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

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SUMMARY

Arenaviruses and hantaviruses cause severe human disease. Little is known regarding host proteins required for their propagation. We identified human proteins that interact with the glycoproteins (GPs) of a prototypic arenavirus and hantavirus and show that the lectin endoplasmic reticulum (ER)-Golgi intermediate compartment 53 kDa protein (ERGIC-53), a cargo receptor required for glycoprotein trafficking within the early exocytic pathway, associates with arenavirus, hantavirus, coronavirus, orthomyxovirus, and filovirus GPs. ERGIC-53 binds to arenavirus GPs through a lectin-independent mechanism, traffics to arenavirus budding sites, and is incorporated into virions. ERGIC-53 is required for arenavirus, coronavirus, and filovirus propagation; in its absence, GP-containing virus particles form but are noninfectious, due in part to their inability to attach to host cells. Thus, we have identified a class of pathogen-derived ERGIC-53 ligands, a lectin-independent basis for their association with ERGIC-53, and a role for ERGIC-53 in the propagation of several highly pathogenic RNA virus families.

INTRODUCTION

Arenaviruses and hantaviruses are rodent-borne, negative-sense RNA viruses that cause significant morbidity and mortal-ity in humans (Buchmeier et al., 2007; Schmaljohn and Nichol, 2007). Most pathogenic arenaviruses are associated with severe hemorrhagic fever syndromes in humans. Examples include the New World arenaviruses Junin virus (JUNV), Machupo virus (MACV), and Guanarito virus (GTOV), which are the etiologic agents of Argentine, Bolivian, and Venezuelan hemorrhagic fevers, respectively, as well as Lassa virus (LASV), an Old World arenavirus that causes Lassa fever along the coast of West Africa (Buchmeier et al., 2007). Additionally, lymphocytic choriomeningitis virus (LCMV) can cause aseptic meningitis in immunocompetent individuals and is a potent teratogen (Buchmeier et al., 2007). LCMV and Dandenong virus (DANV), an LCMV-like virus, are also responsible for a nearly uniform lethality in immunosuppressed recipients of virus-infected tissues (Fischer et al., 2006; Palacios et al., 2008). Hantaviruses cause two human illnesses: hemorrhagic fever with renal syndrome in the Old World and hantavirus cardiopulmonary syndrome (HCPS) in the New World (Schmaljohn and Nichol, 2007). Sin Nombre virus (SNV) and Andes virus (ANDV) are the primary etiologic agents of HCPS in North and South America, respectively, and are associated with a fatality rate of 35%–39% (da Rosa Elkhoury et al., 2012; MacNeil et al., 2011). US Food and Drug Administration (FDA)-approved vaccines or effective antivirals do not currently exist for the prevention and/or therapeutic treatment of arenavirus or hantavirus disease.

Arenaviruses and hantaviruses each encode an envelope glycoprotein (GP) that decorates the surface of the virion and functions to mediate attachment and entry of virions into permissive host cells. Each GP is encoded as a precursor (GPC) that is proteolytically processed into mature subunits. The arenavirus GPC is posttranslationally modified to yield a stable signal peptide (SSP) as well as GP1 and GP2 subunits (Lenz et al., 2001), whereas the hantavirus GPC is cotranslationally processed

into G1 and G2 subunits (Löber et al., 2001). In each case, the GP subunits form a mature GP complex (SSP-GP1-GP2 for arenaviruses; G1-G2 for hantaviruses) that facilitates receptor binding and entry (Buchmeier et al., 2007; Schmaljohn and Nichol, 2007).

Relatively little is known regarding interactions that arenavirus or hantavirus GPs have with host proteins or the importance of such interactions for viral replication and disease pathogenesis. Herein, we utilized a proteomics approach to comprehensively identify human proteins that interact with GPs encoded by a prototypic arenavirus or hantavirus. We show that the endoplasmic reticulum (ER)-Golgi intermediate compartment 53 kDa protein (ERGIC-53), an intracellular cargo receptor that facilitates the anterograde transport of a limited number of glycoprotein ligands in the early exocytic pathway (Appenzeller et al., 1999), has a conserved interaction with GPs encoded by multiple families of RNA viruses and is essential for the formation of infectious arenavirus, coronavirus, and filovirus particles in a GP-specific manner. Our results suggest that loss of ERGIC-53 or its functionality leads to the formation of GP-containing virions that are defective in their ability to attach to permissive host cells.

RESULTS

Identification of Cellular Proteins that Associate with Arenavirus and Hantavirus GPs and Choice of ERGIC-53

To identify human proteins that associate with arenavirus and hantavirus GPs, we used an approach that featured affinity purification (AP) of biotinylated viral proteins (LCMV GP to represent arenaviruses or ANDV GP for hantaviruses) in complex with host proteins, followed by mass spectrometry to identify host protein partners as described in Figure S1A and the Supplemental Experimental Procedures (available online). We identified a number of host proteins that associated with LCMV GP (n = 309), ANDV GP (n = 134), or both GPs (n = 51) (Figures 1A–1C, S1B, and S1C; Tables S1A–S1C). As shown in Figure S1D and Table S1D, host proteins that associated with both GPs were enriched for processes involving the ER, protein folding, and vesicular transport. The LCMV GP-only partners were enriched for processes that included the ER, the proteasome, and nuclear import, while the ANDV GP-only partners were enriched for protein translation and ribosome biogenesis. We were particularly interested in the subset of proteins that interacted with both GPs, as they could serve as broad-spectrum antiviral targets. Of these, we chose ERGIC-53 for further study based upon several criteria. First, the ERGIC-53-viral GP interaction is physiologically plausible (e.g., each protein traffics within the exocytic pathway); ERGIC-53 is a mannose-specific lectin (Itin et al., 1996) and the viral GPs are mannosylated (Schmaljohn et al., 1986; Wright et al., 1990). Second, based on its identification as a cargo receptor within the exocytic pathway (Appenzeller et al., 1999), we hypothesized that ERGIC-53 could be required for GP maturation and therefore might be critical for viral propagation. Finally, ERGIC-53 is an attractive target because loss of this protein or its normal function is well tolerated both in vitro (Mitrovic et al., 2008; Nyfeler et al., 2006; Vollenweider et al., 1998) and in vivo (Khoriaty et al., 2012).

Confirmation that ERGIC-53 Has a Conserved Association with GPs Encoded by Multiple Pathogenic Arenaviruses and Hantaviruses

We next wished to determine whether ERGIC-53 could associate with additional GPs encoded by arenaviruses (LASV, MACV, JUNV strain XJ, JUNV strain Candid #1 [C#1], and Whitewater Arroyo virus [WWAV]) or hantaviruses (SNV). Each viral GP tested, when serving as bait, was able to coprecipitate ERGIC-53 (Figures 1D, 1F–1H, S1E, and data not shown). Likewise, ERGIC-53 was able to coprecipitate each GP screened (Figures 1E, 1I–1K, S1F–S1I, and data not shown). For the arenavirus GPs, only the full-length GPC was coprecipitated as prey. A full-length hantavirus GPC cannot be recovered, as this protein is cotranslationally cleaved into G1 and G2 subunits prior to synthesis of a full-length GPC species (Löber et al., 2001). As shown in Figure 1L, we further verified the specificity of the ERGIC-53-JUNV C#1 GP interaction by showing that both proteins strongly colocalize within a structure that we putatively identify as the ERGIC; both proteins also preferentially concentrate in this structure.

ERGIC-53 Is Required for Arenavirus Propagation

We conducted a series of viral challenge studies to determine how various manipulations of ERGIC-53 might impact the ability of arenaviruses (JUNV C#1 or DANV) to release infectious progeny. Partial silencing of ERGIC-53 expression via siRNA transfection led to a considerable reduction in the release of infectious JUNV C#1 (24 hr postinfection [pi], 39.6% decrease, p = 0.03; 48 hr pi, 64% decrease, p = 0.02; 72 hr pi, 51% decrease, p = 0.01) (Figures 2A and S2A), whereas increased expression of WT ERGIC-53 enhanced release of infectious JUNV C#1 (24 hr pi, 48% increase, p = 0.025; 48 hr pi, 68% increase, p = 0.004) (Figures 2B and S2B). Expression of an ER-restricted, dominant-negative (DN) mutant of ERGIC-53 (Vollenweider et al., 1998) resulted in a pronounced reduction in the release of infectious JUNV C#1 (48 hr pi, 90.5% decrease, p = 1.4 ×10^{-8}; 72 hr pi, 99.8% decrease, p = 3.2 × 10^{-4}) (Figures 2C and S2C), DANV (95% reduction, p = 0.003) (Figures 2D and S2D), and LCMV strain Armstrong 53b (data not shown). These experiments clearly demonstrate that ERGIC-53 is required for the propagation of New World and Old World arenaviruses.

