Mechanistic understanding of N-glycosylation in Ebola virus glycoprotein maturation and function

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The Ebola virus (EBOV) trimeric envelope glycoprotein (GP) precursors are cleaved into the receptor-binding GP1 and the fusion-mediating GP2 subunits and incorporated into virions to initiate infection. GP1 and GP2 form heterodimers that have 15 or two N-glycosylation sites (NGSs), respectively. Here we investigated the mechanism of how N-glycosylation contributes to GP expression, maturation, and function. As reported before, we found that, although GP1 NGSs are not critical, the two GP2 NGSs, Asn563 and Asn618, are essential for GP function. Further analysis uncovered that Asn563 and Asn618 regulate GP processing, demannosylation, oligomerization, and conformation. Consequently, these two NGSs are required for GP incorporation into EBOV-like particles and HIV type 1 (HIV-1) pseudovirions and determine viral transduction efficiency. Using CRISPR/Cas9 technology, we knocked out the two classical endoplasmic reticulum chaperones calnexin (CNX) and/or calreticulin (CRT) and found that both CNX and CRT increase GP expression. Nevertheless, NGSs are not required for the GP interaction with CNX or CRT. Together, we conclude that, although Asn563 and Asn618 are not required for EBOV GP expression, they synergistically regulate its maturation, which determines its functionality.

The 2014 outbreak of Ebola hemorrhagic fever in West Africa has resulted in the deaths of over 11,000 people and represents the worst epidemic of this disease in history (1). Ebola hemorrhagic fever is caused by Ebola viruses (EBOVs),4 members of Filoviridae, which can have a 90% rate of mortality in humans and nonhuman primates (2). Filoviridae is composed of two genera: EBOVs and Marburg viruses, which are enveloped and have single, non-segmented, negative-sense RNA genomes with a size of ~19 kb (3). So far, five EBOV species have been identified, including Zaire, Sudan, Bundibugyo, Tai Forest, and Reston, which share ~60% amino acid identity. All of these isolates can cause diseases in humans, except for the Reston strain, which is pathogenic in nonhuman primates; the Zaire, Sudan, and Bundibugyo strains have been very pathogenic in humans (2).

The EBOV genome encodes seven structural proteins and one non-structural protein (3). As the only structural component on the viral surface, the glycoprotein (GP) is responsible for viral entry and initiation of infection (4, 5). EBOV GPs are class I viral fusion proteins, a classification category that also includes influenza viruses, paramyxoviruses, and retroviruses (6). EBOV GP is unique because it also functions as an important virulence factor that induces cytotoxic effects both in vivo and in vitro (7). Thus, EBOV has evolved an RNA-editing mechanism to regulate its GP expression through two open reading frames (8, 9). Most transcripts (80%) are not RNA-edited and thus produce a truncated, secreted GP because of a premature stop codon that plays a role in immune evasion (10). The remaining transcripts are edited, which produces the full-length precursor GP (GPpre) in the endoplasmic reticulum (ER). GPpre is further processed into a fully glycosylated uncleaved GP0 that assembles into trimers in the ER. Each GP0 subunit is then cleaved by the convertase furin in the trans-Golgi to produce the surface subunit GP1 (~130 kDa) and transmembrane subunit GP2 (~24 kDa), which are linked by a disulfide bond (11). Although the incorporation of GP trimers into viral particles is necessary for infection, many of these trimers are released into culture medium because of cleavage of the GP2 transmembrane anchor by tumor necrosis factor α-converting enzyme (12).

The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains supplemental Table 1.

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4 The abbreviations used are: EBOV, Ebola virus; GP, glycoprotein; GPpre, precursor glycoprotein; ER, endoplasmic reticulum; MLD, mucin-like domain; RIPA, radioimmune precipitation analysis.

