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Lassa virus entry requires a trigger-induced receptor switch

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Abstract

Lassa virus spreads from rodents to humans and can lead to lethal hemorrhagic fever. Despite its broad tropism, chicken cells were reported to resist infection thirty years ago. We show that Lassa virus readily engaged its cell surface receptor α-dystroglycan in avian cells, but virus entry in susceptible species involved a pH-dependent switch to an intracellular receptor, the lysosome-resident protein LAMP1. Iterative haploid screens revealed that the sialyltransferase ST3GAL4 was required for the interaction of the virus glycoprotein with LAMP1. A single glycosylated residue in LAMP1, present in susceptible species but absent in birds, was essential for interaction with the Lassa virus envelope protein and subsequent infection. The resistance of *Lamp1*-deficient mice to Lassa virus highlights the relevance of this receptor switch *in vivo*.

Lassa virus binds to glycosylated α-dystroglycan (α-DG) at the cell surface to enter cells (1–2). Over 30 years ago it was reported that Lassa virus infects a broad suite of cells from different species with the exception of chicken (3). This was recapitulated by a recombinant vesicular stomatitis virus (VSV) that enters cells using the Lassa virus glycoprotein (rVSV-GP-LASV, (4)). Because wild-type VSV was unaffected this indicated a defect in Lassa glycoprotein-mediated entry (fig. S1A). Birds, however, generate glycosylated α-DG (5) and the Lassa virus glycoprotein (Lassa-GP) recognized avian α-DG (figs. S1B–C).

To identify host factors affecting virus entry independent of α-DG binding we carried out a haploid screen in receptor-deficient cells. For this we made use of their incomplete resistance phenotype (fig. S2). This showed that neither α-DG nor factors glycosylating α-

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DG act as host factors under these conditions (Figs. 1A, S3A–B and tables S1–2). Instead, we found genes involved in glycosylation, Golgi function and heparan sulfate biosynthesis. The latter were not identified in wild-type cells (fig. S3C and table S3) (4), suggesting that in the absence of α-DG, Lassa virus utilizes heparan sulfate, a commonly used virus attachment factor (6). Importantly, the lysosomal transmembrane protein LAMP1 and factors involved in N-glycosylation and sialylation, including the α-2,3-sialyltransferase ST3GAL4, stood out in both genotypes. Cells deficient for *LAMP1* or *ST3GAL4* were equally resistant to wild-type Lassa virus as those lacking α-DG (Figs. 1B and S4A–B). Expression of human but not chicken LAMP1 sensitized chicken fibroblasts to infection with rVSV-GP-LASV (Figs. 1C and S4C) and imposed virus susceptibility in *LAMP1*-deficient human cells (Figs. 1D and S5). This requirement for LAMP1 was specific for Lassa virus and not shared by the related lymphocytic choriomeningitis virus (fig. S6). Thus, LAMP1 and ST3GAL4 were important for Lassa virus infection independent of α-DG and host factor function of human LAMP1 was not shared by its chicken ortholog.

Because LAMP1 deficiency neither causes pronounced phenotypes in mice (7) nor detectably impaired the endolysosomal compartment in cultured cells (fig. S7) we asked if Lassa-GP could bind to LAMP1. As the majority of LAMP1 is localized in the acidic interior of lysosomes (8–9) these experiments were carried out at neutral and acidic pH. Immobilized virus glycoprotein (GP) bound α-DG at neutral pH, but this interaction was lost at acidic pH, where Lassa-GP instead strongly bound to LAMP1 (Figs. 2A and S8). Likewise, intact virions were captured by the luminal region of LAMP1 at acidic but not at neutral pH (fig. S9). Finally, this interaction was observed for both human and mouse LAMP1 but not chicken LAMP1 or human LAMP2 (Figs. 2B and S10).

Virus particles expressing eGFP fused to the VSV matrix (M) protein (rVSV-GP-LASV-MeGFP) were internalized in cells lacking *LAMP1* or *ST3GAL4* (fig. S11) but accumulated in vesicles of *LAMP1*-deficient cells (Fig. 2C). In wild-type cells fusion of viral and cellular membranes leads to release of MeGFP protein into the cytoplasm (10) but in *LAMP1*-deficient cells MeGFP remained localized to vesicles (figs. S12–13), suggesting that the association of Lassa-GP with LAMP1 precedes membrane fusion. In agreement with this, LAMP1 interacted with Lassa-GP in a pre-fusion configuration when the GP1 subunit of the viral envelope protein is still part of the complex (11–12) but not when GP1 was fully released from GP2 by low pH (Figs. 2D and S14). To test if Lassa GP-mediated membrane fusion was affected by LAMP1 we carried out cell-cell fusion experiments in the presence of a LAMP1 mutant that localizes to the cell surface (LAMP1d384) ((8), fig. S15A). Expression of this mutant led to increased syncytia formation as a consequence of membrane fusion (Figs. 2E and S15B–C). This effect was independent of α-DG (fig. S15D). Collectively, these experiments suggest that Lassa virus engaged α-DG at the cell surface, entered the endocytic pathway and bound to LAMP1 upon reaching the acidic interior of lysosomes, prior to membrane fusion.

To test this model we engineered an artificial virus infection scenario. α-DG knockout cells expressing LAMP1d384 were not sensitive to rVSV-GP-LASV under normal conditions but lowering the extracellular pH during virus exposure led to productive infection (fig. S16A–E). Lassa virus entry normally depends on acidification of endosomes (13) and is sensitive

to bafilomycin (14). The engineered entry route was, however, bafilomycin-insensitive (fig. S16F–G). Thus, the requirement for α-DG could be bypassed by re-routing LAMP1 to the cell surface and triggering binding to Lassa-GP.

Besides LAMP1 the screens identified the α-2,3-sialyltransferase ST3GAL4 as an α-DG-independent host factor. Because proteins modified by this enzyme could display genetic interactions we searched for host factors depending on ST3GAL4. *ST3GAL4*-deficient cells were mutagenized and selected with rVSV-GP-LASV. Like experiments in wild-type cells, this screen identified *DAG1* and its modifiers (4). Expectedly, the disrupted *ST3GAL4* locus did not act as a host factor under these conditions, but neither did *LAMP1* (Figs. 3A, S3B–C and S17A). Thus we investigated a putative biochemical connection between them. LAMP1 is glycosylated (15), comprising both N- and O-glycans (16). LAMP1 derived from *ST3GAL4*-deficient cells showed reduced binding to lectins that preferentially capture α-2,3-linked sialic acid (17) (fig. S17B–C) and lost its ability to bind to Lassa-GP (Fig. 3B). This demonstrated that LAMP1 was only able to act as a host factor in the context of ST3GAL4 proficiency.