The Release of Infectious JUNV C#1 Is Restricted in Cell Lines Derived from ERGIC-53 Null Individuals

Humans with homozygous null mutations in *LMAN1* (lectin, mannose binding 1), the gene encoding ERGIC-53, have combined deficiency of factor V (FV) and FVIII (F5F8D, Online Mendelian Inheritance in Man [OMIM] 227300), a mild bleeding disorder characterized by reduced levels of circulating FV and FVIII (Nichols et al., 1998). We therefore conducted challenge studies featuring B cells derived from *LMAN1*$^{+/+}$ (2829-D) and *LMAN1*$^{-/-}$ (CRC-78 and CRC-79) individuals. The two *LMAN1*$^{-/-}$ individuals, despite being from separate families (A2 and A12 in Neerman-Arbez et al., 1999), encode an identical null mutation (c.822-1G > A splice site mutation) that abrogates expression of ERGIC-53. Significantly less infectious virus was released from each of the *LMAN1*$^{-/-}$ cell lines when compared to the *LMAN1*$^{+/+}$ control cells (96% and 97% decrease for
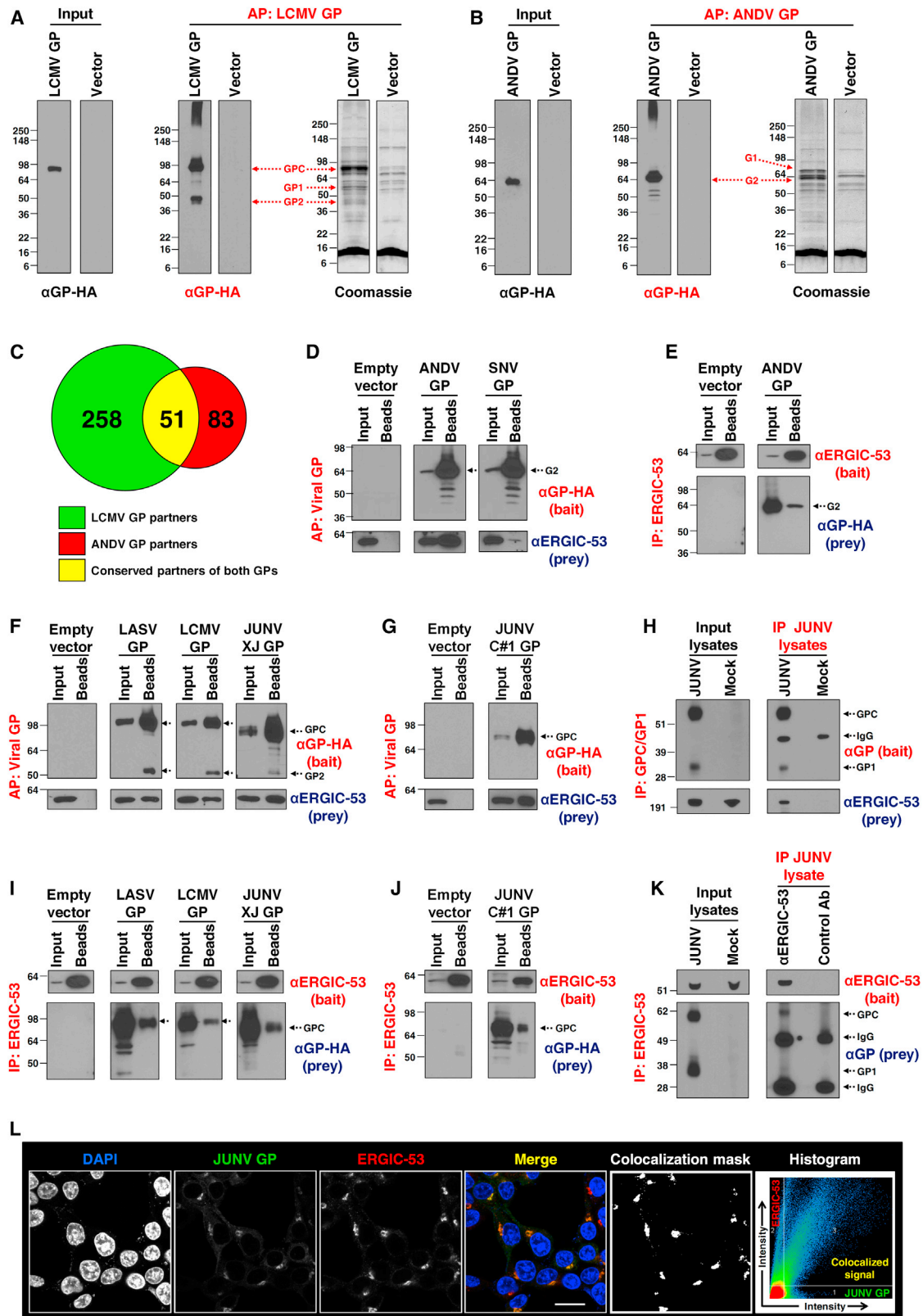
Figure 1. Identification of Human Proteins that Associate with Arenavirus and Hantavirus GPs

(A and B) HEK293T cells were cotransfected with a pCAGGS plasmid encoding either LCMV GP (A) or ANDV GP (B) 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

(legend continued on next page)

CRC-78 and CRC-79, respectively; p < 0.0001 for each) (Figures 2E and S2E).

ERGIC-53's Influence on JUNV Replication Is Specific and Can Be Minimally Mapped to the JUNV GP

To determine the specificity of ERGIC-53's impact on arenavirus replication, we employed a pseudotyping approach, featuring vesicular stomatitis virus (VSV) strain ΔG, a recombinant VSV that does not encode its native glycoprotein (G) and accordingly cannot form infectious particles unless a suitable viral glycoprotein (e.g., VSV G or JUNV GP) is provided in *trans* (Whitt, 2010). There was no difference in the formation of infectious VSVΔG particles decorated with native VSV G between the WT and DN ERGIC-53 backgrounds (Figures 3A and S3A). This may be due to the fact that VSV G does not associate with ERGIC-53 in coimmunoprecipitation assays (Figures 3E and 3F). The formation of infectious VSVΔG decorated with JUNV XJ GP was significantly impaired in cells expressing DN ERGIC-53 compared to the WT ERGIC-53 cells (80% reduction; p = 0.001) (Figures 3B and S3B). These experiments demonstrate that ERGIC-53's impact on arenavirus propagation is specific and can be minimally restricted to the GP itself. Importantly, the fact that ERGIC-53 does not associate with VSV G demonstrates the specificity of its interaction with arenavirus and hantavirus GPs.

ERGIC-53 Broadly Associates with Viral Class I Fusion Proteins and Is Required for the Propagation of Coronaviruses and Filoviruses

We next screened for an association between ERGIC-53 and class I fusion GPs encoded by coronaviruses (the severe acute respiratory syndrome coronavirus [SARS CoV] spike protein [S]), orthomyxoviruses (the H1N1 influenza virus A/WSN/33 hemagglutinin protein [HA]), and filoviruses (the Ebola [EBOV] and Marburg virus [MARV] GPs). Each GP, when used as bait, was able to coprecipitate ERGIC-53 (Figures 3G and 3I). Reciprocally, ERGIC-53 was able to coprecipitate the uncleaved, precursor GP from each virus (Figures 3H and 3J). The formation of infectious VSVΔG particles decorated with either the SARS CoV S or EBOV GP was significantly impaired in cells expressing DN ERGIC-53 compared to the WT ERGIC-53 cells (81% reduction, p = 0.03 for SARS CoV; 70% reduction, p = 0.0002 for EBOV) (Figures

3C, 3D, S3C, and S3D). In summary, ERGIC-53 has a conserved interaction with class I fusion GPs and is required for the propagation of coronaviruses and filoviruses in a GP-specific manner.