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GP₁ is divided into core domain and mucin-like domain (MLD), and the core domain is further divided into the receptor-binding domain (RBD) and glycan cap (Cap) regions in FL GP are indicated. Numbers indicate amino acid residues. NGSs are marked as Y, B, FL-GP and ΔMLD-GP expression in cells and their incorporation into HIV-1 virions were detected by Western blotting. GP₁awa EBOV GP precursor; p24 and p55, HIV-1 Gag antigens. GP proteins were detected by a FLAG antibody, and actin and HIV-1 Gag proteins were detected by specific antibodies. C, comparison of GP-mediated HIV-1 transduction. Virions were produced from 293T cells after transfection with the pNL-LucΔEnv and ΔMLD-GP or FL-GP expression vectors. Vero cells were infected with these viruses after normalization of the p24 Gag proteins, and viral transduction efficiency was quantitated by measuring the cellular luciferase activities. Results are presented as relative values. Error bars represent standard deviations calculated from three experiments.

Results

The role of Asn⁵⁶³/Asn⁶¹⁸ in GP processing and virion incorporation

To reduce cytotoxicity, we used MLD-deleted (ΔMLD) GP in most of our experiments to study GP activity. We first compared ΔMLD-GP expression and activity with the full-length (FL) GP. All GP proteins were fused to an N-terminal FLAG tag, so their expressions were detectable with an anti-FLAG antibody. 293T cells were transfected with an FL-GP or ΔMLD-GP expression vector plus a ΔEnv HIV-1 proviral vector expressing the firefly luciferase report gene (pNL-LucΔEnv). After 48 h, pseudovirions were collected and quantified, and the viral transduction efficiency was measured by infecting Vero cells. In addition, transected cells as well as pseudovirions were lysed, and the levels of viral protein expression were determined by Western blotting.

As reported, we found that, because of the heavy O- and N-glycosylation of the MLD in the Golgi, FL-GP₁ exhibited a higher molecular weight than FL-GP₁, whereas ΔMLD-GP₁ exhibited a lower molecular weight than ΔMLD-GP₁ (Fig. 1B). In addition, ΔMLD-GP exhibited higher levels of protein expression and were more effectively processed and pack-
aged into HIV-1 particles than FL-GP (Fig. 1B). Moreover, ∆MLD-GP dramatically increased HIV-1 transduction compared with FL-GP (Fig. 1C). These results demonstrate that ∆MLD-GP provides a more effective and convenient system to study GP biochemistry and function.

Next, each of the nine NGSs on ∆MLD-GP were deglycosylated by replacing Asn with Gln, creating nine mutants: N40Q, N204Q, N228Q, N238Q, N257Q, N296Q, N563Q, and N618Q. When their expression in cells and HIV-1 pseudovirions was determined, it was found that none of these single mutations reduced GP expression and processing, except for N563Q and N618Q, which reduced processing (Fig. 2A, lanes 1–10). In addition, both the N618Q and N563Q mutations dramatically reduced GP incorporation (Fig. 2B, lanes 9 and 10).

To continue this investigation, a number of combinative and accumulative mutations were created. For example, N40Q/204Q, N296Q/563Q, and N563Q/618Q have the two indicated NGSs mutated; N40Q-228Q, N40Q-238Q, N40Q-257Q, N40Q-268Q, N40Q-296Q, N40Q-563Q, N40Q-618Q, N257Q-618Q, and N257Q-296Q have three to nine NGSs mutated. A more detailed description of these mutations is provided in supplemental Table 1. It was found that the five N-terminal NGSs (Asn40 to Asn257) were not required for GP cleavage (Fig. 2A, lanes 11–16). However, when the accumulated mutations were extended to Asn257 and beyond, GP1 expression either decreased or became undetectable in both cells and virions (Fig. 2, A and B, lanes 17–20). When the five C-terminal NGSs (Asn257, Asn268, Asn296, Asn563, and Asn618) were mutated (N257Q–N618Q), GP1 became undetectable in cells and virions (Fig. 2, A and B, lane 23); when Asn663 and Asn618 were mutated (N563Q/N618Q), GP1 showed the same defect (Fig. 2, A and B, lane 25). Together, these results demonstrate that Asn563/Asn618 are crucial for EBOV GP proteolytic cleavage and incorporation.