LAMP1 consists of three luminal domains: a membrane-proximal domain, an O-glycosylated hinge region and a distal domain. The distal domain contains 11 N-glycosylation sites (UniProt, P11279) and was sufficient to support rVSV-GP-LASV infection by itself (fig. S18). Reconciling that genes for N-glycosylation and sialylation acted as host factors and that LAMP1 derived from *ST3GAL4*-mutant cells was not recognized by Lassa-GP, we speculated that one of these glycosylation sites was important for host factor function. We found that only Asn^{76} was essential for VSV-GP-LASV infectivity (Fig. 3C). This residue is present in LAMP1 from species susceptible to Lassa virus but absent in birds (Fig. 3D) (15). Substitution of this amino acid in human LAMP1 for the respective avian residue (N76S) was sufficient to block infection (fig. S19) and binding to Lassa-GP (Fig. 3E). Reciprocally, insertion of a region surrounding human Asn^{76} into chicken LAMP1 converted the avian protein into a host factor (fig. S20). Thus, we identified the sialyltransferase ST3GAL4 as a critical enzyme required for LAMP1 to function as a host factor, and mapped the interaction between sialylated LAMP1 and Lassa-GP to a single N-glycan present in sensitive species but absent in birds.

Because Lassa virus has a rodent reservoir we examined if LAMP1 is required for the propagation of wild-type Lassa virus *in vivo*. Following intraperitoneal injection virus was cleared in *Lamp1*-knockout mice, whereas infection was manifest in all organ samples taken from wild-type or heterozygous animals (Figs. 4A and S21).

Here we elucidated that Lassa virus entry requires a pH-regulated engagement of α-DG and LAMP1, both of which need to be glycosylated. However, the glycan structures that are required for host factor function are unrelated and constructed by distinct enzymes (Figs. 4B and S22). Unlike in rodents (18), the human upper airway mainly contains α-2,6-linked sialic acid moieties rather than α-2,3-linked sugars (19) generated by enzymes such as ST3GAL4 (20). It has been proposed that this is an adaptation to evade pathogens such as avian influenza but it may also limit human-to-human spread of Lassa virus (21). Lassa virus has been described as a ‘late-penetrating’ virus (22) requiring low pH (23). Our

findings rationalize these observations and emphasize the emergence of intracellular receptors for virus entry.

Materials and Methods

Cells

HAP1 cells (10) and isogenic knockout derivatives were cultured in IMDM supplemented with 10% fetal calf serum (FCS), penicillin–streptomycin and L-glutamine. HEK-293T cells and knockout derivatives, Vero cells (American Type Culture Collection, Manassas, Virginia, USA), mouse embryonic fibroblasts (MEFs) and chicken embryonic fibroblasts (Cell Lines Service GmbH, Eppelheim, Germany) were cultured in DMEM supplemented with 10% FCS, penicillin–streptomycin and L-glutamine. DF1 chicken fibroblasts (kind gift from Dr. M. Verheije) were grown in DMEM supplemented with 10% FCS, penicillin–streptomycin, L-glutamine and 1mM sodium pyruvate. HAP1 cells and isogenic knockout clones were utilized for haploid genetic screens (see below) and follow-up experiments. HEK-293T cells and isogenic knockout derivatives were used for the generation of recombinant retroviruses (see below), production of Flag-tagged Lassa virus glycoprotein (Lassa-GP) and LAMP1 proteins, as well as follow-up experiments. Vero cells were used for the amplification of rVSV-GP-LASV and rVSV-G. Chicken embryo fibroblasts and DF1 chicken fibroblast cells were used for follow-up experiments.

Recombinant vesicular stomatitis viruses (rVSVs)

Generation of recombinant vesicular stomatitis virus (rVSV) expressing eGFP and the Lassa virus glycoprotein (rVSV-GP-LASV), the LCMV glycoprotein (rVSV-GP-LCMV) or the eGFP-expressing control virus (rVSV-G) have been described previously (4, 25). A functional fluorescent VSV M protein was constructed by introducing the eGFP coding sequence in between Arg^{39}-Gly^{40} of VSV M. MeGFP was cloned into pVSV-G and pVSV-LASV-GP (4) replacing the native VSV M sequence and replication-competent fluorescent virus (rVSV-LASV-GP-MeGFP) was recovered as described previously (26).

Infectivity assays with rVSV, rVSV-GP-LASV and rVSV-GP_LCMV

Cells were challenged with ca. 6.7×10^6 plaque forming units (PFU)/ml (multiplicity of infection (MOI) ≈ 2) of the respective virus and infectivity was assessed by the fraction of eGFP-positive cells 4–6h after challenge using a fluorescence microscope (Zeiss, Oberkochen, Germany). The average number of eGFP-positive cells ± standard-deviation (SD) per field was calculated using ImageJ. In some experiments, cell nuclei were visualized by staining with Hoechst33342 (Invitrogen, Carlsbad, California, USA) or 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen) and the percentage of infected cells per field was calculated using the ratio of eGFP-positive cells and nucleus counts.

Infectivity assays with wild-type LCMV

Wild-type and *LAMP1*-deficient cells were infected with LCMV-Armstrong (kind gift of Allan Zajac, University of Alabama-Birmingham) at an MOI of 3 and infection was allowed to proceed for 16h at 34°C. Cells were pretreated with 100nM bafilomycin A1 or mock-treated for 1h before infection. Where applicable bafilomycin A1 was maintained present

throughout the infection. Cells were then fixed with 4% paraformaldehyde (PFA) and immunofluorescence was performed using anti-LCMV polyclonal antisera. Secondary antibodies were coupled to Alexa Fluor 488 and nuclei were visualized using a DAPI stain.

Internalization assays with rVSV-GP-LASV-MeGFP

Cells grown on coverslips coated with poly-L-lysine (Sigma-Aldrich, St. Louis, Missouri, USA) were inoculated with gradient-purified rVSV-GP-LASV-MeGFP (MOI ≈ 300) at 4°C for 30min to allow binding of virus particles to the cell surface. Cells were subsequently incubated for 2h at 37°C in the presence of 10mM NH_4Cl to allow accumulation of internalized viral particles. Cells were washed with citric acid buffer (pH 3.0) to remove residual surface-bound virus and fixed in 2% PFA. The cellular plasma membrane was labeled by incubation of cells with 1μg/ml wheat germ agglutinin (WGA) conjugated to Alexa Fluor 568 (Molecular Probes, Invitrogen) in PBS for 15min at room temperature. After washing with PBS, cells were mounted onto glass slides and fluorescence was monitored by spinning-disk confocal microscopy (Zeiss). Representative images were collected with Slidebook 4.2 software (Intelligent Imaging Innovations).

LysoTracker staining

MEFs were plated on live cell imaging chambers and incubated in normal DMEM the day before imaging. Cells were washed with PBS before incubation with LysoTracker (LysoTracker® Red DND-99, Invitrogen) diluted 1:2000 in DMEM at 37°C for 30min. Before imaging, cells were washed 3× with PBS to remove excess dye.

DQ Red BSA degradation

MEFs were plated on coverslips the day before the experiment. The cells were incubated with the BODIPY labeled bovine serum albumin in DMEM at a final concentration of 50μg/ml for 5 hours. Cells were subsequently 3× washed with PBS, fixed with 4% PFA for 15min and imaged with a confocal microscopy.

Transferrin uptake

MEFs were plated on coverslips the day before the experiment. Cells were pulse-labeled with Alexa Fluor 488 conjugated Transferrin (Invitrogen) at 50 μg/ml for 30min at 37°C. After pulse cells were washed 2× with PBS and chased for the indicated time with medium lacking conjugated Transferrin and subsequently washed 2× with PBS and fixed with 4% PFA for 15min.

