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2 Proximity interactome analysis of Lassa polymerase reveals 3 eRF3a/GSPT1 as a druggable target for host directed antivirals.

4 Jingru Fang^{1,2}, Colette Pietzsch^{3,4}, George Tsaprailis⁵, Gogce Crynen⁵, Kelvin Frank Cho⁶, Alice
5 Y. Ting^{7,8}, Alexander Bukreyev^{3,4,9}, Erica Ollmann Saphire^{2*} and Juan Carlos de la Torre^{1*}

6 ¹ Department of Immunology and Microbiology, Scripps Research, La Jolla CA 92037

7 ²La Jolla Institute for Immunology, La Jolla CA 92037

8 ³Department of Pathology, University of Texas Medical Branch, Galveston, TX 77550

9 ⁴Galveston National Laboratory, University of Texas Medical Branch, Galveston, TX 77550

10 ⁵Proteomics Core, Scripps Research, Jupiter, FL 33458

11 ⁶Cancer Biology Program, Stanford University, Stanford, CA 94305

12 ⁷Department of Genetics, Department of Biology and Department of Chemistry, Stanford
13 University, Stanford, CA 94305

14 ⁸Chan Zuckerberg Biohub, San Francisco, CA 94158

15 ⁹Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston,
16 TX 77550

17 *Erica Ollmann Saphire; Juan Carlos de la Torre.

18 **Email:** erica@lji.org and juanct@scripps.edu

19 **Author Contributions:** J.F., E.O.S., and J.C.T designed research; J.F. performed all non-BSL4
20 experiments and analyzed data; C.P. performed all BSL-4 experiments; A.B. provided supervision
21 to BSL4 research; G.T. and G.C performed proteomic experiments and analyses; K.F.C. and
22 A.Y.T provided a critical resource; J.F., E.O.S., and J.C.T wrote the paper. All authors reviewed
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Abstract

Completion of the Lassa virus (LASV) life cycle critically depends on the activities of the virally encoded RNA-dependent RNA polymerase in replication and transcription of the negative-sense RNA viral genome in the cytoplasm of infected cells. We hypothesized that interactions with an array of cellular proteins may enable LASV polymerase to execute distinct viral RNA biosynthetic processes. To investigate this hypothesis, we applied proximity proteomics to define the interactome of LASV polymerase in cells, under conditions that recreate viral transcription and replication. We engineered a LASV polymerase-biotin ligase TurboID fusion protein that retained polymerase activity and successfully biotinylated the proximal proteome, which allowed us to identify 42 high-confidence hits that interact with LASV polymerase. We performed an siRNA screen to evaluate the role of the identified interactors in LASV infection, which uncovered six host factors for which their depletion affected LASV infection. We found that one polymerase interactor, eukaryotic peptide chain release factor subunit 3a (eRF3a/GSPT1), physically and functionally associated with LASV polymerase, exhibiting proviral activity. Accordingly, pharmacological targeting of GSPT1 resulted in strong inhibition of LASV infection. In summary, our work demonstrates the feasibility of using proximity proteomics to illuminate and characterize yet to be defined, host-pathogen interactomes, which can reveal new biology and uncover novel targets for the development of antivirals against LASV.

Significance Statement

Lassa virus (LASV), the causative agent of Lassa fever (LF), poses an important public health problem in Western Africa, and current therapeutic interventions are very limited. Due to its limited genome coding capacity, LASV proteins are often multifunctional and orchestrate complex interactions with cellular factors to execute the steps required to complete its viral life cycle. LASV polymerase is essential for the replication and expression of the viral genome, and it is consequently an attractive target for antiviral intervention. Here we present the first host interactome of LASV polymerase, which can guide the identification of novel druggable host cellular targets for the development of cost-effective antiviral therapies against LF.

Main Text

Introduction

Lassa virus (LASV) is a mammarenavirus highly prevalent in Western Africa, where it infects several hundred thousand individuals annually, resulting in a substantial number of Lassa fever (LF) disease cases, associated with high morbidity and significant mortality with high case-fatality rates (CFRs) among hospitalized LF patients (WHO). While rodent-to-human transmission is the main contributor to human LASV infection, a significant number of LF cases can arise from human-to-human transmission (1). Moreover, LASV expansion outside its traditional endemic areas and increased travel have resulted in exported LF cases from endemic Western African countries to non-endemic countries (2). To date, no US Food and Drug Administration (FDA) licensed countermeasures are available to prevent or treat LASV infections, and current anti-LASV therapy is limited to an off-label use of ribavirin that has limited efficacy and can cause significant side effects. Hence, there is an unmet need for therapeutics to combat LASV infections, a task that would be facilitated by a better understanding of virus-host cell interactions that modulate replication and gene expression of LASV in infected cells.