Role of Asn563/Asn618 in GP demannosylation

During N-glycosylation, the preassembled oligosaccharide (Glc3Man9GlcNAc2) needs to be further processed after being attached to NGSs. Among the nine Man residues, four are α1,2-linked and are sequentially removed by class I α-mannosidases. The other five are either α1,3- or α1,6-linked, and two of these are removed by a class II α-mannosidase. Endoglycosidase H (Endo H) cleaves each structure of these oligosaccharides as they are processed until the class II enzyme removes the two Man residues. Thus, Endo H-sensitive and -resistant glycoproteins contain high-Man or low-Man sugars, respectively.

We used Endo H treatment to determine how NGSs regulate GP demannosylation. As expected, GPpre from both ∆MLD-GP and FL-GP were sensitive to Endo H because it contains unprocessed Man residues; GP1 was resistant to Endo H.
The role of Asn563/Asn618 in GP oligomerization

To understand whether the N563Q and N618Q mutations have altered the GP conformation, we used flow cytometry to determine GP interaction with two conformation-dependent anti-EBOV GP mAbs, KZ52 (21) and 13C6 (22). We first used a conformation-independent anti-FLAG antibody to detect WT, N563Q, N618Q, and N563Q/N618Q ΔMLD GP proteins and found a similar level of surface expression (Fig. 4, A and B). However, the N563Q mutation reduced KZ52 and 13C6 binding by 25% or 50%, and the N618Q mutation also did so at relatively modest levels (Fig. 4, A and B). These results are consistent with a previous report (17), suggesting that Asn563/Asn618 are important for maintaining the natural conformation.

Next we tested whether Asn563 and Asn618 play a role in GP oligomerization. Asn563 is located in the proximity of the trimer interface and may play a role in trimer stabilization (23, 24). The Asn618 glycan is an unusual sugar that is present in the HR2 region, and the currently solved crystal structures of GP have not extended to this region. However, it is possible that this sugar might create some steric hindrance to trimer formation or stabilization. ΔMLD-GP and its mutants N563Q, N618Q, or N563/N618Q were expressed in 293T cells, and soluble GPss were purified from the culture medium. It was found that a single monomer GP band was detected at a similar level in the reducing gel, indicating that Asn563 and Asn618 are not required for GP shedding (Fig. 4C). However, a striking difference was found in the non-reducing gel. Trimmers and monomers were detected from ΔMLD-GP proteins at a ratio of 1.75:1, monomers were exclusively detected from N563Q, trimers were exclusively detected from N618Q, and trimmers and monomers were also detected from N563Q/N618Q but at a ratio of 0.44:1 (Fig. 4, C and D). These results demonstrate that Asn563 increases, whereas Asn618 inhibits, trimer formation, and the N563Q/N618Q double mutation results in an overall reduction in trimer formation.

Role of CNX/CRT in GP expression

CNX is a type I transmembrane protein with 592 amino acids, and CRT is its soluble paralog, sharing ~39% sequence.

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**Figure 3. Effect of demannosylation on EBOV function.** A, analysis of GP sensitivity to Endo H. 293T cells were transfected with the indicated GP expression vectors. Cell lysate was either treated with Endo H or left untreated and analyzed by Western blotting. B, KIF increases GP sensitivity to Endo H. 293T cells transfected with the ΔMLD-GP expression vector were treated with 50 μM KIF or remained untreated. Lysate from these cells was treated with Endo H or left untreated and analyzed by Western blotting. C, KIF inhibition of GP-mediated HIV-1 transduction. 293T cells producing HIV-1 luciferase reporter viruses pseudotyped with EBOV ΔMLD-GP were treated with the indicated amounts of KIF. Viral transduction was measured by infecting Vero cells. Error bars represent standard deviation from three experiments. D, KIF does not affect GP expression in HIV-1 virions. HIV-1 pseudovirions produced in C were purified by ultracentrifugation, and viral protein expression in these virions was determined by Western blotting.
identity with 417 amino acids. Their role in EBOV GP biosynthesis has not been tested.