Endosomal fusion assay

MEFs were plated on coverslips the day before the experiment. The cells were incubated in FITC-labelled Dextran with a molecular weight of 3000 Dalton (Invitrogen) (0.5 μg/ml in DMEM) for 16h, washed 3× with PBS and subsequently chased in DMEM for 3h to ensure transport to lysosomes. Cells were then stained for Lamp2 by immunofluorescence, fixed and mounted onto glass slides.

Antibodies used for immunofluorescence

The following antibodies were used: Lamp1 (clone 1D4B) and Lamp2 (clone Abl93) (Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242), Ctsd (27), Lbpa (clone 6C4, gift from Jean Gruenberg), EEA1 (Cell Signaling Technology, Danvers, Massachusetts, USA), Rab7 (Cell Signaling Technology). For detection of cells infected with Influenza A virus (strain WSN) an anti-NP antibody was used (kind gift of C. de Haan).

Confocal microscopy

Photographs were acquired with an FV1000 confocal laser scanning microscope (Olympus) equipped with an oil immersion objective (U Plan S Apo 100×, N.A. 1.40) and Olympus Fluoview Software (3.0a) and a Leica-Microsystems microscope. Representative images were acquired with LCS software (Leica-Microsystems, Vienna, Austria).

MeGFP protein release assay

Cells grown on coverslips were pre-treated with 10μg/ml puromycin (Invivogen, San Diego, California, USA) for 30min (in the presence or absence of 100nM bafilomycin A1) and inoculated with rVSV-GP-LASV-MeGFP or rVSV-MeGFP control virus at an MOI of ≈ 300. Puromycin and bafilomycin A1 were maintained throughout the experiment. After incubation for 4h at 37°C, cells were washed twice with PBS and fixed with 2% PFA in PBS for 15min at room temperature. Cells were washed with PBS and mounted onto glass slides after which MeGFP localization images were acquired. To visualize the cell membrane, cells were stained with Alexa Fluor 647 labeled wheat germ agglutinin (WGA, Invitrogen, Molecular Probes).

Flow cytometry

Cells were detached using PBS supplemented with 5mM EDTA prior to incubation with murine antibodies directed against glycosylated α-DG (IIH6-C4, Millipore, Billerica, Massachusetts, USA) or against LAMP1 (H4A3, Santa Cruz biotechnology, Dallas, Texas, USA) in PBS 5% bovine serum albumin (BSA). Primary antibodies were labeled with a goat anti-mouse antibody coupled to Alexa Fluor 568 (Invitrogen). Samples were measured on a BD Fortessa flow-cytometer (BD, Franklin Lakes, NewJersey, USA). Fluorescence-activated cell sorting was carried out on a BD FACS Aria flow-cytometer (BD). Data was analyzed and assembled using FlowJo (TreeStar Inc, Ashland, Oregon, USA).

Genome engineering

HAP1 cells with a disrupted *DAG1*, *LAMP1* or *ST3GAL4* locus were generated previously (4). In HEK-293T cells *DAG1*, *LAMP1* or both loci in combination were targeted using transcription activator-like effector nucleases targeting exonic sequences within these genes as described before (4). HEK-293T cells were subcloned and individual subclones were analyzed for the absence of the respective gene products by immunoblot analysis.

Immunoblot analysis

Proteins from cell pellets or lysates were denatured using sample buffer containing 100mM dithiothreitol (DTT) and 2% sodium dodecyl sulfate (SDS), separated by SDS-polyacrylamid-gel-electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore) by wet Western blotting. Membranes were subsequently blocked using PBS 0.1% Tween-20 supplemented with 5% BSA or non-fat milk powder. Glycosylated α-DG of different species was detected using the IIH6-C4 antibody (Millipore). The peptide backbone of dystroglycan (core) was visualized using the GTX105038 antibody (GeneTex, San Antonio, Texas, USA). Flag-tagged Lassa-GP was detected with the anti-Flag M2 antibody (Sigma-Aldrich). Human LAMP1 was detected using antibody AB2971 (Millipore). In experiments including mouse and/or chicken LAMP1, an antibody capable of detecting human, chicken and mouse LAMP1 (ab79821, Abcam, Cambridge, UK) was used. HA-tagged proteins were visualized using antibody H6908 (Sigma-Aldrich). Fc-fusion proteins were detected with horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies (Invitrogen). eIF4G of different species was visualized using antibody #2498 (Cell Signaling Technology). Tubulin was detected using the B-7 antibody (Santa Cruz biotechnology). Lassa-GP1 was detected using a mouse monoclonal antibody (kind gift of R. Schoepp). Human LAMP2 was detected using a specific antibody (Abcam). Primary antibodies were detected using HRP-conjugated anti-mouse (Bio-Rad, Hercules, California, USA and Rockland, Gilbertsville, Pennsylvania, USA) or anti-rabbit (Invitrogen) secondary antibodies. Antibody-bound proteins were visualized using enhancer and peroxide solutions (Thermo-Scientific, Waltham, Massachusetts, USA) and a gel imaging system (Bio-Rad).

Cloning of genes and retroviral transduction of cells

HAP1 cDNA was generated using the SuperScript® III First-Strand Synthesis System (Invitrogen). Human LAMP1 cDNA was amplified from this library using primers (5′-gatcGAATTCaccatggcggcccccggcagcgcc-3′ and 5′-gatcCTCGAGctagatagtctggtagcctgc-3′) and cloned into the retroviral expression vector pMX-IRES-BLAST using EcoRI and XhoI restriction enzymes. Human LAMP1d384 was generated from the wild-type cDNA using an altered reverse primer (5′-gatcCTCGAGctagatagtctggtagccgtgactcctcttcctgccgac-3′). Chicken LAMP1 cDNA was gene-synthesized based on the Ensembl transcript ENSGALT00000027170 and cloned into pMX-IRES-BLAST. HA-tagged variants of human and chicken LAMP1 were created by gene-synthesis of variants, C-terminally encoding a short linker followed by a triple HA tag as present in the pCMV6-AC-3HA vector (Addgene) and XhoI site (AAVYPYDVPDYAGYPYDVPDYAGSYPYDVPDYAVGSLE) and cloned into an altered version of the pMX-IRES-BLAST vector in which the IRES sequence was previously replaced with a 2A sequence (pMX-2A-BLAST), creating a single open reading frame containing the cloned genes and the blasticidin resistance cassette. pMX-2A-BLAST was generated by ligation of a gene-synthesized blasticidin cassette in which the translation start site (ATG) was replaced by a self-cleaving P2A sequence (GSGATNFSLLKQAGDVEENPGP, (28)) into pMX-IRES-BLAST using NotI and SalI. Domain mutants of human LAMP1 (LAMP1distal, LAMP1Δdistal and LAMP1Δhinge) were generated by gene-synthesis and cloned into pMX-2A-BLAST using EcoRI and XhoI.

