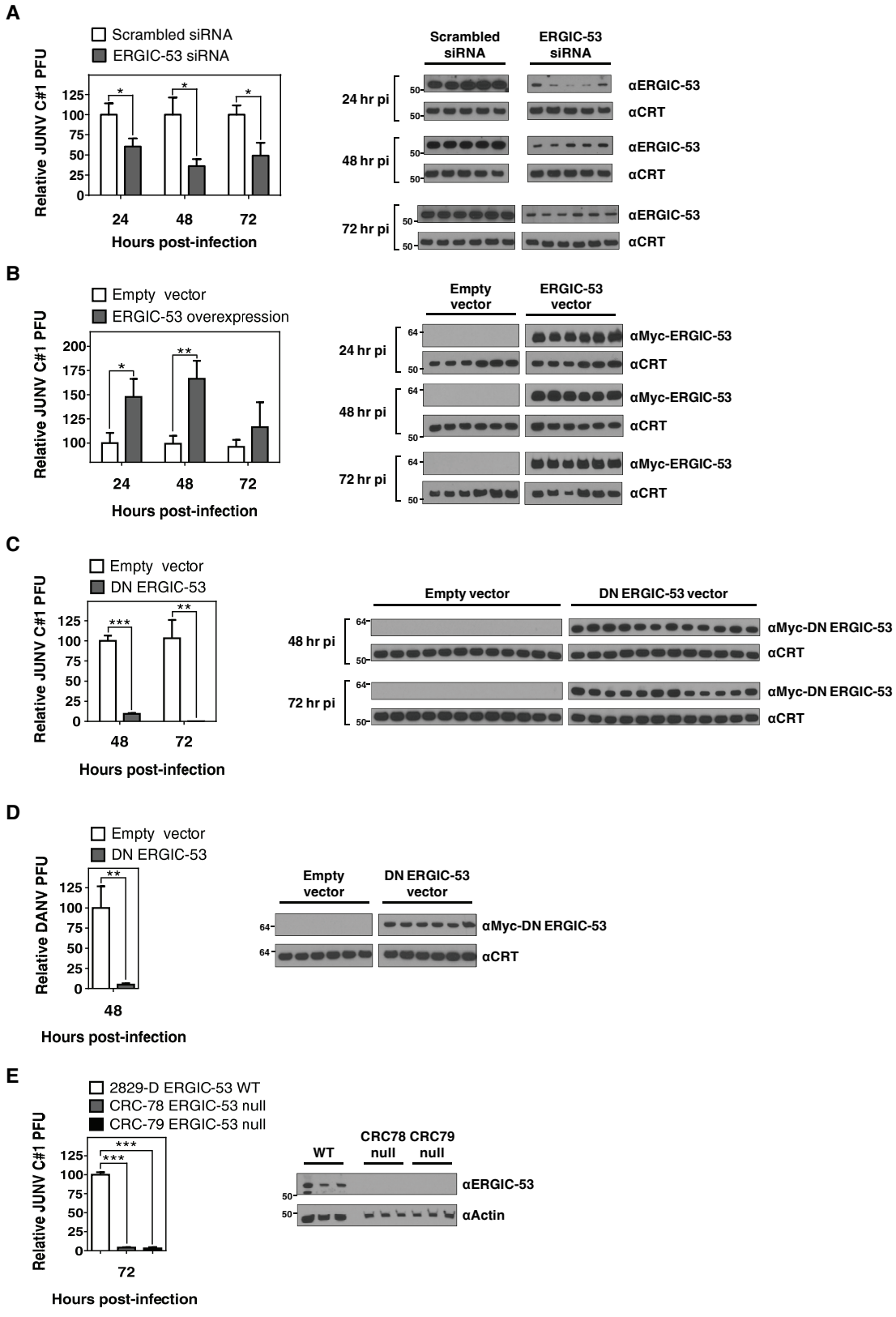
A
Scrambled siRNA
ERGIC-53 siRNA
Relative JUNV C#1 PFU
125
100
75
50
25
0
*
*
*
24
48
72
Hours post-infection
Scrambled siRNA
ERGIC-53 siRNA
24 hr pi
48 hr pi
72 hr pi
50
αERGIC-53
αCRT
B
Empty vector
ERGIC-53 overexpression
Relative JUNV C#1 PFU
200
175
150
125
100
*
**
24
48
72
Hours post-infection
Empty vector
ERGIC-53 vector
64
50
αMyc-ERGIC-53
αCRT
C
Empty vector
DN ERGIC-53
Relative JUNV C#1 PFU
***
**
48
72
Hours post-infection
Empty vector
DN ERGIC-53 vector
48 hr pi
72 hr pi
αMyc-DN ERGIC-53
αCRT
D
Empty vector
DN ERGIC-53
Relative DANV PFU
**
48
Hours post-infection
Empty vector
DN ERGIC-53 vector
αMyc-DN ERGIC-53
αCRT
E
2829-D ERGIC-53 WT
CRC-78 ERGIC-53 null
CRC-79 ERGIC-53 null
Relative JUNV C#1 PFU
***
***
72
Hours post-infection
WT
CRC78 null
CRC79 null
αERGIC-53
αActin

Figure S2. ERGIC-53 is Required for Arenavirus Propagation, Related to Figure 2

(A) Silencing ERGIC-53 expression impairs the release of infectious JUNV C#1 (related to Figure 2A). HEK 293T cells were transfected with an ERGIC-53-specific siRNA or a scrambled, negative control siRNA and challenged 72 hr later with JUNV C#1 at an MOI of 0.1. Supernatants and cell protein lysates were collected at 24, 48, and 72 hr post-inoculation (pi) and screened for JUNV C#1 plaque forming units (PFU) via plaque assay and ERGIC-53 or CRT (loading control) via Western blot (each lane represents an individual well). Data are presented as mean PFU ± SEM relative to the empty vector transfected wells and are the summation of 2 independent experiments (24 & 48 hr pi n = 5, 72 hr n = 6).

(B) Overexpression of WT ERGIC-53 enhances infectious JUNV release (related to Figure 2B). HEK 293T cells were transfected with a plasmid encoding Myc-tagged, WT ERGIC-53 or, as a control, an empty plasmid; 48 hr following transfection these cells were challenged with JUNV C#1 at an MOI of 0.1. Supernatants and cell protein lysates were collected at 24, 48, and 72 hr pi and screened for JUNV C#1 PFU via plaque assay and Myc-ERGIC-53 or CRT (loading control) via Western blot (each lane represents an individual well). Data are presented as mean PFU ± SEM relative to the empty vector transfected wells and are the summation of 2 independent experiments (n = 6 per time point).

(C and D) Restriction of ERGIC-53 to the ER impairs the release of infectious JUNV C#1 and DANV (related to Figures 2C and 2D, respectively). HEK 293T cells were transfected with a plasmid encoding Myc-tagged DN ERGIC-53 or, as a control, an empty plasmid; 24 hr later cells were challenged with JUNV C#1 (C) or DANV (D) at an MOI of 0.1 or 0.001, respectively. Supernatants and cell protein lysates were collected at the indicated times pi and screened for

PFU via plaque assay and Myc-DN ERGIC-53 or CRT (loading control) via Western blot (each lane represents an individual well). Data are presented as mean PFU ± SEM relative to the empty vector transfected wells and are representative of 2 independent experiments (n = 12 or n = 6 per experiment for JUNV C#1 or DANV, respectively).

(E) Release of infectious JUNV C#1 is impaired in ERGIC-53 (*LMAN1*$^{-/-}$) null cells (related to Figure 2E). B cells from *LMAN1*$^{+/+}$ (2829-D) and *LMAN1*$^{-/-}$ (CRC-78 and CRC-79) individuals were challenged with JUNV C#1 at an MOI of 1. Supernatants and cell protein lysates were collected at 48 and 72 hr pi and screened for JUNV C#1 PFU via plaque assay and ERGIC-53 or actin (loading control) via Western blot (each lane represents an individual well). Data are presented as mean PFU ± SEM relative to the *LMAN1*$^{+/+}$ cells and are representative of 2 independent experiments (n = 3 per condition per experiment).