Trafficking of JUNV C#1 GP or hTfR1 to the Plasma Membrane Is Not Influenced by ERGIC-53

To determine how the loss of ERGIC-53 function impaired the formation of infectious arenavirus particles, we first tested whether ERGIC-53 could be a bona fide cargo receptor required for the proper anterograde trafficking of JUNV GP (Appenzeller et al., 1999). Cells expressing WT or DN ERGIC-53 were infected with JUNV C#1 and screened for surface expression of JUNV C#1 GP. Interestingly, GP surface staining was equivalent in both the WT and DN ERGIC-53 transfected cells in terms of the frequency of cells with GP expression (46% WT versus 47% DN) as well as the intensity of GP staining (median fluorescence intensity [MFI] of 348 for WT versus 363 for DN) (Figure 4B). Confocal microscopy analysis revealed a similar result (Figure 4A). The DN ERGIC-53 had no impact on the ability of hTfR1, the surface receptor required for GP-mediated JUNV entry into host cells (Radoshitzky et al., 2007), to traffic to the cell surface (Figure S4). In summary, cells expressing DN ERGIC-53 have normal surface expression of hTfR1, manifest no defect in viral entry of WT JUNV C#1 particles, and display no defect for GP synthesis or its trafficking to the plasma membrane.

The DN ERGIC-53 Mutant Does Not Impair Proteolytic Processing of JUNV GPC or the Incorporation of GP Species into Virus-like Particles

Cells expressing WT or DN ERGIC-53 were transfected with plasmids encoding the JUNV matrix protein (Z) and JUNV XJ GP to allow for virus-like particle (VLP) formation and release. Expression of DN ERGIC-53 did not impair the generation of GP2 from GPC in cells or the incorporation of GP2 into VLPs (Figure 4C).

Loss of ERGIC-53 Leads to the Formation of Virus Particles that Are Noninfectious

In Figures 2E and S2E, we show that JUNV C#1 is impaired in its ability to release infectious progeny from *LMAN1*$^{-/-}$ cells. To determine whether this was due to (i) a general deficiency in JUNV C#1 particle release or (ii) the release of defective

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cotransfected with the BirA plasmid and an empty vector. Biotinylated GPs and associated host proteins were affinity purified (AP) from cell lysates (input) using magnetic streptavidin beads and separated on polyacrylamide gels for western blot analysis to verify purification of the various GP species (GPC, GP1, and GP2 for LCMV; G1 and G2 for ANDV) and Coomassie staining for mass spectrometry analysis. Each Coomassie-stained gel lane was cut into sections for in-gel, tryptic digestion, and mass spectrometry analysis, as described in the Supplemental Experimental Procedures (see Figure S1A for the proteomics workflow, Figures S1B and S1C for cut maps, Tables S1A–S1C for a list of the proteins, and Table S1D and Figure S1D for results of a functional clustering analysis).
(C) Venn diagram representing the number of identified host proteins associated with LCMV GP, ANDV GP, or both GPs.
(D–G, I, and J) ERGIC-53 has a conserved association with arenavirus and hantavirus GPs. HEK293T cells were transfected with a pCAGGS plasmid encoding either ANDV GP or SNV GP (D); ANDV GP (E); LASV GP, LCMV GP, or JUNV XJ GP (F); JUNV C#1 GP (G); LASV GP, LCMV GP, or JUNV XJ GP (I); or JUNV C#1 GP (J) with a C-terminal HA epitope tag and a BAP or, as a control, an empty pCAGGs plasmid. In (D), (F), and (G), cells were also transfected with the BirA plasmid to ensure biotinylation of each GP. Viral GPs (D, F, and G) or ERGIC-53 (E, I, and J) were affinity purified or immunoprecipitated, respectively, as bait from cell lysates. Input lysates and purified bead fractions were screened for ERGIC-53 (D, F, and G) or viral GP species (G2 for hantaviruses; GPC and GP2 for arenaviruses) (E, I, and J) as prey via western blot. See Figures S1E–S1I for screening of additional GPs.
(H and K) HEK293T (H) or Vero E6 (K) cells were infected with JUNV C#1 and JUNV GP (H), or ERGIC-53 (K) was immunoprecipitated from cell lysates. Input lysates and purified bead fractions were screened for ERGIC-53 (H) or JUNV C#1 GP species (GPC and GP1) (K) as prey via western blot.
(L) ERGIC-53 and JUNV C#1 GP concentrate in the same intracellular structure. JUNV C#1-infected HEK293T cells were fixed, permeabilized, and stained for JUNV C#1 GP (green) and ERGIC-53 (red). Colocalization between GP and ERGIC-53 is displayed as white pixels in the colocalization mask. The histogram shows background gating (white lines) and specific immunofluorescence signal for JUNV GP (region 1), ERGIC-53 (region 2), or colocalized GP and ERGIC-53 (region 3) (78.6% of ERGIC-53 signal colocalized with GP signal; 52.2% of GP signal colocalized with ERGIC-53 signal). Scale bar = 20 μm.
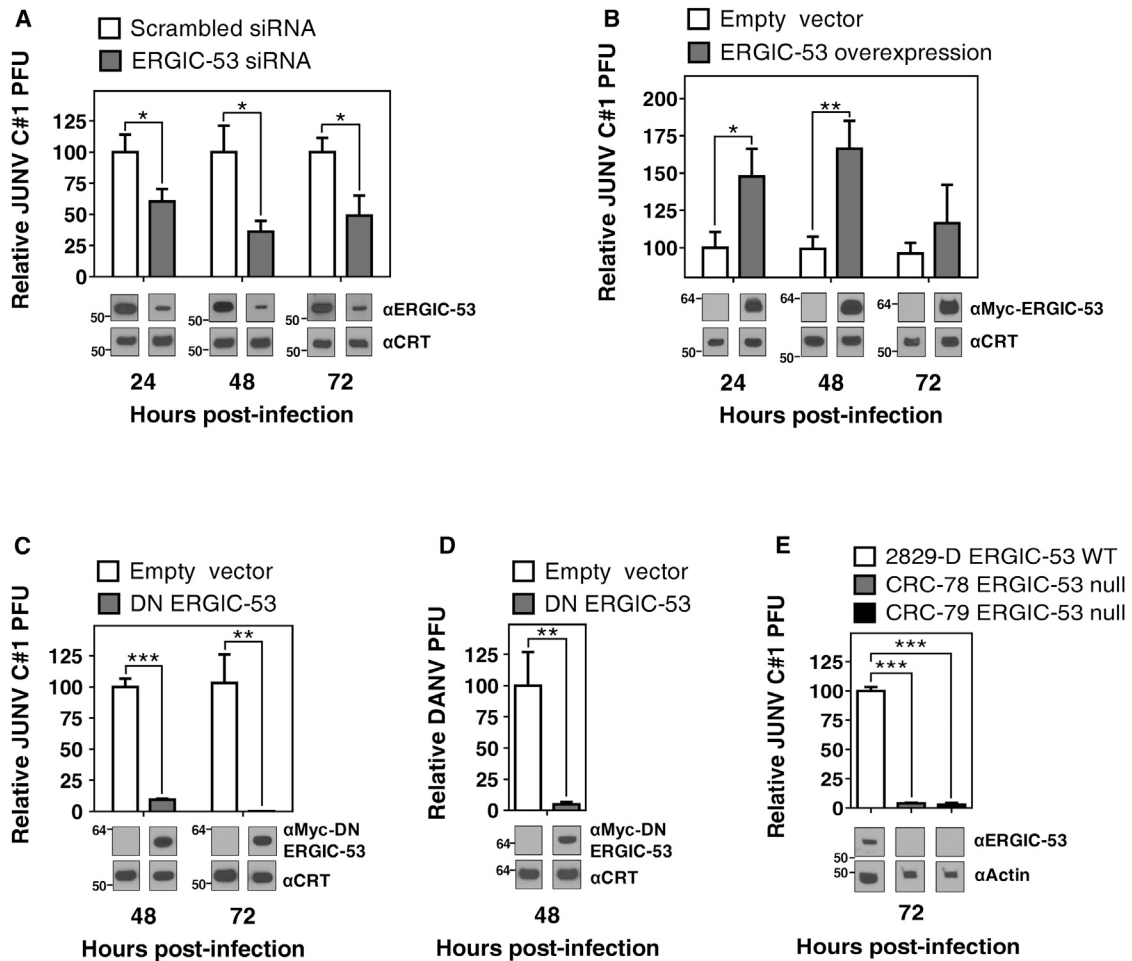
Figure 2. ERGIC-53 Is Required for Arenavirus Propagation

(A) Silencing ERGIC-53 expression impairs the release of infectious JUNV C#1. HEK293T cells were transfected with an ERGIC-53-specific siRNA or a scrambled, negative control siRNA and challenged 72 hr later with JUNV C#1. Supernatants and cell protein lysates were screened for JUNV C#1 plaque-forming units (pfu) via plaque assay and ERGIC-53 or calreticultin (CRT) via western blot. Data are presented as mean pfu ± SEM relative to the empty vector-transfected wells and are the summation of two independent experiments (24 and 48 hr pi, n = 5, 72 hr n = 6).