For generation of LAMP1distal, amino acids 1–197 of the human protein, comprising the membrane-distal ordered domain and three additional adjacent amino acids for flexibility, were fused to amino acids 381–417, encompassing the transmembrane region and lysosomal targeting sequence and two adjacent amino acids. LAMP1Δdistal was generated by deleting amino acids 29–197 from the full-length sequence. For the generation of LAMP1Δhinge, amino acids 198–217 comprising the proline-rich hinge region were deleted from the full-length protein.

Asparagine-mutants of LAMP1distal were generated by gene-synthesis (replacing the asparagine-encoding triplets with GCA, encoding alanine) and cloned into the pMX-2A-BLAST vector using EcoRI and XhoI. ‘Chickenized’ human LAMP1-N76S was generated from the wild-type cDNA by introduction of a point mutation in the sequence by overlap-extension PCR using the following internal primers: 5′-ctgccatcagatgccacagtggtgctcTCAcgcagctcctgtggaaaagagaac-3′ and 5′-gttctcttttccacaggagctgcgTGAgagcaccactgtggcatctgatggcag-3′. ‘Humanized’ chicken LAMP1 was generated by gene-synthesis introducing residues 63–101 of human LAMP1 into the chicken protein at the corresponding position. ‘Chickenized’ human and ‘humanized’ chicken LAMP1 were cloned into pMX-IRES-BLAST using EcoRI and XhoI restriction enzymes. Full length human LAMP2 was cloned from the HAP1 cDNA library using primer sequences 5′-gcaacgGGATCCaccatggtgtgcttccgcctcttc-3′ and 5′-gcaacgCTCGAGaaattgctcatatccagcatgatg-3′ and cloned into pMX-2A-BLAST using BamHI and XhoI.

A retroviral expression vector encoding LAMP2-RFP was constructed as follows: Overlap-extension PCR was performed to C-terminally ligate RFP to the human LAMP2 coding sequence. A 6× alanine-linker was introduced as a spacer. The first PCR was performed with the following primers: 5′-gcaacgGGATCCatggtgtgcttccgcctcttc-3′ and 5′-gctgctgcagcagcagatctaaattgctcatatccagcatgatg-3′. The second PCR was carried out with the following primers: 5′-agatctgctgctgcagcagctgctatggtgagcaagggcgagga-3′ and 5′-gcaacgGTCGACttacttgtacagctcgtccatg-3′. The resulting PCR product was cloned into pBABE-PURO using BamHI and SalI.

Retroviral vectors were transfected into HEK-293T cells together with pAdvantage (Clontech, Mountain View, California, USA) and the packaging plasmids pCMV-VSV-G and pGAG-POL. Virus was harvested 48h post transfection, filtered and applied to the respective cells. Transduced cells were selected with blasticidin S (Invivogen) or puromycin (Invivogen).

Cloning of constructs to direct the expression of Fc-fusion proteins

LAMP1-Fc and LAMP2-Fc expression plasmids were generated by overlap-extension PCR of DNA fragments encoding LAMP1 and LAMP2 extracellular parts (human LAMP1; residues Ala^{29}–Ser^{351} (PCR1), chicken LAMP1; residues Ser^{17}–Ser^{377} (PCR2), human LAMP1distal; residues Ala^{29}–Ser^{140} (PCR3), chicken LAMP1distal; residues Ser^{17}–Ser^{185} (PCR4), human LAMP2; residues Leu^{29}-Q^{342} (PCR5)) and a fragment encoding the Fc domain of rabbit IgG1 (PCR6–10). The resulting products were cloned (using BglII and BamHI restriction enzymes) into a phCMV soluble expression vector (a kind gift from Dr.

E. Ollmann-Saphire), introducing an IgK signal sequence at the N-terminus. Sequences of the overlap-extension primers: 5′-gcaacgAGATCTcgagcagcaatgtttatggtgaaaaatggc-3′ and 5′-cgctgttctctgcgccttcgtgacacg-3′ (PCR1) with 5′-aggcgttttcagagaacagcgagacacactac-3′ and 5′-ggtttaGGATCCtttacccgg-3′ (PCR6); 5′-gcaacgAGATCTtcctcttcatttgacgtgagaga-3′ and 5′-cgctgttctcgttttcatccagctgacattctt-3′ (PCR2) with 5′-tggatgaaaacgagaacagcgagacacactac-3′ and 5′-ggtttaGGATCCtttacccgg-3′ (PCR7); 5′-gcaacgAGATCTcgagcagcaatgtttatggtgaaaaatggc-3′ and 5′-cgctgttctcggaaggcctgtcttgttcaca-3′ (PCR3) with 5′-cgctgttctcggaaggcctgtcttgttcaca-3′ and 5′-ggtttaGGATCCtttacccgg-3′ (PCR8); 5′-gcaacgAGATCTtcctcttcatttgacgtgagaga-3′ and 5′-cgctgttctcagagaccatatcttccctacatt-3′ (PCR4) with 5′-atatggtctctgagaacagcgagacacacta-3′ and 5′-ggtttaGGATCCtttacccgg-3′ (PCR9); 5′-gcaacgAGATCTcgagcattggaacttaatttgacagattcag-3′ and 5′-cgctgttctcctgaaatgctccagacactgaa-3′ (PCR5) with 5′-gagcatttcaggagaacagcgagacacactac-3′ and 5′-ggtttaGGATCCtttacccgg-3′ (PCR10) Fc-fusion proteins were expressed by transfection of the plasmids into HEK-293T cells and affinity purified from the culture supernatant using protein A sepharose beads (GE Healthcare, Little Chalfont, UK).

Immunoprecipitations

Flag-tagged Lassa-GP was produced in HEK-293T cells engineered to lack both α-DG and LAMP1. Cells were transiently transfected with pCAGGS-LASV-GP-Flag (kindly provided by Dr. S. Kunz) and lysates were prepared 48h post transfection in NETN buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, 0.5% NP-40) adjusted to pH 8.0, 6.5 or 5.5 and supplemented with complete protease inhibitor cocktail (Roche, Basel, Switzerland). Lysates were sonicated and Lassa-GP was immobilized on anti-Flag M2 agarose beads (Sigma-Aldrich). Beads were washed and incubated with whole cell lysates prepared in NETN buffer adjusted to the respective pHs. After incubation beads were washed with NETN buffer adjusted to the respective pH. Bound proteins were eluted and subjected to immunoblot analysis. For incubation of Flag-tagged GP with cells, beads were washed and bound proteins were eluted using 3×Flag peptide (Sigma Aldrich) and added to cells that were harvested in PBS containing 10% FCS. Flag-GP was incubated with the cells at 4°C for 1h and subsequently cells were pelleted and washed 3 times with PBS.

Lectin bead protein capture and PNGase F treatment

Cells were lysed in Lectin lysis buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% Triton-X 100, 2mM $CaCl_2$, 2mM $MgCl_2$) complemented with complete protease inhibitor cocktail and samples were sonicated. Lysates were supplemented with 1% β-mercaptoethanol, 1% NP-40 and 0.5% SDS, boiled and incubated with 40μg/ml PNGase F or mock-treated at 37°C. Lysates were then incubated with Maackia amurensis lectin (MAA) agarose beads (US Biological, Salem, Massachusetts, USA) or Sambucus Nigra lectin (SNA) agarose beads (Vector Labs, Burlingame, California, USA). Beads were washed bound proteins were subjected to SDS-PAGE and immunoblot analysis (see above).