(A - E) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, determined using the unpaired Student's *t* test.

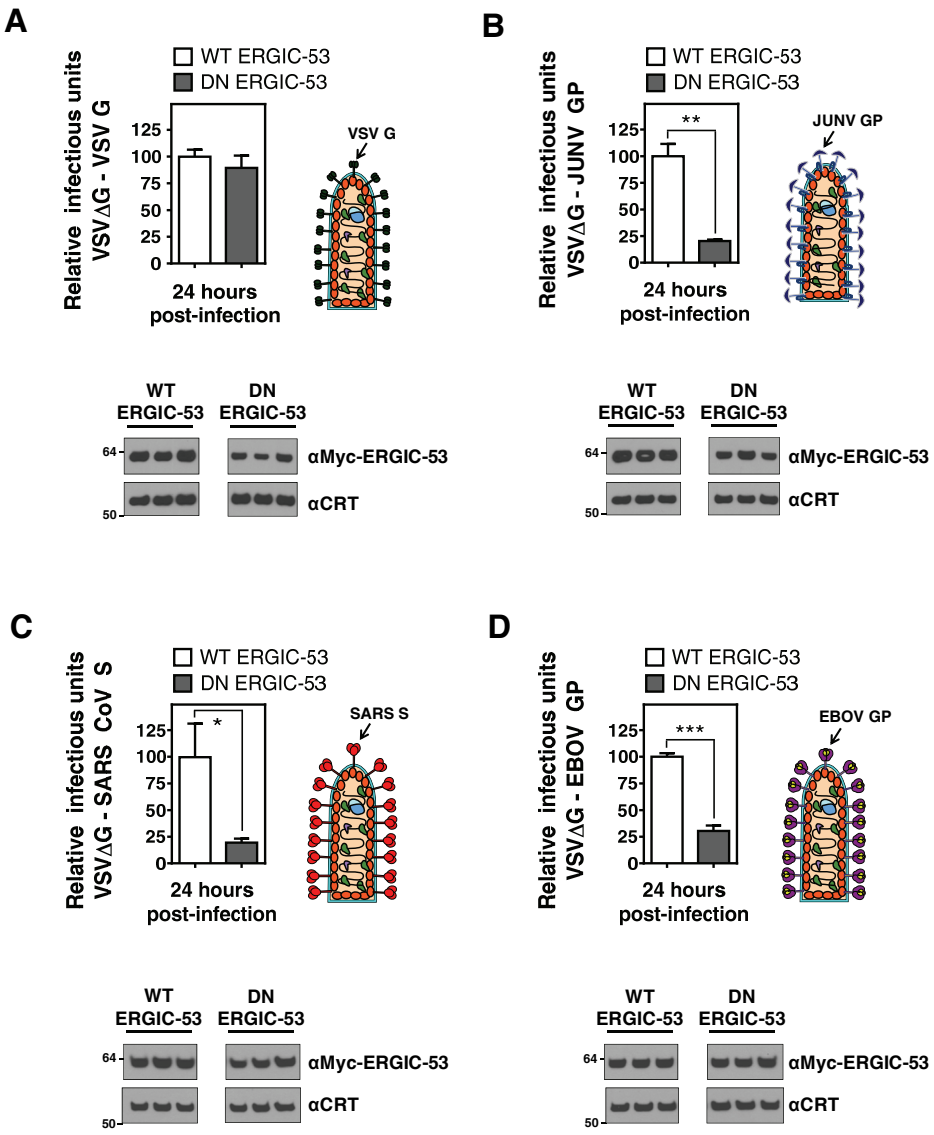

Figure S3. ERGIC-53's Influence on JUNV, SARS CoV, and EBOV Propagation is Specific and Can Be Minimally Mapped to the viral GP, Related to Figure 3

(A - D) HEK 293T cells were initially transfected with a plasmid encoding Myc-tagged WT or DN ERGIC-53, then 24 hr later the WT and DN ERGIC-53 cells were transfected with a plasmid encoding VSV G (A), JUNV XJ GP (B), SARS CoV S (C), or EBOV GP (D). Twenty-four hr following the final transfection, cells were challenged with VSVΔG at an MOI of 2. Supernatants and cell protein lysates were collected 24 hr later and screened for infectious VSVΔG particles pseudotyped with VSV G, JUNV XJ GP, SARS CoV S, or EBOV GP via focus assay and Myc-

ERGIC-53 (WT or DN) or CRT (loading control) via Western blot (each lane represents an individual well), respectively. Data are presented as mean infectious units ± SEM relative to the WT ERGIC-53 vector transfected wells and are representative of 2 independent experiments (n = 3 wells per condition per experiment). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, determined using the unpaired Student's *t* test.

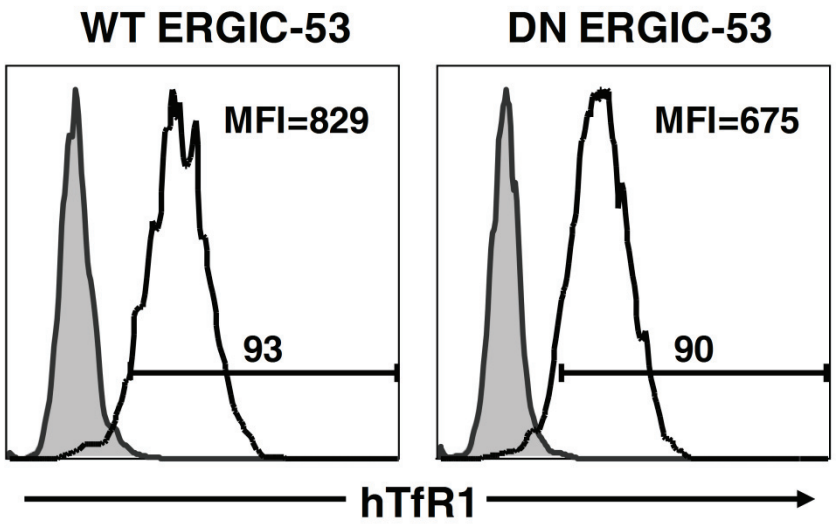

Figure S4. The ER-Restricted, DN ERGIC-53 Does Not Impair Trafficking of hTfR1 to the Plasma Membrane, Related to Figure 4

HEK 293T cells were transfected with a plasmid expressing WT ERGIC-53 or the DN ERGIC-53 mutant and 72 hr later incubated at 4°C with an anti-hTfR1 antibody to stain for surface expression of hTfR1 and then fixed, permeabilized, and incubated with an anti-Myc antibody to stain for internal Myc-ERGIC (WT or DN). The histograms are gated on Myc-positive cells and show the percentage of transfected cells with hTfR1 staining (grey shaded = isotype-matched IgG control antibody signal; white = hTfR1 signal). The median fluorescence intensity (MFI) is reported for each condition.