(B) Overexpression of WT ERGIC-53 enhances infectious JUNV C#1 release. HEK293T 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. Supernatants and cell protein lysates were screened for JUNV C#1 pfu via plaque assay and Myc-ERGIC-53 or CRT via western blot. Data are presented as mean pfu ± SEM relative to the empty vector-transfected wells and are the summation of two independent experiments (n = 6 at each time point).

(C and D) Restriction of ERGIC-53 to the ER impairs the release of infectious JUNV C#1 and DANV. HEK293T 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). Supernatants and cell protein lysates were screened for pfu via plaque assay and Myc-DN ERGIC-53 or CRT via western blot. Data are presented as mean pfu ± SEM relative to the empty vector-transfected wells and are representative of two 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. EBV-transformed B cells from $LMAN1^{+/+}$ (2829-D) and $LMAN1^{-/-}$ (CRC-78 and CRC-79) individuals were challenged with JUNV C#1. Supernatants and cell protein lysates were screened for JUNV C#1 pfu via plaque assay and ERGIC-53 or actin via western blot. Data are presented as mean pfu ± SEM relative to the $LMAN1^{+/+}$ cells and are representative of two independent experiments (n = 3 per condition per experiment).

For (A)–(E), *p < 0.05, **p < 0.01, and ***p < 0.001, as determined using the unpaired Student's t test. Note that western blot results from representative lysates are shown; the full panel of lysates is displayed in Figure S2.

particles, we concentrated virions from the supernatants of $LMAN1^{+/+}$ (2829-D) or $LMAN1^{-/-}$ (CRC-78) cells and screened them for infectious virus, viral genome, and viral structural proteins. We found no discernible difference in the quantity of viral proteins (GP1, nucleoprotein [NP], or Z) released from $LMAN1^{+/+}$ or $LMAN1^{-/-}$ cells (Figure 4D), despite a nearly 10-fold reduction in infectious virus titer from $LMAN1^{-/-}$ cells

(Figure 4E). Additionally, the $LMAN1^{-/-}$-derived particles contained viral genomic RNA with a 6.4-fold higher ratio of genome to infectious virus compared to $LMAN1^{+/+}$ particles (Figure 4F). Lastly, the $LMAN1^{-/-}$ particles also exhibited a specific defect in attachment to host cells (52% reduction compared to $LMAN1^{+/+}$ particles) (Figure 4G). In summary, loss of ERGIC-53 expression does not impact the ability of
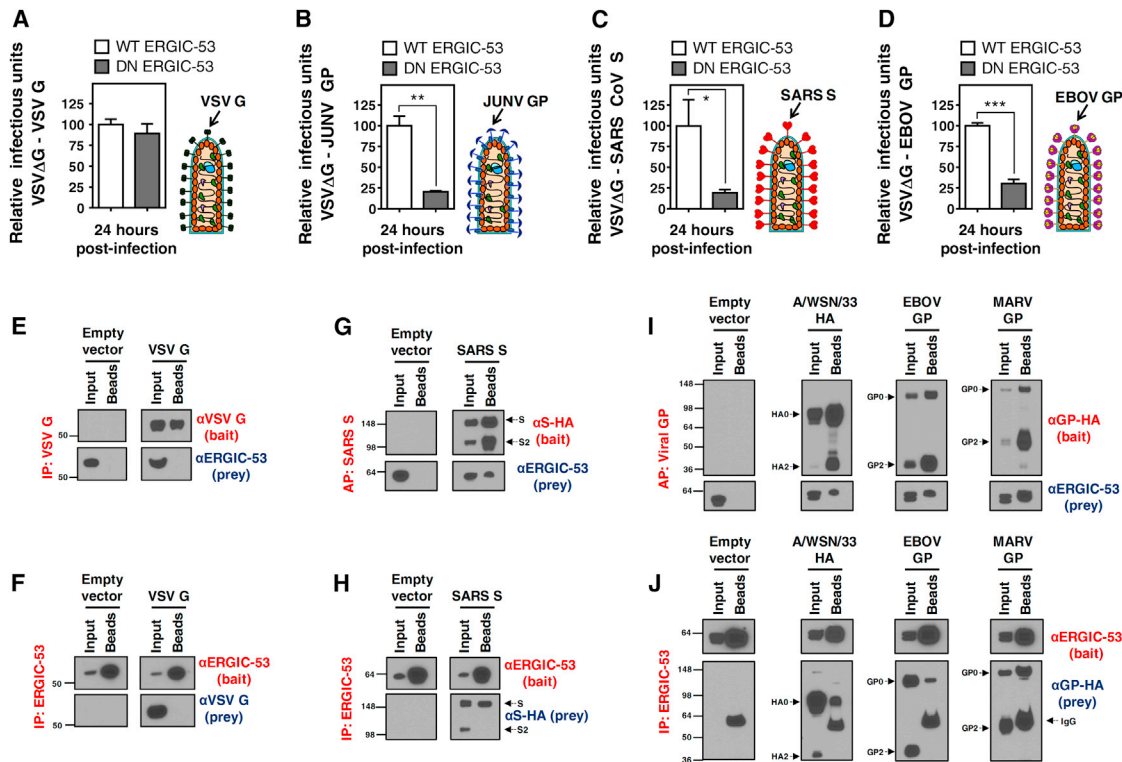
Figure 3. ERGIC-53 Broadly Associates with Class I Viral Fusion GPs and Influences the Propagation of JUNV, SARS CoV, and EBOV in a GP-Specific Manner

(A–D) ERGIC-53 is required for the production of infectious viral particles in a GP-specific manner. HEK293T cells were initially transfected with a plasmid encoding Myc-tagged WT or DN ERGIC-53, then 24 hr later with a plasmid encoding VSV G (A), JUNV XJ GP (B), SARS CoV S (C), or EBOV GP (D). Following the final transfection (24 hr), cells were challenged with VSVΔG. Supernatants and cell protein lysates were screened for infectious VSVΔG particles pseudotyped with the indicated viral GP via focus assay and Myc-ERGIC-53 (WT or DN) or CRT via western blot (see Figure S3 for blots), respectively. Data are presented as mean infectious units ± SEM relative to the WT ERGIC-53 vector-transfected wells and are representative of two independent experiments (n = 3 wells per condition per experiment). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, as determined using the unpaired Student's t test.

(E and F) ERGIC-53 does not associate with VSV G. HEK293T cells were transfected with a plasmid encoding VSV G or an empty plasmid, and either VSV G (E) or ERGIC-53 (F) was immunoprecipitated as bait from cell lysates (input). Immunoprecipitated bead fractions were screened for ERGIC-53 (E) or VSV G (F) as prey via western blot.

(G–J) ERGIC-53 has a conserved association with class I viral fusion GPs. HEK293T cells were transfected with a plasmid encoding WT ERGIC-53 and a pCAGGS plasmid encoding either SARS CoV S (G and H) or influenza A/WSN/33 HA, EBOV GP, or MARV GP (I and J) with a C-terminal HA epitope tag and BAP or an empty pCAGGs plasmid. In (G) and (I), cells were also transfected with the BirA plasmid to ensure biotinylation of each GP. Viral GPs (G and I) or ERGIC-53 (H and J) were affinity purified or immunoprecipitated, respectively, as bait from cell lysates. Input lysates and purified bead fractions were screened for ERGIC-53 (G and I) or viral GP species (full-length S and the processed S2 subunit for SARS CoV; full-length HA0 and the processed subunit HA2 for influenza virus A/WSN/33; and full-length GP0 and the processed GP2 subunit for EBOV and MARV) (H and J) as prey via western blot. Data are representative of two independent experiments.

JUNV to generate particles containing viral structural proteins or genome, but rather renders the particles themselves noninfectious, due in part to a defect in their ability to attach to permissive host cells.