Fc-pull-down assays

Lysates from Lassa-GP transfected HEK-293T cells (see above) were incubated with protein A sepharose bead-bound Fc fusion proteins and incubated for 2h at 4°C under slow rotation.

Beads were washed four times with lysis buffer after which proteins were taken up in sample buffer and subjected SDS-PAGE and immunoblot analysis (see above).

Radiolabeling and capture of recombinant rVSV-GP-LASV

Vero cells were cultured in T150 flasks and inoculated with rVSV-GP-LASV (MOI ≈ 3). 2.5h post inoculation the medium was replaced with methionine-free and cysteine-free medium containing 10μg/ml Actinomycin D (labeling medium). 3.5h post inoculation the medium was replaced with labeling medium supplemented with 200 μCi ^{35}S-Met/Cys. Virus was propagated O/N at 34°C. Rabiolabeled virus was harvested, filtered through a 0.2μm filter, pelleted and resuspended in NTE buffer (10mM Tris-HCl pH 7.4, 100mM NaCl, 1mM EDTA) O/N on ice. 5μg of radiolabeled virus was incubated for 2h with protein A sepharose bead-bound LAMP1-Fc in neutral (pH 8.0) or acidic (pH 5.5) medium under slow rotation. Beads were washed four times and subjected to SDS gel electrophoresis under reducing conditions. Gels were dried and radiolabeled proteins were detected using a Typhoon 9400 imager (GE Healthcare).

Electron microscopy (EM)

Cells were fixed in Karnovsky's fixative (2% paraformaldehyde + 2.5% glutaraldehyde in 0.1M cacodylate buffer at pH 7.2). Postfixation was performed with 1% osmiumtetroxide in 0.1M cacodylatebuffer. After washing, pellets were stained en bloc with Ultrastain 1 (Leica, Vienna, Austria), followed by an ethanol dehydration series. Finally, the cells were embedded in a mixture of DDSA/NMA/Embed-812 (EMS, Hatfield, Pennsylvania, USA), sectioned and stained with Ultrastain 2 (Leica) and analyzed with a CM10 electron microscope (FEI, Eindhoven, Netherlands).

Cell-cell fusion assays (polykarion assays)

HEK-293T cells and genome-engineered derivatives were transduced with empty vector, human LAMP1 or human LAMP1d384 and selected with blasticidin S. Selected cells were then transiently transfected with an expression vector for GFP together with pCAGGS-LASV-GP-Flag or an empty vector. 48h post transfection, the pH was dropped to 5.5 for 5min. 1h later cells were incubated with fluorescent wheat germ agglutinin (WGA) to visualize cell boundaries and examined for membrane fusion events by confocal microscopy. Fused cells lack intercellular WGA staining and instead show increased homogenously GFP-positive areas as a result of mixing of their cytoplasms. The fusion index was calculated by comparing the number of unfused cells (as determined by WGA staining) per field in mock versus Lassa-GP transfected cells of the respective genotype.

rVSV-GP-LASV infectivity assays at acidic pH and bafilomycin treatment

HAP1 or HEK-293T cells of the respective genotypes were seeded prior to infection and, where applicable, pre-treated with 100nM bafilomycin A1 or mock-treated with DMSO for 1.5h. rVSV-GP-LASV was diluted to 6.7×10^6 PFU/ml in regular IMDM medium or IMDM adjusted to pH 5.5 and added to the cells (MOI ≈ 2). In some experiments, 100nM bafilomycin A1 was added along with the virus. Virus containing medium was removed 1–

1.5h post challenge and cells were scored for infection with rVSV-GP-LASV 5–7h post infection by eGFP expression using a fluorescence microscope.

Alignment of LAMP1 polypeptides from different species

Human LAMP1 (ENSGALT00000027170, Ensembl), LAMP1 from Rhesus macaque (I0FRN0, UniProt), from dog (F1Q260, UniProt), mouse (P11438, UniProt) and chicken (P05300, UniProt) were aligned and phylogenetically related using UniProt. In multi-species comparison, identity (dark grey) and similarity (light grey) were indicated.

Haploid genetic screens

The generation of gene-trap retrovirus and mutagenesis have been described previously (4). Approximately 10^8 mutagenized HAP1 cells or mutagenized HAP1 cells engineered to lack *DAG1* or *ST3GAL4* were used for the respective screens. Non-engineered mutagenized HAP1 cells were selected with ca. 4.5×10^4 PFU/ml of rVSV-GP-LASV (MOI $\approx$ 0.1) in the presence of 5mM NH_4Cl. Mutagenized HAP1 cells lacking *DAG1* or *ST3GAL4* were exposed to higher titers (ca. 6.7×10^6 PFU/ml, MOI $\approx$ 12.5) of rVSV-GP-LASV for selection. Following selection, the surviving colonies were expanded to ca. 3×10^7 total cells and their genomic DNA was isolated using a QIAamp DNA mini kit (Qiagen, Venlo, Netherlands).

Sequence analysis of gene-trap insertion sites

Recovery of gene-trap insertion sites from unselected control cells and cells selected with rVSV-GP-LASV has been described previously (4). For rVSV-GP-LASV-selected mutagenized HAP1 cells lacking *DAG1* or *ST3GAL4* enzymatic digest of the recovered genomic DNA was omitted. Processing of sequencing data from virus-selected populations was carried out as described before (4) with the exception that close read filtering (two reads on the same strand being within 2bp from one another) was omitted. For every gene, enrichment of inactivating gene-trap insertions in the rVSV-GP-LASV-selected populations over an unselected control data set (4) was assessed by applying a one-sided Fisher's exact test. Genes with *P*-value $\leq 10^{-4}$ were considered enriched (tables S2–4, first tab). Furthermore, genes found enriched for insertion sites had to pass a second statistical test. Enriched genes needed to show a significant bias ($P \leq 0.05$, binomial test) for gene-trap insertions in sense orientation of the affected gene (disruptive) versus insertions in antisense orientation (see tables S2–4, second tab). Because gene-trap insertions in exons are expected to disrupt the gene regardless of their orientation, this test could only be applied to genes for which the majority of insertions (≥70%) mapped to intronic regions. Genes for which less than 70% of the insertions affected introns could only be tested for enrichment of disruptive insertions compared to the unselected control dataset (see above), but were therefore required to meet a stricter cut-off ($P \leq 10^{-20}$, see tables S2–4, third tab). All *P*-values were corrected for false discovery rate (FDR). If the reported *P*-value was smaller than what the software (R) could report, its numerical value was set to the smallest non-zero normalized floating-point number R was capable of listing on the computer used for data analysis (ca. 10^{-314}).