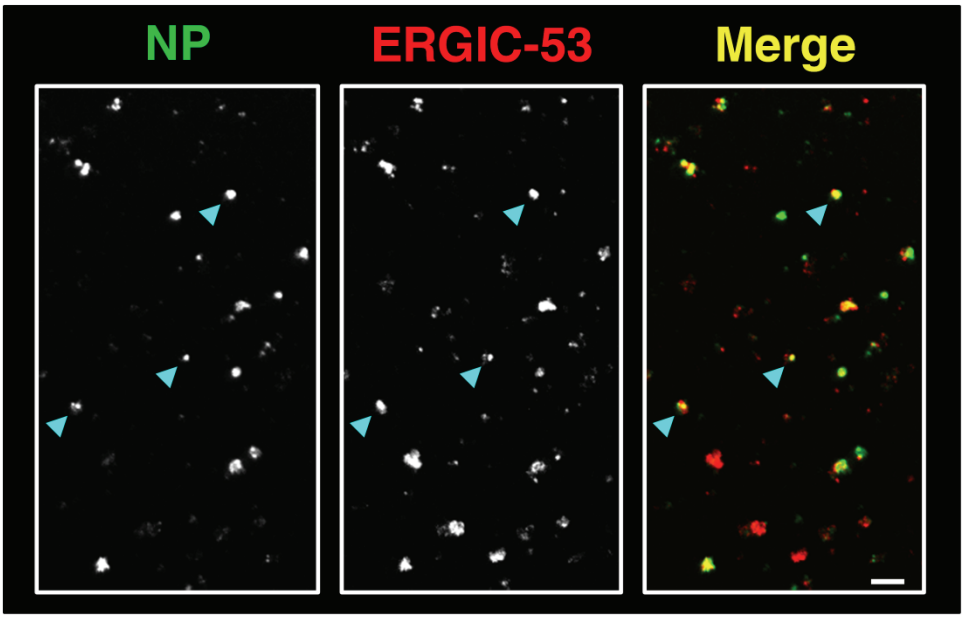

Figure S5. ERGIC-53 is Incorporated into Arenavirus Particles, Related to Figure 5D

JUNV C#1 particles generated in Vero E6 cells were adhered onto glass cover slips, permeabilized, and screened for JUNV NP (green) and ERGIC-53 (red) via confocal microscopy as described in the Extended Experimental Procedures. The data presented are representative of 2 independent experiments. The arrowheads highlight JUNV particles that contain ERGIC-53. Scale bar, 5 μm.

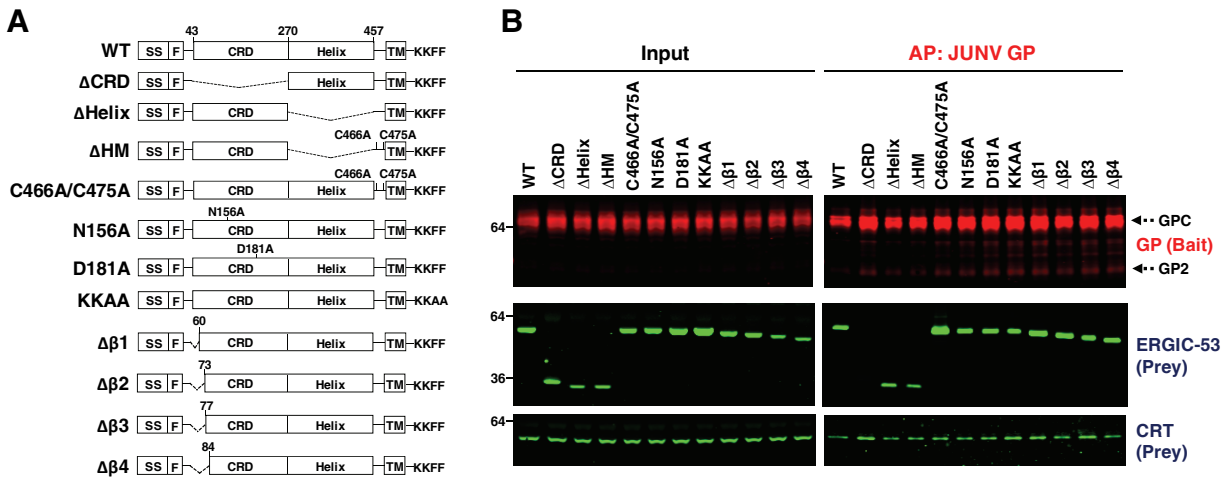

Figure S6. The C-Terminal Region of ERGIC-53's CRD Is Required for the ERGIC-53 - JUNV GP Interaction, Related to Figure 6

(A) Depiction of ERGIC-53 mutants used in this study. ERGIC-53 is a nonglycosylated, type I transmembrane protein that forms homohexamers and consists of an ER-lumenal domain, a transmembrane domain, and a cytoplasmic domain (for review see (Hauri et al., 2000)). The ER-lumenal portion of the protein contains a carbohydrate recognition domain (CRD) that selectively binds high mannose glycans in a Ca^{2+}- and pH-dependent manner (Appenzeller-Herzog et al., 2004; Appenzeller et al., 1999; Itin et al., 1996). Lectin binding can be disrupted by deletion of the entire CRD (ΔCRD) (residues 44 - 269) or specific β-strands within the CRD (e.g. strands 1 & 2 (Δβ2) (residues 43-72), 1 through 3 (Δβ3) (residues 43 - 76), or 1 through 4 (Δβ4) (residues 43-83)) (Zheng et al., 2010), or through mutation of individual amino acids (N156A or D181A) (Itin et al., 1996; Velloso et al., 2003; Zheng et al., 2010) within the CRD that are critically required for coordinating Ca^{2+}-binding. The ER-lumenal region of ERGIC-53 also encodes an alpha helical domain and two cysteine residues (C466 & C475) that are all required for the formation of ERGIC-53 homohexamers. Deletion of the helical domain (ΔHelix) (residues 271 - 457) (Neve et al., 2005) or mutation of the cysteine residues to alanine

(C466A/C475A) (Nufer et al., 2003) results in the loss of noncovalently-associated or disulfide-linked homohexamers, respectively. Deletion of the helical domain combined with mutation of C466 and C475 to alanine (Δhelix monomer (ΔHM)) (Zheng et al., 2010) completely abolishes ERGIC-53 oligomerization and yields monomeric ERGIC-53, which, like the DN mutant (KKAA) (Kappeler et al., 1997), cannot traffic beyond the ER. Additionally, several of these constructs (ΔCRD, Δβ1, Δβ2, Δβ3, Δβ4, and ΔHM) abolish ERGIC-53's ability to interact with MCFD2 (Zheng et al., 2010). SS, signal sequence; F, Flag epitope tag; TM, transmembrane.

(B) HEK 293T cells were co-transfected with the BirA plasmid, a plasmid encoding JUNV C#1 GP with a C terminal HA tag and BAP, and a plasmid encoding the indicated ERGIC-53 mutants with an N-terminal FLAG epitope tag. JUNV GP species (GPC and GP2) were AP as bait from cell lysates. Input lysates and captured bead fractions were screened for ERGIC-53 (prey) and, as a control, CRT (prey) via Western blot. The data are representative of 2 independent experiments.

Supplemental Experimental Procedures

Cells and Viruses

HEK 293T/17 cells (CRL-11268, American Type Culture Collection, Manassas, VA) (referred to as HEK 293T cells in the manuscript) were grown in Dulbecco's Modified Eagle Medium (DMEM) (11965-118) supplemented with 10% fetal bovine serum, 1% Penicillin-Streptomycin (15140-163), 1% MEM Non-Essential Amino Acids Solution (11140-050), 1% HEPES Buffer Solution (15630-130), and 1% GlutaMAX (35050-061) purchased from Invitrogen (Carlsbad, CA). Vero E6 cells were provided by J. L. Whitton (The Scripps Research Institute, La Jolla) and grown in DMEM supplemented with 10% FBS, 1% Penicillin-Streptomycin, and 1% HEPES Buffer Solution. The EBV-immortalized B lymphoblastoid cell lines derived from normal (*LMAN1*$^{+/+}$; 2829-D) or ERGIC-53 null (*LMAN1*$^{-/-}$; CRC-78 and CRC-79) individuals have been described previously (the null lines are described as A2 and A12, respectively, in (Neerman-Arbez et al., 1999)) and were maintained in RPMI 1640 Medium (22400-105, Invitrogen) containing 10% FBS and 1% Penicillin-Streptomycin. The two *LMAN1*$^{-/-}$ individuals are from different families but encode an identical null mutation (c.822-1G>A splice site mutation) that completely abrogates expression of ERGIC-53. All cell lines were cultured at 37°C in a humidified incubator containing 5% CO_2. JUNV C#1 was provided by R. Tesh (The University of Texas Medical Branch at Galveston) and M. J. Buchmeier (University of California, Irvine) and DANV by W. I. Lipkin (Columbia University). JUNV C#1, which is an attenuated vaccine strain, was originally derived from WT JUNV strain XJ and differs by 12 amino acids (Chosewood et al., 2009; Goni et al., 2006). Working stocks of infectious JUNV C#1 and DANV were generated in Vero E6 cells. Infectious titers of these viruses were determined via plaque assay on Vero E6 cells. VSVΔG encoding a GFP reporter has been described elsewhere (Takada

et al., 1997) and was provided by M. Whitt (The University of Tennessee Health Science Center, Memphis, TN). An infectious stock of VSVΔG pseudotyped with VSV G was generated by first transfecting HEK 293T cells with a pCAGGS plasmid encoding VSV G and then 24 hr later inoculating these cells with infectious VSVΔG (which had previously been pseudotyped with VSV G) at a multiplicity of infection (MOI) of 3. Supernatants were collected 24 hr following inoculation and infectious titer was determined by enumerating green foci via focus assay in Vero E6 cells.