ERGIC-53 Traffics to Sites of JUNV Assembly and Is Incorporated into Virions

Based on our finding that ERGIC-53 was detectable in concentrated supernatant preparations from JUNV C#1-infected cells (Figure 4D), we hypothesized that ERGIC-53 might be packaged into viral particles. We first addressed whether ERGIC-53 trafficking was altered during infection by surface labeling cells with antibodies specific for JUNV C#1 GP and ERGIC-53. We observed discrete JUNV C#1 GP puncta of ∼200–400 nm at the plasma membrane that we suggest are putative sites of viral

assembly and budding (Figure 5B). Strikingly, ERGIC-53 formed puncta of the same size, shape, and position as GP (Figure 5B). In contrast, ERGIC-53 was not detectable at the plasma membrane of uninfected cells (Figure 5B). This altered trafficking is specific, as calreticulin (CRT), another Ca^{2+}-binding lectin of the exocytic pathway, did not similarly redistribute to the plasma membrane following infection (Figure 5C). The intracellular distribution of ERGIC-53 did not change between mock- or JUNV C#1-infected cells (Figure 5A). We next captured JUNV particles using an anti-GPC/GP1 antibody and found that ERGIC-53 was detectable in JUNV particles via western blot (Figure 5D). ERGIC-53 also colocalized with JUNV NP in viral particles adhered onto coverslips (Figure S5). Thus, during arenavirus infection, ERGIC-53 traffics to sites of viral assembly and budding and is incorporated into virions.
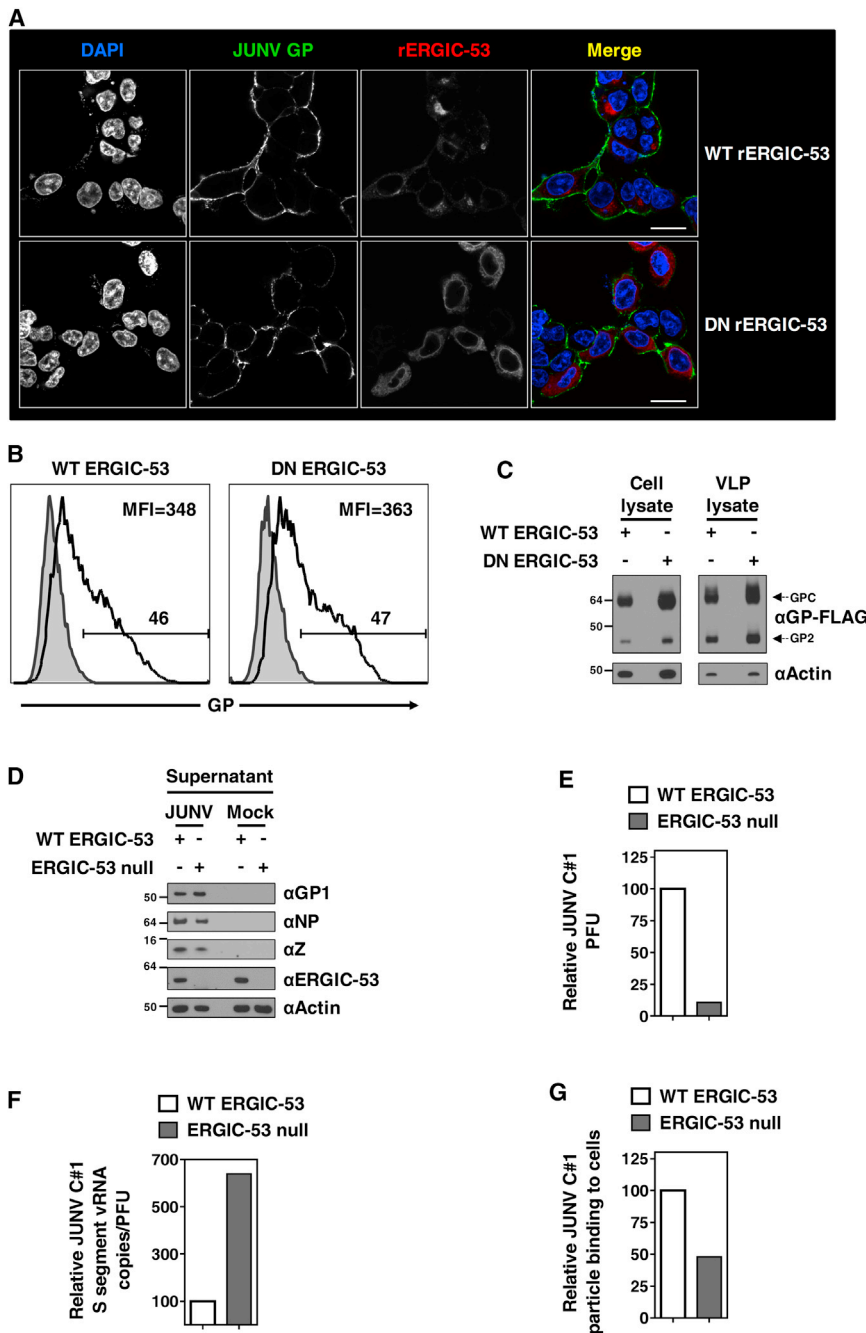
Figure 4. Loss of ERGIC-53 Function Does Not Inhibit the Formation of GP-Containing Arenavirus Particles, but Instead Renders Them Noninfectious

(A and B) The ER-restricted, DN ERGIC-53 does not impair trafficking of JUNV C#1 GP to the plasma membrane. HEK293T cells were transfected with a plasmid expressing WT or DN ERGIC-53, then inoculated 24 hr later with JUNV Candid #1 or not (mock), and collected 72 hr later to visualize internal rERGIC-53 and surface JUNV GP staining via confocal immunofluorescence microscopy (GP, green; Myc-ERGIC-53, red) (A) or fluorescence-activated cell sorting (FACS) (B). In (B), the histograms are gated on Myc-positive cells and show the percentage of transfected cells with GP staining (gray, mock infected; white, JUNV C#1 infected). The median fluorescence intensity (MFI) is reported. Scale bars = 20 μm. Surface expression of hTfR1 is not altered by DN ERGIC-53 (Figure S4).

(C) The DN ERGIC-53 mutant does not impair proteolytic processing of JUNV GPC or the incorporation of GP species into VLPs. HEK293T cells were transfected with a plasmid expressing WT or DN ERGIC-53, then 24 hr later with plasmids encoding the JUNV Z and XJ GPC proteins, respectively, to permit the formation of VLPs. Cells and supernatants (concentrated via ultracentrifugation through sucrose) were screened for the presence of various GP species (the C-terminally FLAG-tagged precursor GPC or proteolytically processed GP2) or actin via western blot. The data are representative of two independent experiments.

(D–G) JUNV C#1 generates GP-containing virus particles that are noninfectious in ERGIC-53 null cell lines. B cells derived from $LMAN1^{+/+}$ (2829-D) and $LMAN1^{-/-}$ (CRC-78) individuals were challenged with JUNV C#1 or not, and supernatants from these cells were concentrated through sucrose and screened for viral proteins (GP1, NP, and Z), ERGIC-53, or actin via western blot (D), JUNV C#1 PFU via plaque assay (E), the ratio of S segment genomic RNA copies, as measured by quantitative RT-PCR, to pfu (F), or attachment to host cells (G). All values are reported relative to the $LMAN1^{+/+}$ particles. Statistics are not shown because the values in (D)–(G) were derived from the same individual preparation of either $LMAN1^{+/+}$- or $LMAN1^{-/-}$-derived viral particles. For the attachment assay, virions were allowed to bind to cells at 4°C for 1.5 hr. Following washes to remove unbound particles, bound virus was enumerated on the basis of viral S segment genomic RNA copies detected via quantitative RT-PCR. The data in (D)–(F) are representative of two independent experiments.