First, we determined whether EBOV GP expression depends on CNX/CRT. The CNX and/or CRT genes were knocked out from 293T cells using CRISPR/Cas9 technology (Fig. 5A), generating two single KO cell lines, ΔCNX and ΔCRT, and one double KO cell line, ΔΔ (Fig. 5B). When EBOV GP expression was determined in these KO cells, it was found that GP expression was reduced in both ΔCNX and ΔCRT cells, and the expression was further reduced in ΔΔ cells (Fig. 5C). In addition, when CNX and CRT expression were gradually restored in ΔΔ cells by co-transfection with their expression vectors, GP expression was increased in a dose-dependent manner (Fig. 5, D and E). These results demonstrate that both CNX and CRT increase EBOV GP expression.

Second, we determined whether EBOV GPs interact with CNX or CRT. ΔMLD-GPs were expressed in WT, ΔCNX, ΔCRT, and ΔΔ cells, and protein complexes were pulled down using anti-FLAG antibodies. It was found that the endogenous CNX could be pulled down from WT and ΔCRT but not ΔCNX and ΔΔ cells (Fig. 6A). These results demonstrate that GPs interact with CNX in a CRT-independent manner. To detect the interaction with CRT, these cells were co-transfected with a CRT expression vector, and the experiment was repeated. It was found that, although similar levels of CRT were detected in these cells, CRT could only be pulled down from cells expressing GPs (Fig. 6B). In addition, more CRT proteins were pulled down from WT and ΔCNX than ΔCRT and ΔΔ cells. These results demonstrate that GPs also interact with CRT in a CNX-independent manner.
The role of Asn\textsuperscript{563}/Asn\textsuperscript{618} in GP-mediated HIV-1 transduction

To understand how these mutations affect GP function, we determined the transduction efficiency of HIV-1-EBOV GP particles produced from 293T WT, ΔCNX, ΔCRT, or ΔΔ cells. Viruses were pseudotyped with FL-GP or ΔMLD-GP bearing N40Q–N618Q, N40Q–N238Q, N563Q, N618Q, or N563Q/N618Q mutations. First, we analyzed how CNX/CRT affects GP function. It was found that the transduction efficiency by ΔMLD GP was synergistically reduced by CNX and CRT KO (Fig. 8A). This result confirms the important role of CNX/CRT in EBOV GP biosynthesis. Second, we analyzed how these glycans affect GP function in WT cells. It was found that the N40Q–N238Q and N563Q/N618Q mutations increased, the N618Q mutation decreased, and the N40Q–N618Q and N563Q/N618Q mutations almost completely disrupted the FL-GP and ΔMLD-GP activities (Fig. 8, B and C). Last, we analyzed how these glycans affect GP function in CNX and/CRT KO cells. Similar results were found in these KO cells as in WT cells. For example, the N40Q–N238Q and N563Q mutants showed a comparable activity to the WT protein, which was higher than the N618Q mutant, and both the N40Q–N618Q and N563Q/N618Q mutants almost completely lost their activities (Fig. 8C). In addition, the difference between the active and inactive mutant activities was much smaller in double KO than in single KO cells (Fig. 8C). Together, these results support that Asn\textsuperscript{563} and Asn\textsuperscript{618} synergistically regulate EBOV GP maturation, which determines its function.

Discussion

In this report, we have collected new insight into how Asn\textsuperscript{563} and Asn\textsuperscript{618} contribute to the functionality of EBOV GP. \textsuperscript{Gp1} is divided into MLD, RBD, and glycan cap domain (Fig. 1A). Its crystal structure shows that the heavily glycosylated glycan cap and MLD surround the RBD, and a thick layer of oligosaccharide cloaks most of the structure (24). It was suggested that N-linked glycans attached to \textsuperscript{Gp1} might not be absolutely required for GP folding and that their function could be complemented by the other glycans in the MLD (16). When we deleted these N-linked glycans in ΔMLD-GP (N40Q–N296Q), the glycan-null N40Q–N618Q mutant was poorly expressed or incorporated, N40Q–N238Q mutant expression, processing, and incorporation were not affected, and the N563Q and N618Q mutations synergistically reduced FL-GP processing and incorporation into E-VLPs (Fig. 7, lanes 1–6). These results further confirm the important role of Asn\textsuperscript{563} and Asn\textsuperscript{618} in GP processing and incorporation.