Like other mammarenaviruses (*Bunyvirales: Arenaviridae*), LASV is an enveloped virus with a bi-segmented, single-stranded RNA genome. Each viral genome segment uses an ambisense coding strategy to direct the synthesis of two viral proteins from open reading frames separated by non-coding intergenic regions (IGRs). The small (S) segment encodes the nucleoprotein (NP), which is responsible for genome encapsidation and immune evasion, and the glycoprotein precursor (GPC), which is co- and post-translationally processed to generate the mature GP that

mediates virion cell entry via receptor-mediated endocytosis (3). The large (L) segment encodes the large (L) protein, which functions as a viral RNA-directed RNA polymerase (RdRp), and the matrix Z protein. NP encapsidates the viral genome and antigenome RNA species to form the viral nucleocapsid (NC) to which L associates to form the two viral ribonucleoprotein complexes (vRNPs) (of L and S segments). The resulting vRNP is responsible for directing replication and transcription of the viral RNA genome. Cellular polymerases and translation machinery can neither read nor translate the viral RNA genome. Instead, the LASV polymerase initiates viral transcription from the genome promoter located at the 3' end of the viral genome, which is primed by a small fragment of host-cell-derived, capped RNA fragment via a mechanism called cap-snatching. Primary transcription results in synthesis of NP and L mRNAs from the S and L segments, respectively. Subsequently, the virus polymerase adopts a replicase mode and moves across the intergenic region (IGR) to generate a copy of the full-length antigenome RNA (cRNA). This cRNA serves as a template for the synthesis of the GPC and Z mRNAs from the S and L segments, respectively, as well as a template for the amplification of the corresponding genome vRNA species (4, 5).

Coordination of multiple domains with distinct enzymatic functions through conformational rearrangement and the engagement of vRNA promoter elements with LASV polymerase have been proposed to mediate the functional transitioning of LASV polymerase between a replicase and transcriptase (6). In the present paper, we have investigated the possibility that host cell factors can critically contribute to the distinct steps of viral RNA synthesis driven by LASV polymerase. As a first and necessary step, we applied the recently developed proximity labeling technology to characterize the cellular interactomes of LASV L polymerase in the context of viral RNA synthesis in living cells. For the first time, we generated a functional LASV polymerase fusion to the engineered promiscuous biotin ligase TurboID, which retains its polymerase activity and can biotinylate its cellular interactors *in situ*. This approach allowed us to affinity-capture biotinylated interactors, leading to the discovery of 42 high-confidence candidate interactors of the LASV polymerase. We implemented a high-content imaging-based, siRNA screen using infection with live LASV in the human hepatocyte Huh7 cell line, which identified six host factors that functionally contributed to LASV infection. We further characterized one of these factors, eukaryotic peptide chain release factor subunit 3a (eRF3a/GSPT1), and determined that it physically interacts with LASV polymerase and is required for LASV infection. Accordingly, pharmacological targeting of GSPT1 strongly inhibits LASV infection. Our results have improved our understanding of the cellular proteins and pathways involved in LASV multiplication and illuminate novel therapeutic strategies against a highly lethal human pathogen.

Results

Generating a PL enzyme-LASV L fusion protein with polymerase and proximity labeling activities. We selected TurboID, an engineered promiscuous biotin ligase, for proximity labeling due to its optimized labeling efficiency. TurboID labels exposed lysine residues on neighboring proteins within a ~10 nm radius and is highly active, requiring <10 minutes of labeling time for detectable activity (7). We incorporated TurboID together with an HA tag into the LASV L protein at a site previously shown to tolerate incorporation of epitope tags without severely affecting polymerase activity (8) (**Figure 1A**).

We confirmed that the LASV polymerase-TurboID fusion protein was functionally active using a LASV minigenome (MG) system (**Figure S1**). This cell-based viral MG system recapitulates LASV RNA synthesis using an intracellular reconstituted vRNP expressing a reporter gene of interest, in this case, a fluorescent protein ZsGreen. The expression level of the minigenome-encoded reporter serves as a surrogate measure of vRNP activity and as a comprehensive measurement of LASV MG replication, transcription, and translation of the minigenome reporter transcript (9).