The JUNV GP-ERGIC-53 Interaction Requires a Unique Region of ERGIC-53's Carbohydrate Recognition Domain and Occurs Independently of ERGIC-53's Ability to Oligomerize, Traffic, or Bind Mannose, MCFD2, or Ca²⁺

To determine the molecular basis for ERGIC-53's association with viral GPs, we screened a panel of ERGIC-53 mutants (described in detail in Figure S6 and in Zheng et al., 2010), for their ability to associate with the JUNV C#1 GP. As

shown in Figure 6, of the 11 mutants screened, only the ΔCRD mutant displayed a defect in binding to JUNV C#1 GP. Interestingly, despite the requirement of the carbohydrate recognition domain (CRD) for binding, the association of ERGIC-53 with JUNV C#1 GP does not appear to be lectin mediated, as mutations that disrupt ERGIC-53's ability to bind mannose (N156A, D181A, Δβ2, Δβ3, and Δβ4) do not disrupt its interaction with JUNV C#1 GP. Likewise, the
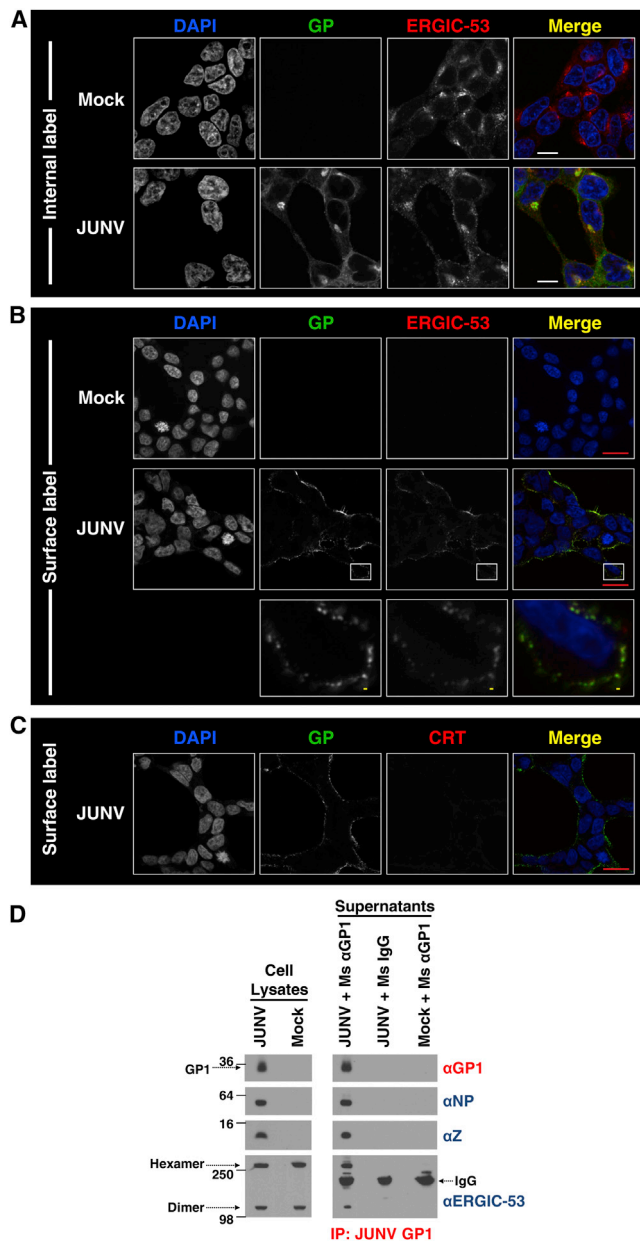
Figure 5. ERGIC-53 Traffics to Sites of Arenavirus Budding and Is Incorporated into Virions

(A–C) ERGIC-53 traffics to sites of arenavirus budding. JUNV C#1- or mock-infected HEK293T cells were screened for internal expression of JUNV GPC/GP1 and ERGIC-53 (A), surface expression of JUNV GPC/GP1 and ERGIC-53 (B), or, as a control, surface expression of JUNV GPC/GP1 and CRT (C) via confocal microscopy. Scale bars = 10 μm (white), 20 μm (red), and 300 nm (yellow).

(D) ERGIC-53 is a component of arenavirus particles. An anti-GP1 antibody was used to immunoprecipitate viral particles from supernatants of JUNV-infected or mock-infected Vero E6 cells. An irrelevant, species-matched antibody was also used for immunoprecipitation from the JUNV-infected supernatants. Cell lysates and immunoprecipitated protein fractions were screened for viral proteins (GP1, NP, and Z) and ERGIC-53 via western blot under nonreducing conditions. Data are representative of two independent experiments. ERGIC-53 was also detectable in JUNV C#1 particles via confocal microscopy (Figure S5).

association does not appear to require Ca^{2+} binding (N156A and D181A), oligomerization (ΔHM), the helix domain (ΔHelix), or the association of MCFD2 (Δβ1, Δβ2, Δβ3, Δβ4, and ΔHM). The ΔHM and DN ERGIC-53 (KKAA) results also suggest that trafficking beyond the ER is not required for the interaction. Finally, the ΔCRD, Δβ4, and ΔHelix results indicate that the GP-interacting domain on ERGIC-53 lies within the C-terminal 185 amino acids of the CRD (residues 84–269).

DISCUSSION

Arenaviruses and hantaviruses are significant human pathogens for which FDA-approved vaccines or effective antivirals do not exist. Their proteomes consist of only four proteins. While functional roles have been defined for each viral protein, their interactions with host proteins, and the importance of these interactions for viral replication and disease pathogenesis, remain largely unknown. In the current study, we addressed this deficiency by providing a comprehensive viral GP-human protein interactome map using GPs encoded by a representative arenavirus and hantavirus. We identified ERGIC-53 as a potential antiviral target based upon its ability to associate with GPs encoded by several families of pathogenic RNA viruses and its clear role in the propagation of arenaviruses, coronaviruses, and filoviruses. We demonstrate that ERGIC-53 is required not for the formation of GP-containing arenavirus particles, but rather for their infectiousness. We also show that ERGIC-53 traffics to sites of arenavirus budding and is incorporated into virions. Finally, we provide insight into the molecular basis for the GP-ERGIC-53 interaction by showing that the C-terminal region of ERGIC-53's CRD is required for the interaction independent of ERGIC's ability to oligomerize, traffic, or bind mannose, MCFD2, or Ca^{2+}.

ERGIC-53 is a nonglycosylated, hexameric type I integral membrane protein that functions as a cargo receptor for soluble glycoproteins within the early exocytic pathway. Its lumenal domain contains a CRD with homology to leguminous lectins and mammalian galectins; it selectively binds to high-mannose glycans in a pH- and Ca^{2+}-dependent manner (Appenzeller-Herzog et al., 2004; Appenzeller et al., 1999; Itin et al., 1996). Only five glycoproteins—FV and FVIII (Moussalli et al., 1999; Nichols et al., 1998), the cathepsins C and Z (Appenzeller et al., 1999; Vollenweider et al., 1998), and alpha-1 antitrypsin (Nyfeler et al., 2008)—have been shown to require ERGIC-53 for their efficient anterograde trafficking. Typically, ERGIC-53 captures its cargo proteins in the ER via its lectin activity and releases them in the ERGIC, presumably due to the lower pH of this compartment (Appenzeller-Herzog et al., 2004; Appenzeller et al., 1999). In the case of FV and FVIII, MCFD2 is also required for the trafficking of these proteins independent of ERGIC-53 (Zhang et al., 2003). Because ERGIC-53 and MCFD2 directly interact, it has been suggested that they form a mature cargo receptor required for efficient FV/FVIII trafficking (Nyfeler et al., 2006; Zhang et al., 2005). Importantly, while loss of ERGIC-53 expression or function impairs movement of its specific ligands, the overall architecture of the exocytic pathway is maintained, and major glycoproteins still traffic normally (Mitrovic et al., 2008; Nyfeler et al., 2006; Vollenweider et al., 1998). Indeed, humans with homozygous null mutations in ERGIC-53 or
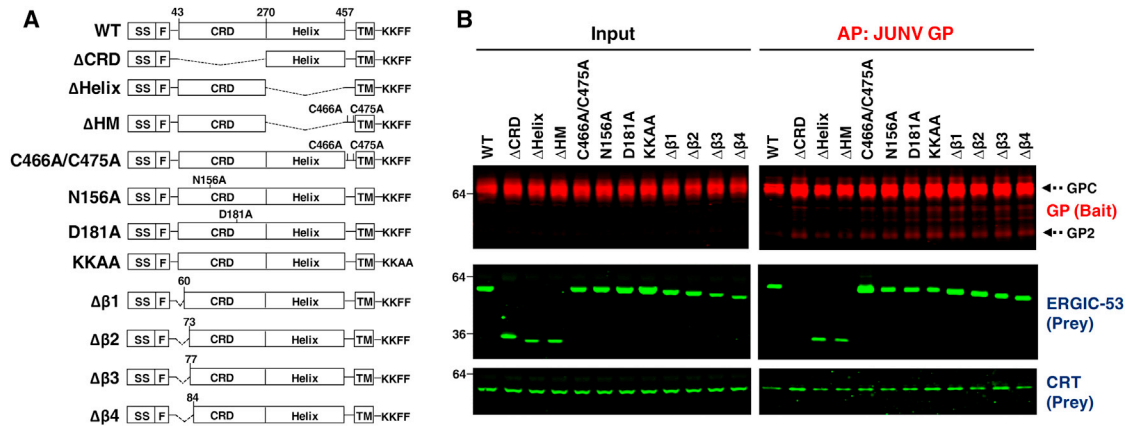
Figure 6. The C-Terminal Region of ERGIC-53's CRD Is Required for the ERGIC-53-JUNV GP Interaction

(A) Depiction of ERGIC-53 mutants used in this study. SS, signal sequence; F, FLAG epitope tag; TM, transmembrane. See Figure S6 for a detailed description of each mutant.