We also performed similar experiments in CNX and/or CRT KO cells after expressing VP40 and ΔMLD-GP bearing N40Q–N618Q, N40Q–N238Q, N563Q, N618Q, or N563Q/N618Q mutations. In general, much lower levels of GP expression were detected from these KO cells, particularly in double KO cells, which confirms the requirement of CNX/CRT for GP expression (Fig. 7, lanes 7–30). In addition, GP processing and incorporation were more strongly inhibited by these mutations in these KO cells, particularly in the double KO cells. Together, these results further demonstrate the important role of Asn\textsuperscript{563} and Asn\textsuperscript{618} in GP processing and incorporation.

Role of Asn\textsuperscript{563}/Asn\textsuperscript{618} in FL-GP processing and incorporation

We have shown that N563Q/N618Q mutations synergistically reduce ΔMLD-GP processing and incorporation into HIV-1 virions. We then used FL-GP and EBOV-like particles (E-VLPs) to confirm these observations.

FL-GP and its mutants N40Q–N618Q, N40Q–N238Q, N563Q, N618Q, or N563Q/N618Q were expressed with the EBOV matrix protein VP40 in 293T WT cells, and E-VLPs were purified from the culture supernatants by ultracentrifugation. After that, viral protein expression in cells and E-VLPs was determined by Western blotting. As shown previously in Fig. 2,
we found that not only were the total levels of GP expression reduced, but GP processing and virion incorporation were also inhibited (Fig. 2, A, lane 18, and B, lane 18). Thus, our results demonstrate that these N-linked glycans become indispensable for EBOV GP expression and function when MLD is not present, confirming that the glycan cap and MLD play a complementary role in GP folding.

Unlike GP₁, and independent of the MLD, N-linked glycans in GP₂ are required for EBOV GP function. Recent reports investigating the removal of NGSs from the FL-GP backbone show that, although an N563D mutation can enhance viral entry by 2-fold and an N618D mutation can decrease viral entry by 2-fold, an N563D/N618D double mutation is able to completely disrupt viral entry (17). Consistently, we also found that...
viral entry was slightly increased by an N563Q mutation, slightly decreased by an N618Q mutation, and almost completely disrupted by an N563Q/N618Q mutation (Fig. 8). Thus, there is conclusive evidence that glycans at Asn563 impede but glycans at Asn618 enhance viral entry, but simultaneous deglycosylation of both residues completely disrupts viral entry.

Although EBOV GP has a thick coating of oligosaccharides, there are vulnerable regions that are exposed to neutralizing antibodies. For example, the neutralizing antibodies KZ52 and 16F6 bind to both GP1 and GP2 in their prefusion complex, which may prevent their conformational rearrangements (23, 24). Their GP1 epitope has been mapped to residues 42–44, and their GP2 epitopes are in the internal fusion loop and HR1. Asn563 and Asn618 are located in HR1 or HR2, respectively. As reported before (17), we found that Asn563 and/or Asn618 deglycosylation reduced GP binding to KZ52 and another neutralizing antibody, 13C6 (Fig. 4, A and B). Thus, glycans at Asn563 and Asn618 should play an important role in GP conformational changes from pre-fusion to post-fusion state.

To understand more about Asn563 and Asn618, we performed several biochemical experiments. First, we found that both the N563Q and N618Q single mutations reduced, but the N563Q/N618Q double mutation completely blocked, GP protease cleavage (Figs. 2A and 7). Second, we found that the N563Q, N618Q, and N563Q/N618Q mutations all reduced GP incorporation (Figs. 2B and 7). Third, we found that the N563Q mutation strongly inhibited, but the N618Q mutation strongly promoted, GP trimerization; the N563Q/N618Q mutation caused a mixed phenotype by reducing trimers and increasing monomers (Fig. 4C). Nevertheless, they are not required for interaction with the ER chaperones CNX/CRT (Fig. 6). Together, these results further demonstrate that Asn563/Asn618 are indeed important for the maintenance of crucial GP conformations for infection.

We also found that the N563Q, N618Q, and N563Q/N618Q mutants became highly sensitive to Endo H, indicating that they should contain high-Man sugars (Fig. 3A). Demannosylation is an essential step during N-glycosylation, which remodels the
Monoclonal antibodies were purchased from Cell Signaling. Western blotting was purchased from Jackson ImmunoResearch Laboratories. The anti-HIV-1 Gag monoclonal antibody (183) was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.