We used a wild-type LASV L (L-WT) and an N-terminal HA tagged L (L-HA) as controls for comparison with LASV L-TurboID in the context of measuring polymerase activity and protein expression levels. Results from the LASV MG assay revealed that LASV L-TurboID fusion polymerase (L-TurboID) retains 70% of WT activity, with slightly lower expression levels compared

to that of the L-HA control (**Figure 1B**). We validated the construct's proximity labeling activity by detecting biotinylated proteins in cells expressing the viral polymerase-TurboID fusion together with vRNP components. We found that LASV L-TurboID produced a broad range of biotinylated proteins in the presence of exogenous biotin, and promiscuous labeling was observed in a time-dependent manner (**Figure 1C**).

Because western blot analysis using soluble cell lysates does not illuminate the subcellular localization of the biotinylated proteins, we used confocal fluorescent microscopy to detect in-cell biotinylation by LASV L-TurboID in fixed cell specimens. For these studies, we transfected HEK293T cells with LASV MG system components, either with LASV L-TurboID or L-HA control, followed by one-hour of biotin labeling, 24 hours post-transfection. We observed that LASV L-TurboID localized to discrete puncta connecting patches in the cytoplasm, similar to the N-terminal HA-tagged L control (L-HA). Furthermore, the biotinylation signal overlapped with the location of L-TurboID, suggesting efficient and localized biotinylation mediated by the LASV L-TurboID (**Figure 1D**).

Defining LASV polymerase interactomes by proximity proteomics. To set up the proximity labeling-based proteomics experiment, we transfected HEK 293T cells with LASV MG system components, including L-WT or L-TurboID followed by biotin labeling. Transfected cells were lysed, and biotinylated proteins were captured with streptavidin (SA) beads. Proteins enriched by SA beads were washed and subjected to on-bead trypsin digestion. Digested peptides in solution were labeled with unique tandem-mass tags (TMTs) for quantitative proteomic analysis (**Figure 2A**).

To assess the efficiency of the enrichment, biotinylated material bound to SA beads (SA-pull down) from equal amounts of starting material (Input) was eluted in SDS loading buffer containing DTT and biotin and analyzed by western blot. We observed enrichment of biotinylated proteins specific to samples with LASV L-TurboID expression. We observed that only a small fraction of LASV NP was biotinylated, which may reflect that only a low percentage of the total NP participates in the formation of a functional vRNP, or that in the vRNP, the majority of NP was not accessible to biotinylation or remained insoluble under the lysis conditions we used to prepare the proteomic samples. LASV L-TurboID fusion polymerase was also detected in the SA-pull down fraction, suggesting self-biotinylation (**Figure 2B**).

To analyze the LASV polymerase interactome, host proteins identified as LASV polymerase interactors were filtered by two criteria: degree of enrichment compared to the control proteome, and the corresponding statistical confidence. The control for the LASV L-TurboID (TurboID_pol) interactome was the interactome of LASV L-WT (WT_pol). For every identified protein, the abundance ratio in the TurboID_pol sample was normalized to that in the WT_pol sample, resulting in the value of fold change. Multiple comparisons were performed across three biological replicates and adjusted *p*-values (adjusted to False Discovery Rate of 0.05) were determined to identify proteins that were enriched in the L-TurboID interactome. A threshold of adjusted *p*-value < 0.05 and log₂(fold change) > 1 was used to narrow down high-confidence hits. In summary, we found 42 high-confidence hits for cellular proteins that interact with the LASV polymerase (**Figure 2C**).

Effect of siRNA targeting high-confidence LASV polymerase interactors on viral infection. Next, we assessed the importance of LASV polymerase-interacting protein candidates identified by proximity proteomics in authentic LASV infection. For this experiment, we used an siRNA-based functional screen based on well-established experimental procedures (10). Briefly, human hepatocyte Huh7 cells were first transfected with a library of individual siRNAs targeting each of the 42 high-confidence hits, and subsequently infected with the recombinant-LASV-eGFP 48 hours post-transfection (11) (**Figure 3A**). siRNA knockdown (KD) of proviral and antiviral candidate host proteins should lead to reduced and enhanced viral infection, respectively. Protein targets with at least two independent siRNA KD resulting in the same infection phenotype across two experiments were considered as true hits.