Plasmids and Transfections

To identify and/or validate the interaction of human proteins, including ERGIC-53, with various viral GPs in Figures 1, 3, 6, S1, and S6, we subcloned each respective viral GP into our previously described pCAGGS expression vector (Cornillez-Ty et al., 2009). This vector expresses each GP as a fusion protein containing 3 C-terminal elements: a hemaglutinin (HA) epitope tag (YPYDVPDYA) followed by the tobacco etch virus (TEV) cleavage site (ENLYFQG) followed by a 23 amino acid biotin acceptor peptide (BAP) (MASSLRQILDSQKMEWRSNAGGS). When co-transfected with a second plasmid that encodes the bacterial biotin ligase BirA, the BAP can be biotinylated for affinity purification with streptavidin beads. GPs were subcloned into this vector using Gateway Technology (Invitrogen) following the manufacturer's instructions. Briefly, each GP was first amplified via PCR using forward and reverse primers containing *att*B1 and *att*B2 sequences, respectively. In each case, the stop codon was excluded. PCR products were subcloned into pDONR221 via a BP recombination reaction. GP genes were then subcloned from pDONR221 into the modified

pCAGGS vector via an LR recombination reaction. The nucleotide sequence of each GP clone was verified by DNA sequencing. GPs were subcloned from the following viral strains (for each GP, an NCBI Gene Identifier number and a Protein Locus number are listed to provide a link to the actual nucleotide sequence cloned for that particular GP and the corresponding translated amino acid sequence, respectively): ANDV strain CHI-7913 (30313864, AAO86638), SNV NMR11 (999407 (note that there are two silent mutations in our clone: G changed to T and A changed to C at positions 60 and 843, respectively, of referenced sequence), AAC42202), LASV strain Josiah (23343509, NP_694870), LCMV strain Armstrong 53b (61655715, AAX49341), JUNV strain XJ (also referred to as Parodi) (see (Reignier et al., 2006) for description of the nucleotide sequence; the amino acid sequence of the cloned gene matches JVU70799), JUNV strain C#1 (52222815, AAU34180), MACV strain Carvallo (see (Reignier et al., 2006) for description of nucleotide sequence, the amino acid sequence of the cloned gene matches AAN09942); GTOV strain INH-95551 (22901284, AAN09938), WWAV strain AV 9310135 (14333982, AAK60497), SARS CoV S from strain Tor2 (JX163924, AFR58686), influenza HA from strain A/WSN/33 (CY010788, ABF47955), EBOV strain Zaire (EBORNA, AAB81004), and MARV strain Musoke (DQ217792, ABA87127). Each of these GPs, with the exception of LCMV GP, JUNV XJ GP, MACV GP, SARS CoV S, influenza virus A/WSN/33 HA, EBOV GP, and MARV GP was synthesized by Bio Basic Inc. (Markhamm, ON). The LCMV strain Armstrong GP gene was provided by J. C. de la Torre (The Scripps Research Institute, La Jolla) while the JUNV strain XJ GP and MACV strain Carvallo GP were provided by P. Cannon (University of Southern California, Los Angeles). The MARV GP in pCAGGS and EBOV GP in pcDNA3.1 were obtained through BEI Resources, NIAID, NIH, Manassas, VA (NR-19815 and NR-19814, respectively). The SARS CoV S was provided by M. J. Buchmeier (University of

California, Irvine) while the influenza virus A/WSN/33 HA in pCAGGS was provided by M. Shaw and A. Garcia-Sastre (Mount Sinai School of Medicine, New York). For the VSV pseudotyping experiments in Figures 3 and S3, we utilized standard pCAGGS vectors encoding VSV G (provided by J. C. de la Torre (Lee et al., 2002)) or EBOV GP (NR-19814, BEI Resources), our modified pCAGGS vector encoding SARS CoV S, and a pSA90 vector encoding JUNV XJ GP provided by P. Cannon (Reignier et al., 2006). The pCAGGS VSV G plasmid was also used for the immunoprecipitation experiments to screen for an interaction between VSV G and ERGIC-53 in Figures 3E and 3F. For the viral challenges (Figures 2, 3, S2, and S3), GP interaction (Figures 3G-3J), GP trafficking (Figures 4A and 4B), hTfR1 trafficking (Figure S4), and VLP experiments (Figure 4C) we utilized pCDNA3 GM (which encodes WT ERGIC-53 with an N-terminal c-Myc tag) and/or pECE KKAA (which encodes the DN ERGIC-53 with an N-terminal c-Myc tag; the 2 C-terminal phenylalanines of this ERGIC-53 gene have been replaced with alanines) plasmids that were provided by H. P. Hauri (University of Basel, Basel, Switzerland) (Itin et al., 1995). For the VLP experiments, we also utilized our modified pCAGGS vector encoding JUNV strain XJ13 Z (this gene was synthesize by Bio Basic Inc. and subcloned via Gateway Technology) (33868610, AAQ55249 as well as JUNV XJ GP with a modified series of C-terminal epitope tags (HA followed by FLAG). We have previously described the panel of ERGIC-53 plasmids that were utilized to determine the molecular basis for the JUNV GP - ERGIC-53 association in Figures 6 and S6 (Zheng et al., 2010). Briefly, the WT and ERGIC-53 mutants were cloned into the pED plasmid by replacing the ERGIC-53 signal sequence with that of calreticulin (CRT) and introducing a FLAG epitope directly after the signal sequence. The mutant plasmids used were ΔCRD (R44-E269), ΔHelix (G271-N457), ΔHM (G271-N457 and C466A/C475A), C466A/C475A, N156A, D181A, KKAA (the 2 C-

terminal phenylalanines were replaced with alanines), Δβ1 (H43-Q59), Δβ2 (H43-N72), Δβ3 (H43-S76), and Δβ4 (H43-A83) (a schematic of each mutant is shown in Figures 6A and S6A; a detailed description of each mutant is provided in the Figure S6A legend). All transfections were done using either Fugene HD (4709713001, Roche Applied Science, Indianapolis, IN) (3 µl Fugene HD per 1 µg DNA) or Polyethylenimine (PEI) (23966, Polysciences, Inc., Warrington, PA) (5 µl PEI (from a 1 mg/ml solution in PBS (10010049, Invitrogen)) per 1 µg DNA).