HAP1 infections with wild-type Lassa virus

HAP1 cells, seeded in 96 well black plates (Greiner Bio-One Cellcoat®), were incubated with Lassa virus, Josiah strain, at MOI ≈ 1 in a Biosafety Level 4 (BSL4) laboratory located at USAMRIID. Following 1h absorption, virus inoculum was removed and cells were washed once with PBS. Fresh HAP1 cell culture media was added to each well and cells were incubated at 37°C, 5% CO_2, 80% humidity. Following 48h incubation, cells were washed once with PBS and submerged in 10% formalin prior to removal from the BSL4 laboratory. Formalin was removed and cells were washed 3 times with PBS. Cells were blocked by adding 3% BSA/PBS to each well and incubating at 37°C for 2h. Lassa GP-specific mAb 52-161-6 was added to each well and incubated at room temperature for 2h. Cells were washed 3 times with PBS prior to addition of goat anti-mouse IgG-Alexa Fluor 488 (Invitrogen, Molecular Probes®) secondary antibody. Following 1h incubation with secondary antibody, cells were washed 3 times prior to addition of Hoechst 33342 (Invitrogen, Molecular Probes®) diluted in PBS. Cells were imaged and Lassa virus infected cells enumerated using the Operetta High Content Imaging System (PerkinElmer, Waltham, Massachusetts, USA) and Harmony® High Content Imaging and Analysis Software (PerkinElmer).

Mouse injections and tissue titer analysis

Lamp1$^{-/-}$ (n=9), Lamp1$^{+/-}$ (n=12), and wild-type (Lamp1$^{+/+}$, n=8) mice, 13–16 weeks of age, were inoculated intraperitoneally with 100 PFU of Lassa virus, Josiah strain. Subsets of mice from each knockout group and wild-type group were euthanized on days 3 and 6 post infection and blood, liver, spleen, kidneys, and lungs were collected. Blood samples were allowed to clot prior to centrifugation and serum fraction was collected for viral titer determination. Tissue samples were weighed and appropriate volumes of Minimal Essential Medium (MEM)/2% FBS were added to yield 10% tissue homogenates. Following homogenization using a gentleMACS™ Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany), homogenates were centrifuged and supernatants collected for viral titer determination. Half of each organ was placed in 10% formalin for immunohistochemical analysis and hematoxylin and eosin (H&E) staining.

Viral titers were determined by plaque assay using Vero cells maintained in MEM/5%FBS. Serum and tissue samples were serially diluted in MEM/5%FBS and added to confluent monolayers of Vero cells. Following 1h incubation at 37°C, 5% CO_2, 80% humidity, cells were overlaid with a mixture of 1 part 1% agarose (Seakem, Lonza, Basel, Switzerland) and 1 part 2× Eagle basal medium (EBME)/5% FBS and incubated at 37°C, 5% CO_2, 80% humidity for 7 days. Cells were again overlaid with a mixture of 1 part 1% agarose (Seakem) and 1 part 2× EBME/30mM HEPES/5% FBS/5% Neutral Red and incubated for 1–2 more days before enumerating the number of plaque forming units per milliliter. Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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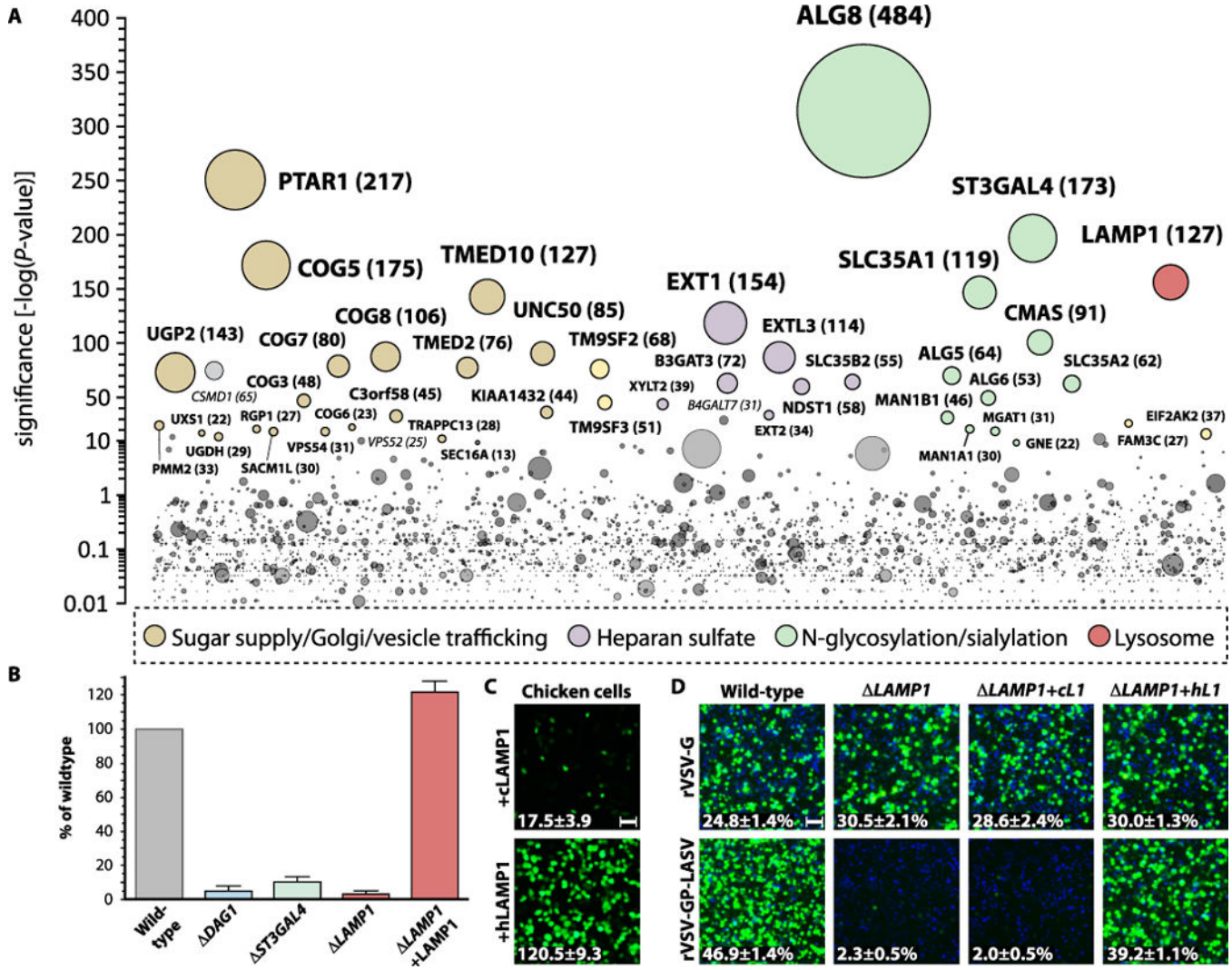

Figure 1. Human LAMP1 is an α-DG-independent host factor for Lassa virus and bypasses an infection barrier in avian cells

(A) Haploid genetic screen for host factors required for infection with rVSV-GP-LASV in cells lacking α-DG. The *y* axis indicates the significance of enrichment of gene-trap insertions in particular genes as compared to a non-selected control cell population. Filled circles represent genes and their size corresponds to the number of insertion sites identified in the virus-selected cell population. Hits were colored if they passed the statistical criteria described in the supplementary experimental procedures. Significant hits were grouped by function horizontally and data is displayed until –log(*P* value) = 0.01. **(B)** HAP1 cell lines with nuclease-generated mutations in the corresponding genes were exposed to wild-type Lassa virus and stained with antibodies specific for viral antigens to measure infected cells. *LAMP1*-deficient cells were complemented with human LAMP1 (L1) cDNA. **(C)** Chicken fibroblasts were transduced with retroviruses expressing chicken (c) or human (h) LAMP1 and challenged with rVSV-GP-LASV. Average number (±SD) of infected cells per field (eGFP-positive) is indicated. **(D)** Wild-type or *LAMP1*-deficient HAP1 cells transduced with

retroviruses expressing cLAMP1 or hLAMP1 were exposed to rVSV-G or rVSV-GP-LASV. Percentage (±SD) of infected cells (eGFP-positive) is indicated. Scale bars: 50μm.