Surprisingly, most high-confidence viral polymerase interactors exhibited an antiviral role, as their siRNA-mediated knockdowns increased normalized %LASV-eGFP infection (**Figure 3B and S2**). Limited (10 - 20% of cells) infection of non-silencing control (NSC) siRNA transfected cells

(**Figure S3**) may have facilitated favoring the detection of enhancement over reduction in infection. Nevertheless, our results were supported by stringent statistical metrics (two independent experiments with two multiplicities of infection (MOIs), four independent siRNAs per target) and guided by quantitative parameters of the resulting phenotype (Relative % infection and cell count).

Potential antiviral factors identified for LASV include EIF3CL, EIF4G2, TAGLN2, TUBA1B, and UPF1. Potential proviral factors for LASV include GSPT1, RPL23, and PLK1 (**Figure 3C**). Knockdown of GSPT1 reduces LASV infection, without affecting the cell count. However, knockdown of the latter two, RPL23 and PLK1 reduces cell count as well as LASV infection, complicating definitive interpretation of a proviral effect. It is possible that these host factors support both LASV replication and cell survival during LASV infection.

Based on the results of the siRNA screen, we highlighted functional hits in our identified LASV polymerase interactome network. We clustered hits based on their STRING-classified (12) cellular pathways in order to extrapolate distinct cellular pathways relevant to LASV infection (**Figure 4A**).

Role of GSPT1 in LASV gene expression. To further validate the biological relevance of the interactome results, we focused on the translation termination factor eRF3a/GSPT1 (13), which has not been previously reported to play any specific role in viral infection.

First, we used co-immunoprecipitation (co-IP) assays to confirm that GSPT1, identified here by proximity proteomics, physically interacts with LASV L protein. To perform the co-IP experiment, we co-transfected HEK 293T cells with plasmids expressing an N-terminal Flag-tagged GSPT1 (long isoform, 68.7 KDa) and LASV L-HA, respectively, in the presence or absence of other viral factors. We found that LASV L-HA consistently immunoprecipitated Flag-GSPT1 in the presence or absence of LASV NP, or LASV NP and LASV minigenome (**Figure 5A**). We next examined the functional consequence of GSPT1 association with both viral polymerases by viral minigenome assays, which measure a combined effect on viral replication, secondary transcription, and the translation of a minigenome reporter. Consistent with results from the siRNA functional screens, we found that depletion of GSPT1 by one experimentally validated siRNA, GSPT1si8, (**Figure S4**) in HEK 293T cells significantly suppresses LASV minigenome activity (**Figure 5B**). In comparison, depletion of GSPT1 did not affect expression of a control eGFP reporter driven by an RNA polymerase II-based expression plasmid.

We also examined whether endogenous GSPT1 interacts with LASV polymerase in a cellular context that recapitulates viral RNA synthesis. In HEK 293T control cells, we observed that endogenous GSPT1 exhibited a diffuse nucleocytoplasmic distribution (**Figure 5C**). However, in HEK 293T cells that successfully reconstituted a functional LASV vRNP, as determined by ZsGreen reporter signal, we detected a fraction of cytoplasmic GSPT1 that clustered with the signal of LASV L-HA (**Figure 5D**), suggesting that GSPT1 can interact with LASV L inside cellular environment.

Role of GSPT1 in LASV multiplication. To validate the role of GSPT1 on LASV multiplication, we examined the phenotype of GSPT1 knock-down (KD) in multi-step LASV growth kinetics in Huh7 cells. Consistent with the results of the siRNA screen, GSPT1 depletion significantly impaired LASV growth in Huh7 cells with at least one log reduction of viral titer 48 hours post-infection (h.p.i.) (**Figure 6A**, 48 h.p.i.). This result supports the proviral role of GSPT1 in LASV infection. Given the known engagement of GSPT1 in cellular nonsense-mediated mRNA decay (NMD) (14, 15), we wondered whether the reduced viral titer was an outcome of decreased LASV mRNA accumulation in infected cells. However, the levels of accumulated LASV vRNA and c/mRNA (s-segment) at 6 h.p.i. were all unchanged in GSPT1-depleted cells (**Figure 6B**). In contrast, we observed that LASV GP2 protein levels were diminished in GPST1-depleted cells at 72 h.p.i. (**Figure 6C**). This finding may reflect the role of GSPT1 as a translation termination factor that mediates nascent peptide-chain release and ribosome recycling. Given unchanged LASV mRNA and decreased LASV GP2 protein levels, we reason that GSPT1 supports LASV infection perhaps by assisting viral protein translation.