Affinity Purification of Viral GPs

To capture biotinylated viral GPs for the identification of human protein partners via mass spectrometry (Figures 1A, 1B, S1B, and S1C) or validation of protein partners via Western blot (Figures 1D, 1F, 1G, and S1E), HEK 293T cells were co-transfected with our modified pCAGGS plasmid that encodes each respective viral GP with a C-terminal HA epitope tag and a BAP, and a second plasmid that encodes BirA to facilitate biotinylation of the viral GPs. As a control for the mass spectrometry studies and APs done to validate an interaction between a given viral GP and endogenous ERGIC-53, cells were co-transfected with the BirA plasmid and an empty pCAGGS plasmid. For the experiments to screen for an association between ERGIC-53 and coronavirus, orthomyxovirus, or filovirus GPs in Figures 3G and 3I, in addition to receiving the GP and BirA plasmids, cells were also transfected with pCDNA3 GM plasmid encoding WT ERGIC-53. For the experiments to map the molecular basis for the interaction between JUNV GP and ERGIC-53 in Figures 6 and S6, in addition to receiving the GP and BirA plasmids, cells were also transfected with a third plasmid encoding either the WT or one of mutant FLAG-tagged, ERGIC-53 proteins. In each case, cells were scraped into the media 48 hr following transfection, pelleted, washed with cold PBS, and then gently lysed on ice in 25 mM Tris-HCL,

pH 7.6 containing 1% Triton X-100 (T9284, Sigma-Aldrich, St. Louis, MO), 0.5% Nonidet P-40 IGEPAL CA-630 (198596, MP Biomedicals, Solon, OH), 140mM NaCl, 1 mM calcium chloride (21115, Sigma-Aldrich), and a Complete Mini EDTA-Free Protease Inhibitor Cocktail tablet (04693159001, Roche Applied Science). Cell lysates were clarified of insoluble material by centrifugation at 10,000 rpm at 4°C followed by incubation with magnetic streptavidin beads (Dynabeads MyOne Streptavidin T1, 65602, Invitrogen) on a rotating platform for 2.5 hours at 4°C. Following 4 washes in ice cold lysis buffer to remove nonspecific proteins, each captured viral GP and its associated cellular protein partners were stripped from the streptavidin beads by boiling the beads in Laemmli buffer containing 5% β-mercaptoethanol and separated by size and charge on Novex 4-20% Tris-Glycine polyacrylamide gels (EC60285BOX, Invitrogen) for either Western blot analysis to confirm bait/prey purification or Coomassie staining for mass spectrometry analysis (described in next section).

Mass Spectrometry

To identify human protein partners of LCMV GP or ANDV GP in Figures 1A, 1B, S1B, and S1C, HEK 293T cells were co-transfected with our modified pCAGGS plasmid encoding each respective viral GP with a C-terminal HA epitope tag and a biotin acceptor peptide (BAP) and a second plasmid encoding BirA to ensure biotinylation of the viral GPs. As a control, cells were co-transfected with the BirA plasmid and an empty vector. Two days later, biotinylated GPs and associated host proteins were affinity purified (AP) from whole cell lysates as described above, run out on a Novex 4-20% Tris-Glycine polyacrylamide gel, stained with Coomassie stain (0.1% Brilliant Blue R (B7920, Sigma-Aldrich) in 40% methanol with 20% acetic acid) diluted in 30%

methanol with 10% acetic acid solution to 20% v/v overnight at room temperature, and then destained for 4 to 6 hr in 30% methanol with 10% acetic acid solution. Each gel lane was cut into 15 (LCMV GP) or 14 (ANDV GP) sections (see Figures S1B and S1C for cut maps) for in-gel digestion of captured proteins using Sequencing Grade Modified Trypsin (V5111, Promega, Madison, WI 6 ng/μL) in 50 mM ammonium bicarbonate overnight at 37°C as previously described (Ballif et al., 2006). Peptides were extracted from gel sections with 50% acetonitrile (MeCN) and 2.5% formic acid (FA) and then dried. Peptides were then resuspended in 2.5% MeCN and 2.5% FA and loaded onto a microcapillary column packed with 12 cm of reversed-phase Magic C18 material (5 μm, 200 Å, Michrom Bioresources, Inc., Auburn, CA) using a MicroAS autosampler (Thermo Scientific, Pittsburgh, PA). Elution was performed with a 5−35% MeCN (0.15% FA) gradient using a Surveyor Pump Plus HPLC (Thermo Scientific) over 40 min, after a 15 min isocratic loading at 2.5% MeCN and 0.15% FA. Mass spectra were acquired in an LTQ-XL linear ion trap mass spectrometer (Thermo Scientific) over the entire run using 10 MS/MS scans following each survey scan. Raw data were searched against the human IPI forward and reverse concatenated databases using SEQUEST software requiring tryptic peptide matches with a 2 Da mass tolerance. Cysteine residues were required to have a static increase in 71.0 Da for acrylamide adduction, and differential modification of 16.0 Da on methionine residues was permitted. Host proteins were considered legitimate GP protein partners if 2 or more unique tryptic peptides were detected from a host protein in samples transfected with a given GP plasmid but not the empty vector or, alternatively, if there was a 5-fold higher quantity of total tryptic peptides detectable from a given human protein in a GP sample compared to the empty vector sample. These filters resulted in a false discovery peptide rate of less than 1%.

Immunoprecipitations

Whole cell protein lysates used for immunoprecipitation studies were generated as described above in the "Affinity Purification of Viral GPs" section. Following centrifugation at 10,000 rpm at 4°C to remove insoluble material, protein lysates were pre-cleared by incubating them with magnetic Protein G beads (Dynabeads Protein G beads, 10004D, Invitrogen) on a rotating platform for 15 minutes at 4°C. Each cleared lysate was then incubated (on a rotating platform) with its respective antibody for 2 hr followed by magnetic Protein G beads for 1 hr. The beads were then washed 4 times with ice cold lysis buffer to remove nonspecific proteins and excess antibody. Captured bait proteins and their associated protein partners (prey) were stripped from the beads by boiling them in Laemmli buffer (with or without 5% β-mercaptoethanol) and separated by size and charge on gradient Novex 4-20% Tris-Glycine polyacrylamide gels for Western blot analysis to confirm bait/prey purification. Immunoprecipitations from cellular protein lysates were carried out using the following antibodies: ERGIC-53 was immunoprecipated with either the mouse anti-ERGIC-53 G1/93 antibody (ALX-804-602-C100, Enzo Life Sciences Farmingdale, NY) (Figures 1E, 1I-1K, 3F, 3H, 3J, S1G and S1H) or a rabbit anti-ERGIC-53 antibody (sc-66880, Santa Cruz, Dallas, TX) (Figure S1F and S1I); VSV G with the mouse anti-VSV G antibody (11 667 351 001, Roche Applied Science) (Figure 3E); JUNV GP with the mouse anti-GP1/GPC antibody GB03-BE08 (NR-2564, BEI Resources) (Figure 1H); or, as a control, a species matched, non-immune Mouse IgG1 Isotype (MAB002, R&D Systems Minneapolis, MN) (Figures 1K and S1H).

To purify intact JUNV C#1 particles in Figure 5D, supernatant from JUNV C#1-infected Vero E6 cells was collected 72 hr post-inoculation, cleared of cells by centrifugation at 1400 RPM, then pre-cleared with magnetic Protein G beads, incubated with the GP1-specific mouse monoclonal antibody QC03-BF11 (NR-2566, BEI Resources) for 2 hr followed by magnetic

Protein G beads for 1 hr. The beads were washed 4 times with PBS containing 0.1% BSA and 1 mM calcium chloride and then boiled in Laemmli buffer to elute/lyse the captured viral particles. The collected lysate was then electrophoresed on a Novex 4-20% Tris-Glycine polyacrylamide gel for Western blot analysis. Controls for this experiment included the use of the GP1-specific antibody with supernatants from mock-infected cells as well as using the MAB002 Mouse IgG1 Isotype Control antibody for immunoprecipitation from the JUNV C#1-infected supernatants.