**Experimental procedures**

**Enzyme, inhibitor, and antibodies**

Endo H was purchased from New England Biolabs. KIF, radioimmune precipitation assay (RIPA) lysis buffer, anti-FLAG M2 antibodies, anti-FLAG affinity gel, and anti-HA antibodies were purchased from Sigma-Aldrich. Anti-actin and anti-C-Myc monoclonal antibodies were purchased from Santa Cruz Biotechnology. Rabbits anti-calnexin polyclonal antibodies were purchased from Enzo Life Science. Anti-calreticulin monoclonal antibodies were purchased from Cell Signaling Technology. Anti-Zaire EBOV VP40 rabbit polyclonal antibody was purchased from Sino Biological. Allophycocyanin-conjugated mouse anti-FLAG antibody was purchased from Columbia Biosciences. KZ52 and 13C6 antibodies were purchased from IBT Bioservices. Phycoerythrin-conjugated goat anti-human IgG secondary antibody was purchased from Santa Cruz Biotechnology. HRP-conjugated secondary antibodies for Western blotting were purchased from Jackson ImmunoResearch Laboratories. The anti-HIV-1 Gag monoclonal antibody (183) was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.

**Plasmids**

The HIV-Luc reporter proviral vector pNL-Luc-ΔEnv was described before (33). The N-terminal FLAG-tagged EBOV ΔMLD GP expression vector was obtained from Shan-Lu Liu (University of Missouri). The human codon-optimized Cas9 expression vector was obtained from George M. Church (Harvard Medical School) through Addgene. pEGFP-C1 was purchased from Clontech. A plasmid expressing the full-length EBOV Zaire subtype matrix VP40 was purchased from Sino Biological.

To create calnexin- and calreticulin-expressing constructs, the nucleotide sequences encoding HA-tagged calnexin and C-Myc-tagged calreticulin were amplified by PCR from pMSCVneo-CANX (34) and pCMV6-CRT (Origene), respectively, and subcloned into the pCAGGS vector. To generate the FL-GP expression vector, the coding sequence for GP MLD was first synthesized and then inserted into the ΔMLD-GP expression vector via overlapping PCR. Single N-glycosylation mutants were constructed by asparagine-to-glutamine substitution using the QuikChange site-directed mutagenesis kit (Agilent Technologies). Single N-glycosylation mutants were subjected to further rounds of mutagenesis to create multiple sites mutants. All vectors were sequenced to confirm the correct sequences. Detailed procedures for the construction of the plasmids are available upon request.

**Cells**

The 293T, HeLa, and Vero cell lines were purchased from the ATCC and maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37 °C in 5% CO₂.

**Virus production**

Lentiviral pseudovirions bearing EBOV GP were generated as described before with minor modifications (35). Briefly, a total of 4 × 10⁶ 293T cells were seeded in 10-cm dishes and cultured overnight. Five micrograms of the plasmid encoding either the WT or mutant EBOV GP and 5 μg of pNL-Luc-ΔEnv were mixed with 30 μl of PEI (Polysciences, 1 μg/μl) in 500 μl of serum-free DMEM. After a 15-min incubation at room temperature, the transfection complexes were added to the cells. The medium was replaced with fresh medium 6 h after transfection. Forty-eight hours later, the culture supernatants were collected, clarified by low-speed centrifugation, and passed through a 0.45-μm syringe filter to remove any cell debris. The virion-containing supernatants were either used to infect target
cells or stored at −80 °C. E-VLPs were generated by the same method, except that the pNL-Luc-ΔEnv vector was replaced with a VP40-encoding plasmid. To analyze the levels of EBOV GP incorporation, supernatants containing virions were centrifuged at 25,000 rpm at 4 °C for 2 h using a Beckman SW-32Ti rotor. Viral pellets were resuspended in RIPA buffer and subjected to Western blotting analysis.