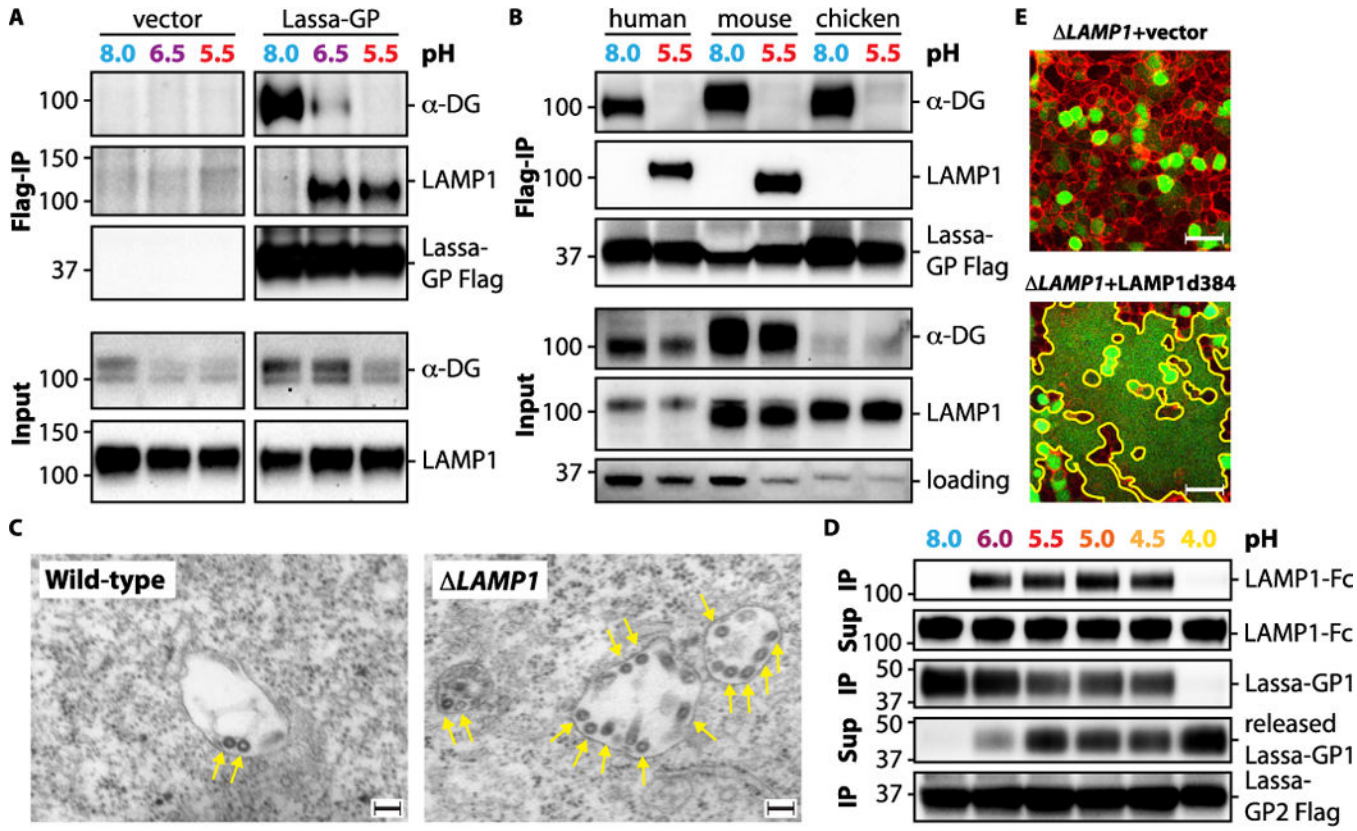

Figure 2. Lassa-GP undergoes a pH-induced switch to engage LAMP1

(A) Flag-tagged Lassa-GP was immobilized on beads and incubated with cell lysates from HEK-293T cells at the indicated pH. Bound proteins were subjected to immunoblot analysis and uncoated beads served as a control. IP = immunoprecipitation. **(B)** Flag-tagged Lassa-GP was immobilized on beads and incubated with lysates from human, mouse and chicken cells at the indicated pH. Bound proteins were subjected to immunoblot analysis. **(C)** Electron micrographs of wild-type and *LAMP1*-deficient HEK-293T cells that were infected with rVSV-GP-LASV. *LAMP1*-deficient cells show an accumulation of the bullet-shaped viral particles (arrows) in intracellular vesicles. Scale bars: 100nm. **(D)** Flag-tagged Lassa-GP was immobilized on beads and incubated with purified LAMP1-Fc at the indicated pH. Complexes (IP) were precipitated and subjected to immunoblot analysis. The supernatant (Sup) was analyzed for the release of Lassa-GP1. **(E)** *LAMP1*-deficient (top) or LAMP1d384 expressing HEK-293T cells (bottom) were transfected with expression vectors for Lassa-GP and GFP and exposed to pH 5.5. Cell boundaries were visualized with fluorescent wheat germ agglutinin (red). Enlarged homogenous green fluorescent areas result from Lassa-GP-induced syncytia formation (yellow outline). Scale bar: 50μm.