SDS-PAGE and Western Blot

Protein lysates were separated by SDS-PAGE using Novex 4-20% Tris-Glycine polyacrylamide gels. Protein transfer to nitrocellulose membranes was accomplished using the iBlot Gel Transfer Device and iBlot Transfer Stack nitrocellulose membranes (IB3010-01) from Invitrogen according to the manufacturer's instructions. Following transfer, membranes were blocked by rocking in a solution of 5% milk in PBS for 1 hour at room temperature followed by 3 washes with Western wash solution (PBS with 0.5% Nonidet P-40 IGEPAL CA-630). Primary antibodies were diluted in antibody diluent (PBS containing 5% milk, 3% FBS, and 0.05% Nonidet P-40 IGEPAL CA-630) and incubated overnight at room temperature. Following 3 washes in Western wash, membranes were incubated with secondary antibodies diluted in antibody diluent for 2 hr at room temperature, followed by 3 final washes in Western wash. Membranes were then developed using chemiluminescence (SuperSignal West Pico (34080) or Femto (34096) Chemiluminescent Substrate, Thermo Scientific). The membranes that were visualized via an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) in Figures 6 and S6 were treated as described above with the following changes: the antibody diluent for the primary antibodies was PBS containing 5% milk and 0.05% Nonidet P-40 IGEPAL CA-630, the antibody diluent for the secondary antibodies was PBS containing 5%

milk, 0.02% SDS, and 0.05% Nonidet P-40 IGEPAL CA-630, while the final wash was done using PBS.

Primary antibodies (and the dilutions they were used at) were: G1/93 mouse anti-ERGIC-53 (1:500), sc-66880 rabbit anti-ERGIC-53 (1:4,000), QC03-BF11 mouse anti-JUNV GPC/GP1 (1:5,000), GB03-BE08 mouse anti-JUNV GPC/GP1 (1:500), 11 667 351 001 mouse anti-VSV G antibody (1:1,000), 9B11 mouse anti-Myc (2276, Cell Signaling, Danvers, MA) (1:3,000), 71D10 rabbit anti-Myc (2278, Cell Signaling) (1:4,000), NA05-AG12 mouse anti-JUNV NP (NR-2582, BEI Resources) (1:200), clone M2 mouse anti-FLAG (F1804, Sigma-Aldrich) (1:3,000), SPA-600 rabbit anti-CRT (Stressgen, Ann Arbor, MI) (1:4,000), Clone AC-15 mouse anti-β-Actin (A5441, Sigma-Aldrich) (1:5,000), HA.11 Clone 16B12 mouse anti-HA (MMS-101P, Covance Emeryville, CA) (1:4,000), and rabbit anti-JUNV Z (1:2,000) (provided by Sandra Goñi and described in (Goni et al., 2010)). Detection of native JUNV C#1 GPC/GP1 using GB03-BE08 or QC03-BF11 was done under non-reducing conditions.

Secondary antibodies used for chemiluminescence were: goat anti-mouse IgG HRP conjugate (H+L) (71045, EMD Millipore, Billerica, MA) (1:10,000), goat anti-mouse light chain IgG HRP conjugate light chain specific (AP200P, EMD Millipore) (1:50,000), Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) (111035045, Jackson, West Grove, PA) (1:10,000), mouse anti-rabbit light chain IgG HRP (211032171, Jackson) (1:50,000).

Secondary antibodies used for LI-COR were: IRDye 800CW Goat Anti-Rabbit IgG (H+L) (926-32211, LI-COR) (1:20,000), IRDye 800CW Goat Anti-Rabbit IgG (H+L) (926-32210, LI-COR) (1:20,000), and IRDye 680CW Goat Anti-Rabbit IgG (H+L) (926-68070, LI-COR) (1:20,000).

Viral Challenge Assays

A series of viral challenge assays were conducted to determine how various manipulations of ERGIC-53 (siRNA silencing of ERGIC-53, overexpression of WT ERGIC-53, expression of DN ERGIC-53, or loss of ERGIC-53 expression due to null mutation of *LMAN1*) would impact the ability of viruses (JUNV C#1, DANV, or VSVΔG pseudotyped with VSV G, SARS CoV S, EBOV GP or JUNV XJ GP) to release infectious progeny. At each time point examined in the various assays, supernatants and cells were collected (from each replicate well) to measure infectious virus or protein expression levels, respectively. Supernatants were clarified via centrifugation at 2000 RPM for 5 minutes and then screened for infectious virus via plaque assay (JUNV C#1 and DANV) or focus forming assay (pseudotyped VSVΔG). To generate protein lysates, cells were scraped into PBS, combined with any cells that pelleted while clarifying the supernatants, pelleted by centrifugation at 2000 RPM, and then lysed on ice in 25 mM Tris-HCL, pH 7.6 containing 1% Triton X-100, 0.5% Nonidet P-40 IGEPAL CA-630, 140 mM NaCl, and a Complete Mini EDTA-Free Protease Inhibitor Cocktail tablet. Lysates were clarified of insoluble material by centrifugation at 10,000 rpm at 4°C and run on Novex 4-20% Tris-Glycine polyacrylamide gels for Western blot analysis. For selected challenge assays described below, we utilized the unpaired Student's *t* test to determine whether statistically significant differences existed between the quantities of infectious virus released from control versus experimental groups as indicated in the Figure legends.

For the siRNA challenge experiments shown in Figures 2A and S2A, HEK 293T cells were plated in 6-well plates, reverse transfected with 25 nM of either an ERGIC-53-specific siRNA (5'-GGACAGAAUCGUAUUCAUCdTdT-3' as sense and 5'-AUGAAUACGAUUCUGUCCdTdT-3' as antisense) (Nyfeler et al., 2006) or a scrambled,

negative control siRNA (Allstars Negative Control siRNA, 1027280, Qiagen, Valencia, CA) using HiPerFect Transfection Reagent (301705, Qiagen) according to the manufacturer's instructions. Cells were challenged 72 hr later with JUNV C#1 at an MOI of 0.1. Supernatants and cell protein lysates were collected at 24, 48, and 72 hr post-inoculation (96, 120, and 144 hr post-transfection with siRNA, respectively) and screened for JUNV C#1 plaque forming units (PFU) via plaque assay or ERGIC-53 and CRT (loading control) expression via Western blot. Prior to carrying out the viral challenge, we determined, using the ERGIC-53-specific siRNA, the timeframe post-transfection that would yield optimal ERGIC-53 knock-down. Using Western blot as a read out, we observed silencing of ERGIC-53 in HEK 293T cells from 48 to 144 hr post-transfection (data not shown).

For the overexpression challenge experiments shown in Figures 2B and S2B, HEK 293T cells were plated in 6-well plates, transfected the next day with a plasmid encoding Myc-tagged, WT ERGIC-53 or an empty vector, and challenged 1 day later with JUNV C#1 at an MOI of 0.1. Supernatants and cell protein lysates were collected at 24, 48, and 72 hr post-inoculation and screened for JUNV C#1 PFU via plaque assay or Myc-WT ERGIC-53 and CRT (loading control) expression via Western blot.

For the DN ERGIC-53 challenge assays shown in Figures 2C, 2D, S2C, and S2D, HEK 293T cells were plated in 24-well plates, transfected the next day with a plasmid encoding Myc-tagged, DN ERGIC-53 or an empty vector, and challenged 1 day later with JUNV C#1 or DANV at an MOI of 0.1 or 0.001, respectively. Supernatants and cell protein lysates were collected at 48 and 72 hr post-inoculation for JUNV C#1 or 48 hr post-inoculation for DANV and screened for PFU via plaque assay or Myc-DN ERGIC-53 and CRT (loading control) expression via Western blot.

For the ERGIC-53 null cell line challenge assays shown in Figures 2E and S2E, EBV-immortalized B lymphoblastoid cell lines derived from 1 normal (*LMAN1*$^{+/+}$; 2829-D) or 2 ERGIC-53 null (*LMAN1*$^{-/-}$; CRC-78 and CRC-79) individuals were seeded in a 24-well plate and challenged with JUNV C#1 at an MOI of 1. Supernatants and cell protein lysates were collected 72 hr post-inoculation and screened for PFU via plaque assay or ERGIC-53 and actin (loading control) expression via Western blot.