**EBOV transduction assay**

A total of 2 × 10^4 Vero cells were seeded in each well of 96-well plates 24 h prior to infection. An equal amount of pseudoviruses (normalized by HIV p24(Cas9)) was inoculated into these cells. After 48 h of infection, the culture medium was removed, and cells were lysed in 100 μl of lysis buffer (Promega). Luminescence was measured with a luciferase assay kit (Promega) and a TD-20/20 luminometer (Turner Designs).

**Western blotting and immunoprecipitation**

After being washed three times with cold PBS, transfected cells were lysed in RIPA buffer supplemented with 1 mg/ml protease inhibitor mixture (Roche) for 30 min on ice. Lysate was centrifuged at 12,000 × g for 10 min at 4 °C to remove any debris. Clarified supernatants were mixed with DTT-containing sample loading buffer (Solarbio), heated at 95 °C for 5 min, and then resolved by PAGE in 10% gels under reducing conditions. Some samples were subjected to Endo H treatment before applying them to the SDS-PAGE. To do so, a total of 20 μg of lysate was first denatured by heating at 100 °C for 10 min in denaturing buffer. Denatured proteins were added to a 20-μl reaction system containing 2 μl of GlycoBuffer and 1000 units of Endo H and incubated at 37 °C for 1 h.

For blue native PAGE, samples were prepared in native PAGE buffer (Life Technology) and separated on Novex 3–12% BisTris gels (Invitrogen) according to the protocol of the manufacturer.

For immunoprecipitation, clarified cell lysate was incubated with antibodies followed by addition of protein G-Sepharose beads (Pierce) or directly with anti-FLAG M2 monoclonal antibody-coupled beads (Sigma) at 4 °C for 4 h with rotation. Beads were washed twice with cold PBS containing 1% Triton X-100, followed by one more washing with PBS alone. Proteins were then eluted from beads by boiling and separated under denaturing conditions.

For Western blotting, gels from reducing or non-reducing electrophoresis were transferred to PVDF membranes. Membranes were briefly blocked with TBS containing 5% skim milk and 0.1% Tween 20, and then incubated with primary antibodies for 1 h at room temperature by the following dilutions: anti-FLAG antibody, 1:2000; anti-HA antibody, 1:3000; anti-Myc antibody, 1:1000; anti-CNX antibody, 1:1000; anti-CRT antibody, 1:1000; and anti-HIV p24 antibody, 1:3000. After being washed further with TBS containing 0.1% Tween 20, membranes were incubated with HRP-conjugated secondary antibodies at a 1:10,000 dilution. Chemiluminescence was detected by incubating the membrane with SuperSignal substrate (Pierce).

**Gene knockout**

The CRISPR/Cas9 system was employed to knock out CNX and/or CRT genes in 293T cells as described before (36, 37). Briefly, a DNA fragment that contained the U6 promoter, a 19-bp target sequence specific for CNX or CRT, a guide RNA scaffold, and a U6 termination signal sequence was synthesized and subcloned into the pGEM-T Easy vector (Promega). The vector was transfected with the human codon-optimized Cas9 expression vector and pEGFP-C1 into 293T cells. Twenty-four hours later, GFP-positive cells were isolated by fluorescence-activated cell sorting and subjected to cloning by limiting dilution. After 10–14 days, knockout clones were identified after screening by Western blotting.

**Flow cytometry**

For cell surface GP staining, a total of 1 × 10^6 293T cells were transfected with EBOV GP expression vector. Forty-eight hours later, cells were harvested and washed twice with PBS containing 0.1% bovine serum albumin. Cells were stained with allophycocyanin-conjugated anti-FLAG antibody for 30 min at 4 °C, washed three times, fixed in 4% paraformaldehyde, and analyzed by FACScan (BD Biosciences). Cells were also incubated with KZ52 or 13C6, followed by staining with phycoerythrin-conjugated anti-human IgG antibodies. The negative gate was defined with untransfected cells stained with the same antibody.

**Author contributions**—B. W. and Y. H. Z. designed the study and wrote the paper. Y. W., B. W., D. A. F., X. Z., X. Y., D. H., Z. Z., C. L., and S. Z. performed the experiments. S. H. X. provided comments regarding the paper. All authors analyzed the results and approved the final version of the manuscript.

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Functional characterization of EBOV GP N-glycosylation

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