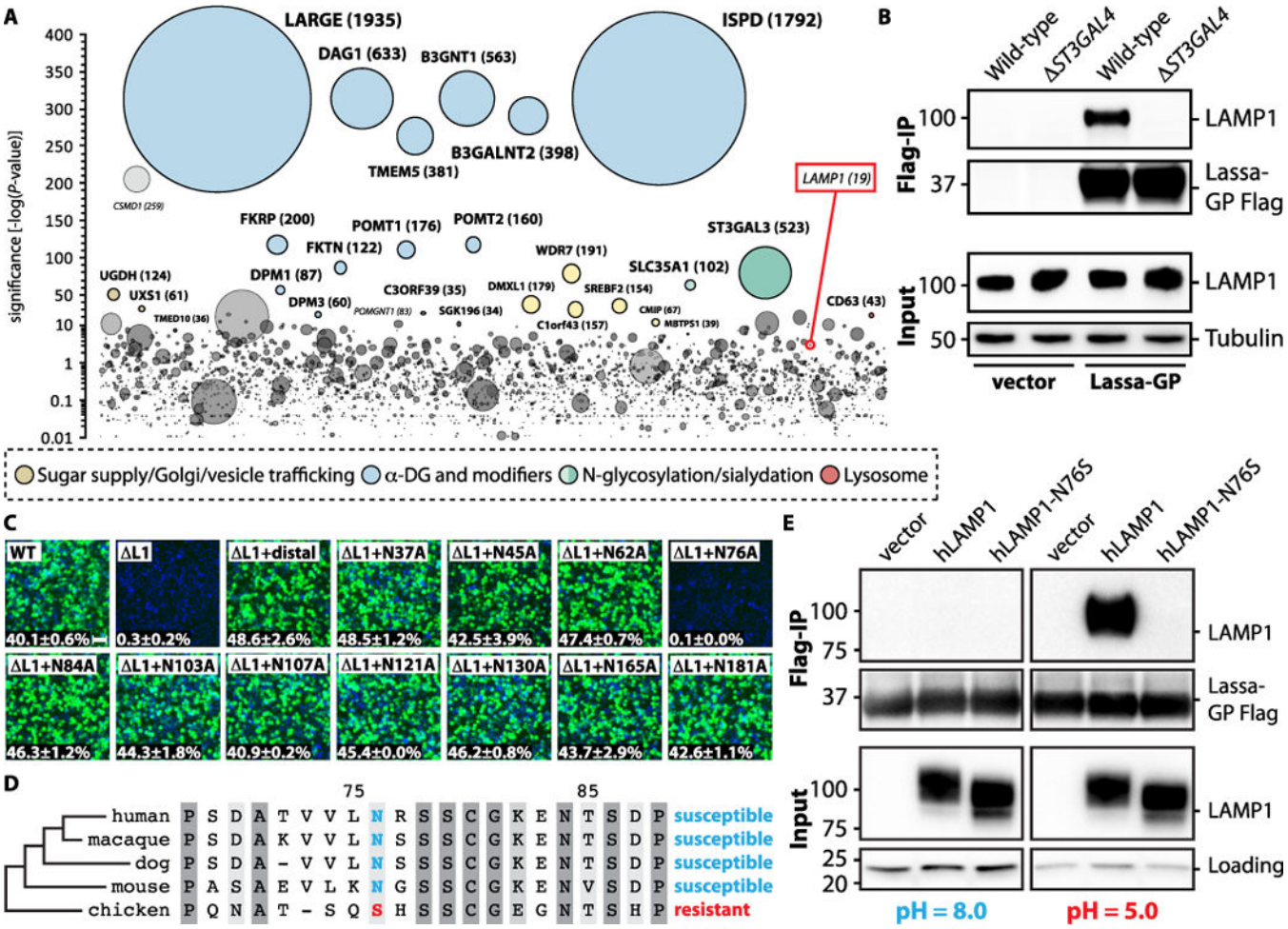

Figure 3. Binding of Lassa-GP to LAMP1 depends on ST3GAL4 and LAMP1-Asn^{76} is critical for host factor function

(A) Haploid genetic screen pointing out genetic interactions between *ST3GAL4* and other Lassa entry factors. *ST3GAL4*-deficient cells were mutagenized and exposed to rVSV-GP-LASV. Gene-trap insertion sites were mapped in the resistant cell population and data was analyzed as in Fig. 1A. **(B)** Flag-tagged Lassa-GP was immobilized on beads and incubated with cell lysates from wild-type and *ST3GAL4*-deficient HAP1 cells at pH 5.5. Bound proteins were subjected to immunoblot analysis. **(C)** Wild-type and *LAMP1*-deficient (ΔL1) HAP1 cells complemented with cDNAs expressing the distal domain of LAMP1 containing mutations at the indicated glycosylation sites were exposed to rVSV-GP-LASV. Percentage (±SD) of infected cells (eGFP-positive) is indicated. **(D)** Comparison of LAMP1 polypeptides from indicated species highlights Asn^{76} as a marker of susceptibility to Lassa virus infection. **(E)** Flag-tagged Lassa-GP was immobilized on beads and incubated with lysates from *LAMP1*-deficient HEK-293T cells expressing human LAMP1 or 'chickenized' LAMP1 carrying the N76S substitution.

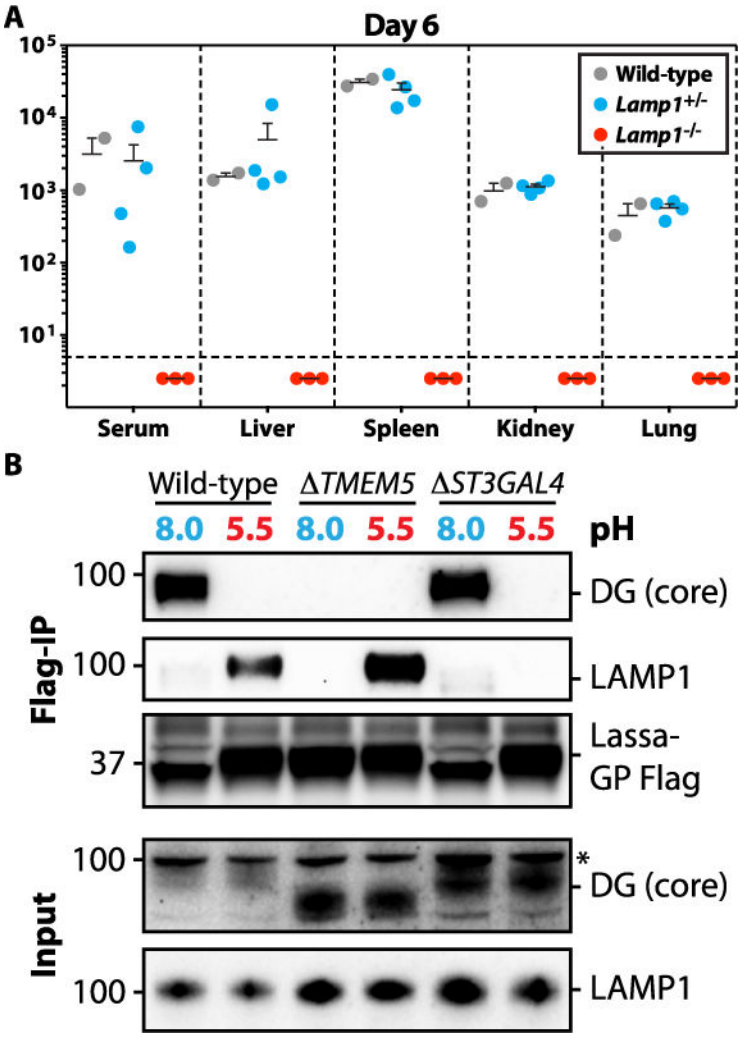

Figure 4. *Lamp1* knockout mice are resistant to wild-type Lassa virus and both host factors require distinct glycosyltransferases

(A) Lassa virus propagation in *Lamp1*$^{+/+}$, *Lamp1*$^{+/-}$ and *Lamp*$^{-/-}$ mice. Mice were injected intraperitoneally with wild-type Lassa virus and viral titers were determined after 6 days in the indicated tissues. The horizontal line marks the detection limit. (B) Flag-tagged Lassa-GP was immobilized on beads and incubated with cell lysates from wild-type, *TMEM5*- or *ST3GAL4*-deficient cells at the indicated pH. The glycosyltransferase TMEM5 is needed to generate an epitope on α-DG that is recognized by Lassa-GP (4). Bound proteins were subjected to immunoblot analysis. * = non-specific background band.