For the VSVΔG pseudotyping challenge assays shown in Figures 3A-3D and S3, HEK 293T cells were initially transfected with a plasmid encoding Myc-tagged WT or DN ERGIC-53, then 24 hr later the WT and DN ERGIC-53 cells were transfected with a plasmid encoding VSV G, JUNV XJ GP, SARS CoV S, or EBOV GP. Twenty-four hr following the final transfection, cells were challenged with VSVΔG (that had been previously pseudotyped with VSV G) at an MOI of 2. Supernatants and cell protein lysates were collected 24 hr later and screened for infectious VSVΔG particles pseudotyped with VSV G, JUNV XJ GP, SARS CoV S, or EBOV GP via focus assay and Myc-ERGIC-53 (WT or DN) or CRT (loading control) via Western blot, respectively.

For the ERGIC-53 null cell line challenge assays shown in Figures 4D-4G, EBV-immortalized B lymphoblastoid cell lines derived from a normal (*LMAN1*$^{+/+}$; 2829-D) or ERGIC-53 null (*LMAN1*$^{-/-}$; CRC-78) individual were seeded in T-75 flasks and challenged or not (mock) with JUNV C#1 at an MOI of 0.1. Supernatants were collected 96 hr post-inoculation, concentrated via ultracentrifugation as described below, and screened for PFU via plaque assay, JUNV proteins (GP1, NP, and Z) or cellular proteins (ERGIC-53 and actin (loading control)) via Western blot, and viral S segment genomic RNA via quantitative RT-PCR (as described below). RNA was extracted from each viral preparation using the QIAamp Viral RNA Mini Kit (52906,

Qiagen) according to the manufacturer instructions. Virion preparations were also screened for their ability to bind host cells in a virus-cell binding assay (described below).

Virus-Cell Binding Assay

To determine whether the viral particles produced in EBV-immortalized B lymphoblastoid cell lines derived from a normal (*LMAN1*$^{+/+}$; 2829-D) or ERGIC-53 null (*LMAN1*$^{-/-}$; CRC-78) individual had differing capacities to bind host cells in Figure 4G, we chilled Vero E6 cells grown in 48-well plates to 4°C, washed them twice with PBS, and then incubated duplicate wells with each viral preparation for 1.5 hrs at 4°C. Unbound virus was then aspirated and each well was washed 3 times in PBS. Following the final wash, total RNA was extracted from each monolayer using the RNeasy Mini Kit (74106, Qiagen) according to the manufacturer instructions. RNA samples were then subjected to quantitative RT-PCR, as described below, to determine the copies of JUNV C#1 S segment genomic RNA.

Quantitative RT-PCR

To enumerate quantities of JUNV C#1 viral S segment genomic RNA, cDNA was generated using 200 nM of primer 5'-AAGGGTTTAAAAATGGTAGCAGAC-3', which is specific for the NP region of the S segment genomic (negative-sense) RNA, with Multiscribe-RT (4311235, Life Technologies, Carlsbad, CA). Reaction conditions were 25ºC for 10 min, 48ºC for 30 min, and 95ºC for 5 min. Quantitative PCR was then performed using a primer-probe set originally described in (Trombley et al., 2010). Specifically, we used forward primer (900 nM) 5'-CATGGAGGTCAAACAACTTCCT-3', reverse primer (900 nM) 5'-GCCTCCAGACATGGTTGTGA-3', and probe (200 nM) 5'-6FAM-ATGTCATCGGATCCTT-MGBNFQ-3'. Note that the forward primer differs by 1 nt from the originally reported sequence.

Reactions were carried out using Taqman Universal PCR Master Mix (4326614, Life Technologies). Reaction conditions were 95ºC for 10 min and 45 cycles of 95ºC for 15 sec and 60ºC for 1 min. Absolute copy numbers of JUNV C#1 S segment genomic RNA were determined by comparison with a series of standard dilutions of the our modified pCAGGS plasmid encoding the JUNV C#1 NP gene. Data was acquired using an Applied Biosystems StepOnePlus Real-Time PCR System and analyzed with the provided StepOne software.

Virus-Like Particle (VLP) Assay

For the VLP assays shown in Figure 4C, HEK 293T cells were initially transfected with a plasmid expressing Myc-tagged WT or DN ERGIC-53, then 24 hr later with a cocktail of 2 plasmids encoding the JUNV Z and XJ GPC proteins, respectively, to permit the formation and release of VLPs. Cells and supernatants were collected 72 hr later and screened for the presence of various GP species (the C-terminally FLAG-tagged precursor GPC or proteolytically processed GP2) or actin (to serve as a loading control) via Western blot. Cells were collected as described above in the "Viral Challenge Assay" section. VLPs were concentrated from supernatants via ultracentrifugation through sucrose (as described below) prior to Western blot analysis.

Ultracentrifugation of VLPs and Authentic JUNV C#1 Particles

The JUNV VLPs in Figure 4C and JUNV C#1 particles in Figure 4D were concentrated via ultracentrifugation through sucrose as previously described (Neuman et al., 2008). Briefly, supernatants were clarified of cellular debris via 2 rounds of centrifugation at 1500 rpm and 2500 rpm, respectively. VLPs/particles were then precipitated by dissolving polyethylene glycol MW 8,000 (81268, Sigma-Aldrich) into clarified supernatants (10% weight/volume) at 4ºC.

Supernatants were then centrifuged at 10,000 RPM for 30 minutes at 4ºC to pellet VLPs/particles. Pellets were gently resuspended in HEPES buffered saline, pH 7.4, layered onto 20% sucrose, and centrifuged at 100,000 x g in a T-865 Rotor (Thermo Scientific) for 2.5 hr at 4ºC. Pelleted virus was gently resuspended in HEPES buffered saline, pH 7.4 for use in plaque and/or Western blot assays.

Confocal Microscopy

Confocal microscopy was used to visualize the localization of JUNV C#1 GP and ERGIC-53 in HEK 293T cells (either internally, at the plasma membrane, or a combination of internal/surface staining) or JUNV C#1 NP and ERGIC-53 in virions. For the internal staining of JUNV C#1 GP and endogenous ERGIC-53 shown in Figure 1L, HEK 293T cells were seeded onto 22 mm glass cover slips (12-541-B, Thermo Scientific) within 6-well dishes, inoculated or not (mock) the next day with JUNV C#1 at an MOI of 0.1, then 72 hr later washed with PBS, fixed with Z-FIX (174, ANATECH, Battle Creek, MI), permeabilized in PBS with 0.1% Triton X-100 and 1% BSA, and then blocked in PBS containing 3% FBS and 10% normal goat serum (005-000-121, Jackson) for 30 minutes at room temperature. The cells were next incubated with a 1:500 dilution of the G1/93 mouse anti-ERGIC-53 antibody in PBS containing 1% BSA and 0.1% Triton X-100 overnight at 4ºC, washed 3 times with PBS containing 0.1% BSA, and incubated with Alexa Fluor 647-conjugated goat anti-mouse IgG (H+L) (A-21236, Invitrogen). After 3 washes in PBS containing 0.1% BSA, cells were incubated with a 1:50 dilution of the GB03-BE08 mouse anti-JUNV GP1/GPC antibody that had been directly conjugated to Alexa Fluor 488. Cover slips were washed 3 times with PBS, stained with 4', 6-diamidino-2-phenylindole hydrochloride

(DAPI) (D9542, Sigma Aldrich), washed 3 times with PBS, and then mounted onto glass slides using ProLong Gold Antifade Reagent (P36934, Invitrogen).