(B) HEK293T cells were cotransfected 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 tag. JUNV C#1 GP species (GPC and GP2) were affinity purified 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 two independent experiments.

MCFD2, despite having F5F8D—a condition that features mild to moderate bleeding symptoms due to reduced levels of circulating FV/FVIII (~5%–30% of normal) —are generally healthy and lead normal lives, provided they receive FV/FVIII supplementation following trauma (Khoriaty et al., 2012). These observations clearly suggest that ERGIC-53 is dispensable in humans and therefore represents a viable antiviral target.

Our studies demonstrate that ERGIC-53 associates with a class of pathogen-derived ligands, specifically GPs encoded by arenaviruses, hantaviruses, coronaviruses, orthomyxoviruses, and filoviruses (Figures 1, 3G–3J, S1E–S1I, and data not shown). We show that, with the exception of the hantavirus GPs, ERGIC-53 preferentially interacts with the uncleaved, precursor GP, but not the proteolytically processed GP subunits (Figures 1I–1K, 3H, 3J, S1F–S1H, and data not shown). In the case of the arenaviruses, this finding strongly suggests that the interaction takes place in the ER and/or ERGIC, prior to proteolytic cleavage of GPC into GP1/GP2 by the SKI-1/SP1 protease, which is thought to occur in the Golgi (Lenz et al., 2001; Wright et al., 1990). Indeed, imaging of JUNV C#1-infected cells revealed that GP and ERGIC-53 both concentrate in the ERGIC (Figures 1L and 5A). It was recently reported that the HIV glycoprotein Env can also associate with ERGIC-53 (Jäger et al., 2012).

Based on previous studies, we initially hypothesized that the ERGIC-53-GP interaction would be mediated by ERGIC-53's CRD binding to one or more high-mannose glycans on the viral GPs. Our results demonstrate that while ERGIC-53's CRD is indeed critical for the interaction, its lectin- and Ca^{2+}-binding functions are completely dispensable (Figures 6 and S6). Consistent with this claim, we also found the interaction to be unaffected by competition with free mannose, manipulation of Ca^{2+}, changes in pH (as low as 5.0), or deglycosylation of the JUNV or ANDV GP (data not shown). In summary, our studies reveal that the molecular basis for ERGIC-53's interaction with JUNV GP is different from any of its previously characterized partners; only the C-terminal region of ERGIC-53's CRD (residues 84–269) is critical for the interaction, whereas ERGIC-53's ability to oligomerize, traffic, or bind mannose, Ca^{2+}, or MCFD2 are not.

Movement of ERGIC-53 within the exocytic pathway is controlled by at least three targeting determinants that work in concert with two types of vesicular coats (COPII and COPI) to mediate ER retention, ER exit, and retrieval from post-ER compartments (Hauri et al., 2000; Nufer et al., 2003). ERGIC-53 preferentially accumulates in the ERGIC and recycles between this compartment and the ER (Ben-Tekaya et al., 2005; Klumperman et al., 1998). Under normal conditions, ERGIC-53 does not appear to traffic beyond the cis-Golgi. However, following its overexpression via plasmid, ERGIC-53 can traffic to the plasma membrane, presumably due to saturation of COPI (Kappeler et al., 1994). JUNV C#1 infection induces a striking redistribution of a portion of the intracellular pool of ERGIC-53 to the plasma membrane, where it strongly colocalizes with GP at putative sites of viral assembly and budding (Figure 5B). Furthermore, ERGIC-53 is packaged into arenavirus particles (Figures 5D and S5), which may indicate that it is required for virion structure and function.

The mechanism by which ERGIC-53 traffics to the plasma membrane during JUNV C#1 infection is unclear, but one possibility is that ERGIC-53 expression increases during JUNV infection and that saturation of COPI allows ERGIC-53 to traffic, perhaps independent of its interaction with JUNV GP, beyond the ERGIC and cis-Golgi to reach the plasma membrane. In support of this idea, infection with the related arenavirus LCMV triggers the activating transcription factor 6 (ATF6) arm of the unfolded protein response (UPR) (Pasqual et al., 2011), which is known to increase ERGIC-53 expression (Nyfeler et al., 2003). Indeed, LCMV infection of nonhuman primates results in increased transcription of ERGIC-53 (Djavani et al., 2009). Alternatively, it is also possible that JUNV GP functions as a cargo receptor to facilitate the movement of ERGIC-53
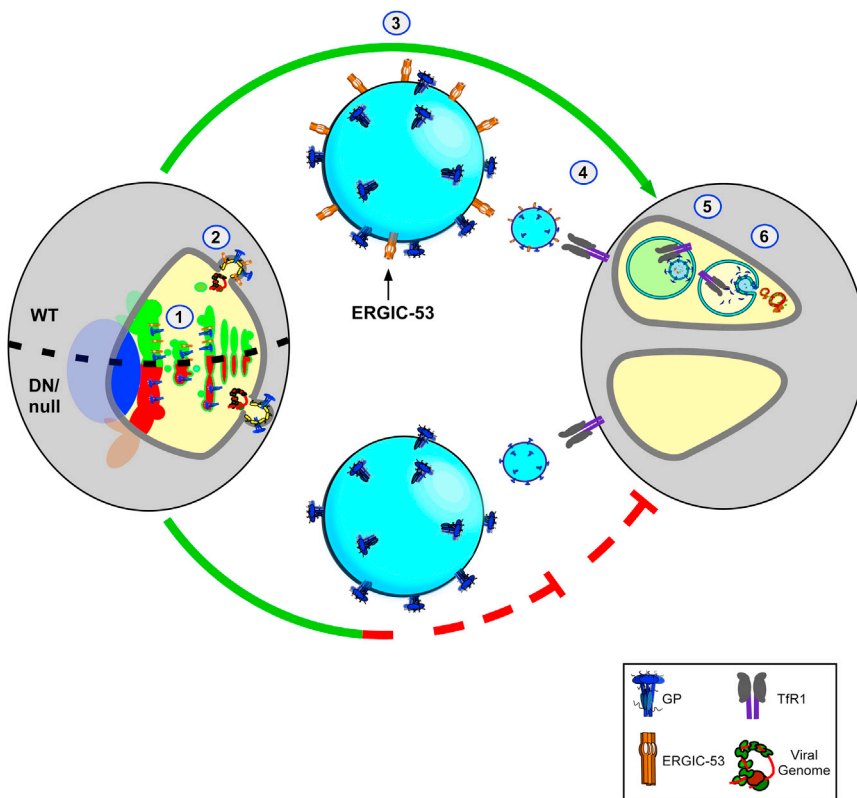
Figure 7. Proposed Model Depicting the Role of ERGIC-53 in JUNV Propagation

Under WT conditions (top half of left cell), the arenavirus GP undergoes a series of maturation steps within the early exocytic pathway (1), including proteolytic cleavage and trafficking to the plasma membrane, where it is incorporated into newly forming viral particles (2) that bud out of the cell (3). The GP on newly formed particles then attaches to its cellular receptor (4), which permits endocytic uptake of particles into endosomes (5), where low pH leads to GP2-mediated fusion of the viral and endosomal membranes and, ultimately, release of viral genome into the cytoplasm (6). In the absence of ERGIC-53 (null) or in the presence of the ER-restricted, DN ERGIC-53 (DN) (bottom half of left cell), GP is still proteolytically processed, trafficked to the plasma membrane, and incorporated, along with other viral structural proteins and viral genome, into budding particles (1–3). These particles, however, lack ERGIC-53 and are defective in their ability to attach to host cells (4). They may have deficiencies in other early replication events (5 and 6) as well.

beyond the ERGIC and *cis*-Golgi to sites of viral assembly and budding.

How does ERGIC-53 impact arenavirus replication? We initially hypothesized that ERGIC-53 was acting as a bona fide cargo receptor required for the anterograde movement of GP out of the ER and ultimately to the plasma membrane. This idea proved incorrect, as expression of the ER-restricted, DN ERGIC-53 mutant had no impact on the ability of GP to reach the plasma membrane (Figures 4A and 4B). Likewise, there was no disruption of either the proteolytic processing of GPC into GP1/GP2 or the ability of these GP species to be incorporated into VLPs (Figure 4C). Additionally, the DN ERGIC-53 mutant had no impact on the ability of WT JUNV C#1 particles to enter cells (Figures 4A and 4B) or on the level of expression of hTfR1 at the plasma membrane (Figure S4). To formally test whether ERGIC-53 is required for the release of viral particles, we challenged normal or ERGIC-53 null cell lines with JUNV C#1 and found similar quantities of viral particles in the supernatants from each cell line (Figure 4D). However, despite equivalent particle release, the null cell-derived particles were ∼10-fold less infectious (Figure 4E), demonstrating that ERGIC-53 is essential for the infectivity of JUNV C#1 particles. Therefore, in the absence of ERGIC-53, arenavirus particles are produced in normal quantities but are defective in the early phase of replication. Our results suggest that this defect minimally exists at the level of virus attachment to the host cell (Figure 4G). It is possible that this defect may also impair other steps of viral entry, such as endocytic uptake of particles into host cells and/or fusion and release of genome into the cytoplasm (see Figure 7 for our proposed model).

How does ERGIC-53 mechanistically impact the infectiousness of arenavirus particles? ERGIC-53 itself may be a critical structural component of the virion, perhaps by acting as a coreceptor required for virion attachment to host cells. Direct support for this idea includes our finding that ERGIC-53 is a component of virions (Figures 5D and S5) and that virions lacking ERGIC-53 are defective (Figures 4D–4G). Additionally, less infectious virus was produced in our challenge studies featuring the ER-restricted, DN ERGIC-53 mutant (Figures 2C, 2D, 3B, S2C, S2D, and S3B) or ERGIC-53 siRNA (Figures 2A and S2A). Conversely, overexpression of WT ERGIC-53 leads to increased trafficking of ERGIC-53 to the plasma membrane (Kappeler et al., 1994), which could lead to more ERGIC-53 being incorporated into particles and explain the increased release of infectious virus seen under these conditions (Figures 2B and S2B). Alternatively, ERGIC-53 could be required to traffic and/or recruit cellular proteins that are critically required for virion structure and function or for the proper maturation of the arenavirus GP (e.g., glycan maturation or other posttranslational modifications).

The protein partners of arenavirus and hantavirus GPs identified in this study help advance our understanding of how these viruses interact with host cell machinery to facilitate GP biogenesis and other aspects of the viral lifecycle. As the GPs themselves are likely to be highly multifunctional due to the small size of their respective proteomes, the identified partners may also help elucidate additional functions for each GP. Each partner represents a candidate target for future antiviral screening. Indeed, four additional proteins identified in our study—stromal cell derived factor 4 (SDF4), archain 1 (ARCN1), coatomer protein complex subunit alpha (COPA), and renin receptor (ATP6AP2)—were recently shown to be required for LCMV and VSV replication (Panda et al., 2011). These examples clearly highlight the feasibility and utility of

using a proteomics-based approach to identify candidate antiviral targets.

In conclusion, ERGIC-53 represents a potential antiviral target because of its clearly demonstrated importance for the replication of pathogenic arenaviruses, coronaviruses, and filoviruses and the fact that loss of this protein or its function is well tolerated in humans (Khoriaty et al., 2012). Furthermore, in the case of the arenaviruses, targeting ERGIC-53 function with an antiviral could be expected to set up an ongoing immunizing therapy, as defective, but presumably immunogenic, viral particles would be released during the course of treatment. While ERGIC-53 represents a potential broad-spectrum antiviral target for arenaviruses, coronaviruses, and filoviruses, it may also be required for additional human pathogens, such as the New World hantaviruses, orthomyxoviruses, or retroviruses, based upon its conserved interaction with their GPs (Figures 1D, 1E, 3I, 3J, and S1I) (Jäger et al., 2012), or for DNA viruses, based on the finding that a murine gamma herpes virus was negatively impacted by silencing of ERGIC-53 (Mages et al., 2008). Based on our finding that JUNV propagation is impaired in cells from ERGIC-53 null individuals, future studies should also address whether exposure to rodent-borne viruses, such as the arenaviruses, has exerted a selective pressure to maintain ERGIC-53 mutations within the human population as a means to confer resistance to infection. Additionally, while bleeding is not a major cause of morbidity or mortality during arenavirus or hantavirus infection, it is interesting to consider that viral GPs, by interacting with ERGIC-53, may disrupt ERGIC-53's normal cargo receptor function for FV and FVIII, contributing to some of the hemorrhagic manifestations seen following infection with these viruses, which can include deficiencies in the levels and/or activity of circulating FV or FVIII (Lee, 1987; Lee et al., 1989; Schwarz et al., 1972). The interaction of ERGIC-53 with the filovirus GPs is particularly intriguing, considering the prominent coagulation abnormalities observed during human infection (Feldmann and Geisbert, 2011). Finally, our studies and others (Gonzalez-Begne et al., 2009) have shown that ERGIC-53 is actively secreted from cells in viral particles and/or cellular exosomes and strongly suggest that it has important roles outside of its normal distribution within the exocytic pathway, perhaps outside of the cell where, in the case of arenaviruses, coronaviruses, and filoviruses, it may influence the endocytic pathway-driven process of viral entry.

EXPERIMENTAL PROCEDURES

Cells, Viruses, Antibodies, Plasmids, siRNAs, and Transfections

A full description of the cells (human embryonic kidney 293T [HEK293T] cells, Vero E6 cells, and B lymphoblastoid cells from either normal or ERGIC-53 null individuals), viruses (DANV, JUNV C#1, and VSVΔG), antibodies, siRNAs, plasmids, and transfection procedures used can be found in the Supplemental Experimental Procedures.

Affinity Purification, Immunoprecipitation, Mass Spectrometry, Virus and VLP Concentration, and Western Blot

To affinity purify viral GPs for the identification of human protein partners via mass spectrometry or validation of protein partners via western blot, HEK293T cells were cotransfected with a plasmid that encodes each respective viral GP with a C-terminal HA epitope tag and a biotin acceptor peptide (BAP) as well as a second plasmid that encodes the bacterial biotin ligase BirA to facilitate biotinylation of the viral GPs. After 2 days, biotinylated GPs and associated host proteins were affinity purified from whole-cell lysates using magnetic streptavidin beads and separated on polyacrylamide gels for either western blot analysis to confirm bait and prey purification or Coomassie staining for mass spectrometry analysis. To determine the identity of cellular proteins captured, each Coomassie-stained gel lane was cut into sections for in-gel, tryptic digestion, and mass spectrometry analysis.

Immunoprecipitation of viral GPs or ERGIC-53 was accomplished by incubating clarified whole-cell protein lysates with antibodies specific for each respective protein followed by magnetic protein G beads. To purify intact JUNV C#1 particles, supernatants were collected at 72 hr postinoculation, clarified, and incubated with either an anti-GP antibody or an isotype control antibody followed by magnetic protein G beads. Immunopurified proteins or viral particles were then washed, eluted from beads, and electrophoresed on polyacrylamide gels for western blot analysis.

JUNV C#1 virions and VLPs were concentrated via ultracentrifugation through a 20% layer of sucrose.

For western blot analysis, 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 from Invitrogen. Proteins were detected using either chemiluminescence or an Odyssey Infrared Imaging System (LI-COR Biosciences).

Full details regarding these approaches are described in the Supplemental Experimental Procedures.

Confocal Immunofluorescence Microscopy

A Zeiss LSM 510 Laser Scanning Confocal Microscope was used to visualize internal or surface expression of ERGIC-53, CRT, JUNV NP, and/or JUNV GP in cells or virions. Colocalization analysis was done using the Zeiss AIM software package as described in the Supplemental Experimental Procedures.

Flow Cytometry

An LSRII (BD Biosciences) was used to enumerate the frequency and intensity of JUNV GP or hTfR1 staining at the plasma membrane or Myc-tagged WT and/or DN ERGIC-53 internally in HEK293T cells, as described in the Supplemental Experimental Procedures.

Viral Challenge Assays

Viral challenge assays were performed to evaluate 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 influence the release of infectious JUNV C#1, DANV, or VSVΔG (pseudotyped with VSV G, JUNV XJ GP, SARS CoV S, or EBOV GP). 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. Infectious virus load was determined via plaque assay for JUNV C#1 and DANV, while GFP-positive foci were enumerated via focus assay for VSVΔG; differences were determined using the unpaired Student's t test. Quantitative RT-PCR was used to determine copy number of JUNV C#1 S segment genomic viral RNAs. Virus attachment to cells was determined through a virus-cell binding assay. A full description of each challenge assay can be found in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.10.010.

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