The intracellular and surface staining of JUNV C#1 GP and endogenous ERGIC-53 shown in Figures 5A and 5B, respectively, was accomplished by seeding HEK 293T cells in T-75 flasks and then inoculating them or not (mock) the next day with JUNV C#1 at an MOI of 0.1. Cells were trypsinized 2 days later (at 48 hr post-inoculation), reseeded onto 22 mm glass cover slips within 6-well dishes, and at 72 hr post-inoculation either a) fixed, permeabilized, and blocked in an identical manner described above for internal staining or b) incubated at 4ºC for surface staining. Following blocking, internal staining was done by incubating cells with a 1:500 dilution of the GB03-BE08 mouse anti-JUNV GP1/GPC antibody and a 1:200 dilution of the sc-66880 rabbit anti-ERGIC-53 antibody in PBS with 1% BSA overnight at 4ºC. After 3 washes in PBS with 0.1% BSA, cells were incubated with a 1:800 dilution of both the Alexa Fluor 488-conjugated Goat Anti-Mouse IgG (H+L) (A-11029, Invitrogen) and Alexa Fluor 647-conjugated Goat Anti-Rabbit IgG (H+L) (A-21245, Invitrogen) antibodies. For the live-cell surface staining, cells were incubated with a 1:50 dilution of the GB03-BE08 mouse anti-JUNV GP1/GPC antibody and a 1:50 dilution of the sc-66880 rabbit anti-ERGIC-53 antibody in PBS with 3% FBS and 10% normal goat serum for 20 minutes at 4ºC. After 3 washes in PBS with 3% FBS, cells were incubated with a 1:200 dilution of both the Alexa Fluor 488-conjugated Goat Anti-Mouse IgG (H+L) and Alexa Fluor 647-conjugated Goat Anti-Rabbit IgG (H+L) antibodies, washed 3 times with PBS, and fixed in 4% paraformaldehyde. In both staining protocols, cells were stained with DAPI, washed 3 times with PBS, and then mounted onto glass slides using ProLong Gold Antifade Reagent. As a control, cells were surface stained for JUNV C#1 GP and CRT using a 1:50 dilution of the SPA-600 rabbit anti-CRT antibody (Figure 5C). The protocol

was the same with the exception of replacing the ERGIC-53-specific antibody with the CRT-specific antibody.

The surface staining of JUNV C#1 GP and intracellular staining of either Myc-tagged WT or DN ERGIC-53 shown in Figure 4A was accomplished by seeding HEK 293T cells in T-75 flasks, transfecting them with plasmids encoding either Myc-tagged WT or DN ERGIC-53, then 1 day later inoculating them with JUNV C#1 at an MOI of 0.1. Cells were trypsinized 2 days later (at 48 hr post-inoculation) and reseeded onto 22 mm glass cover slips within 6-well dishes. At 72 hr post-inoculation cells were washed in PBS and then surface stained for JUNV GP1/GPC via incubation with a 1:50 dilution of the GB03-BE08 mouse anti-JUNV GP1/GPC antibody directly conjugated to Alexa Fluor 488 in PBS containing 3% FBS for 20 minutes at 4°C. Cells were then washed 3 times in PBS and fixed using Z-FIX. Following fixation, cells were washed 2 times with PBS, permeabilized via incubation with PBS containing 0.1% Triton X-100 and 1% BSA for 10 minutes, washed 3 times with PBS containing 0.1% BSA, blocked for 30 minutes at room temperature with PBS containing 1% BSA and 10% normal goat serum, then stained for Myc-tagged WT or DN ERGIC-53 via overnight incubation at 4°C with a 1:200 dilution of the 71D10 rabbit anti-Myc antibody in PBS containing 0.1% BSA. Cells were washed 3 times in PBS containing 0.1% BSA, then incubated with a 1:800 dilution of the Alexa Fluor 647-conjugated goat anti-rabbit antibody in PBS containing 0.1% BSA for 2 hr, washed 3 times in PBS, stained with DAPI, washed 3 times in PBS, then mounted on glass slides using ProLong Gold Antifade Reagent.

Detection of ERGIC-53 in JUNV C#1 particles shown in Figure S5 was accomplished by incubating clarified media from either mock- or JUNV C#1-infected cells for 2 hr at 37°C in 35 mm glass bottom culture dishes (P356-1.5-10-C, MatTek Corporation, Ashland, MA) that had

been coated with 0.01% Poly-L-Lysine (3438-100-01, Trevigen, Gaithersburg, MD). The JUNV C#1-containing media had a titer of ~1 x 10^7 PFU/ml. Viral particles were subsequently fixed by incubation in 4% paraformaldehyde for 30 minutes at room temperature, washed three times with 1X PBS, permeabilized in PBS containing 1% BSA and 0.1% Triton X-100 for 10 minutes at room temperature, and then blocked in PBS containing 1% BSA and 10% normal goat serum for 30 minutes at room temperature. Staining for JUNV NP and ERGIC-53 was done via incubation with a 1:200 dilution of the mouse anti-JUNV NP antibody NA05-AG12 (NR-2582, BEI Resources) and a 1:100 dilution of a rabbit anti-ERGIC-53 antibody (raised by B.Z.) in PBS containing 1% BSA for 2 hours at room temperature. Following 3 washes in PBS with 0.1% BSA, the dishes were incubated with a 1:200 dilution of both the Alexa Fluor 488-conjugated Goat Anti-Mouse IgG (H+L) and Alexa Fluor 647-conjugated Goat Anti-Rabbit IgG (H+L) antibodies. The stained dishes were then washed 3 times in PBS and stored at 4°C prior to imaging in PBS.

Images for all confocal experiments were obtained using a Zeiss LSM 510 Laser Scanning Confocal Microscope. Images were captured using either a 63X or 100X objective lens with a numerical aperture of 1.4. Optical zoom was set to 1.5X and images were obtained at 1.0 Airy unit. The colocalization analysis shown in Figure 1L was performed with the colocalization analysis module contained in the Zeiss Aim software on images that were captured using the 63X objective lens. To determine background gating thresholds for colocalization analysis, we averaged the background signal from triplicate images of either mock-infected cells stained using the GB03-BE08 mouse anti-JUNV GP1/GPC antibody directly conjugated to Alexa Fluor 488 or JUNV C#1-infected cells stained with the Alexa Fluor 647-conjugated goat anti-mouse secondary antibody (gates are shown as white lines in the histogram in Figure 1L).

Flow Cytometry

Flow cytometry was utilized to screen, in cells expressing either WT or DN ERGIC-53, whether JUNV C#1 GP (Figure 4B) or hTfR1 (Figure S4) was detectable at the plasma membrane and, if so, the median fluorescence intensity (MFI) of these respective signals. In both cases, HEK 293T cells were seeded in 6-well dishes and transfected the next day with plasmids encoding either Myc-tagged WT or DN ERGIC-53. To screen for surface expression of JUNV C#1 GP, cells were inoculated the next day with JUNV C#1 at an MOI of 1.0. At 72 hr post-inoculation the media was aspirated and cells detached from the plates by incubating them with Versene (2 mM EDTA in PBS) for 15 minutes at 37°C. Cell pellets were washed 2 times with PBS and then surface stained for JUNV GP1/GPC via incubation with a 1:100 dilution of the GB03-BE08 mouse anti-JUNV GP1/GPC antibody directly conjugated to Alexa Fluor 488 in FACS buffer (PBS containing 2% fetal bovine serum and 0.2% sodium azide) for 20 minutes at 4°C. Cells were then washed 3 times in FACS buffer, fixed/permeabilized in BD Cytofix/Cytoperm solution (554722, BD Biosciences, San Jose, CA) for 20 minutes at room temperature, washed 2 times in BD Perm/Wash buffer (554723, BD Biosciences), then stained for Myc-tagged WT or DN ERGIC-53 via a 20 minute incubation at 4°C with a mouse anti-Myc antibody directly conjugated to Alexa Fluor 647 (2233, Cell Signaling) diluted 1:100 in Perm/Wash buffer. For the experiments looking at surface expression of hTfR1, 72 hr following transfection of the WT or DN ERGIC-53 plasmids, cells were collected and stained as described for JUNV C#1 GP with the exception of replacing the mouse-anti JUNV C#1 GP Alexa Fluor 488 antibody with a primary/secondary antibody combination consisting of a 1:100 dilution of an unlabeled A4A6 mouse monoclonal anti-hTfR1antibody provided by J. Cook (University of Kansas Medical

Center, Kansas City, KS) and a 1:200 dilution of a goat anti-mouse antibody directly conjugated to R-Phycoerythrin (P852, Invitrogen). Data was acquired on an LSR II (BD Biosciences) and analysis was done using FlowJo software (v9.6.2, TreeStar, Inc., Ashland, OR).

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