Based on the effect of GSPT1 knockdown in LASV growth kinetics, we predicted that pharmacological inhibition of GSPT1 would suppress LASV multiplication. We therefore tested the antiviral activity of CC-90009, a cereblon E3 ubiquitin ligase modulator, which selectively targets

GSPT1 for proteasomal degradation (16, 17) (**Figure 6D**). We treated Huh7 cells with CC-90009 immediately following LASV infection and measured the impact of CC-90009 treatment on the LASV growth curve, viral RNAs, and protein accumulation. At 0.1 and 1 μ M, CC-90009 effectively inhibited LASV growth (**Figure 6E**), which correlated with the dose-dependent reduction of both vRNA and c/mRNAs accumulation within 6 hours of viral infection (**Figure 6F**) and diminished viral protein accumulation compared to the DMSO-treated control (**Figure 6G**), without exhibiting cytotoxicity (**Figure 6H**). Together, these results demonstrate that CC-90009 has potent antiviral activity against LASV in human hepatocytes by targeting GSPT1.

Discussion

Here we have presented the first host interactome of LASV polymerase in the context of viral RNA synthesis directed by an intracellular reconstituted vRNP. We first applied proximity-labeling technologies to identify the LASV polymerase interactors *in situ*. We then performed an siRNA screen in human hepatocytes infected with LASV to identify polymerase interactors functionally important during LASV infection. Our finding that most identified polymerase-interactors exhibited an antiviral rather than proviral phenotype could reflect that the viral polymerase itself or the RNA products that it synthesizes are targets of host innate defense mechanisms. Nevertheless, we validated one proviral factor, GSPT1, and demonstrated that it is a druggable target for abrogating LASV infection in cell cultures.

We identified 42 high-confidence LASV polymerase interacting cellular proteins. Among these high-confidence hits, 11 have been previously reported to interact with the NP and L protein of lymphocytic choriomeningitis virus (LCMV), an Old World mammarenavirus related to LASV, via affinity purification-mass spectrometry (AP-MS) approaches in the context of viral infection (18, 19). LCMV is a Biosafety level-2 (BSL2) infectious agent. In contrast, live LASV infection requires the use of BSL4 containment, which complicates proteomic experiments with LASV-infected cells. To overcome this obstacle, we used the TurboID-based, proximity-labeling approach to capture both stable and dynamic protein-protein interactions in the context of a cell-based MG system that recapitulates the biological activities of the LASV vRNP activity. The overlap between the LASV L proximity interactome described here and previously reported LCMV L and NP interactomes (**Figure 4B**) likely reflects conserved molecular recognition interfaces between host cells and Old World mammarenaviruses. Our siRNA-based functional validation studies of the LASV L interactome identified only six, among a total of 42, hits of which depletion significantly impacted LASV infection. This may reflect the limited dynamic range of the siRNA screen in LASV-infected Huh7 cells, despite the stringent thresholds we used to determine all significant functional interactors.

Many RNA viruses have evolved strategies to manipulate the host translational machinery to favor translation initiation of viral mRNAs (20-22). We identified two translation initiation factors in our LASV polymerase interactome, EIF4G2 and EIF3CL, as repressors of LASV infection. EIF4G2 is a functional homolog of EIF4G1 (23), which is known to bridge the high-affinity cytoplasmic cap-binding protein EIF4E, which binds to cap-containing mRNAs, and a scaffolding protein EIF3, which in turn binds to the small ribosomal subunit. In addition, EIF4G2 can enhance the interaction between EIF4E and cap-containing mRNAs (24). As with other mammarenaviruses, transcription of LASV mRNAs uses a 'cap-snatching' mechanism by which viral mRNAs hijack their 5' cap structures from host mRNA species (25), which primes the viral transcription and enables cap-dependent translation mediated by host ribosome (26). Although still controversial, the process whereby mammarenaviruses accomplish "cap-snatching" has been proposed to involve the ability of the virus NP to bind m7GpppN cap structures (27, 28), together with the activity of an endonuclease motif present within the N-terminal region of the LASV polymerase (29) that cleaves cellular mRNAs for acquiring the 5' cap structure. Importantly, the presence of EIF4G2 and EIF3CL but absence of EIF4E in our LASV L interactome suggest that LASV polymerase may not hijack the canonical host cap-binding complex. Instead, based on our results we speculate that L polymerase may compete with the canonical host cap-binding complex (containing EIF4E) for 5' capped cellular mRNAs, and L may bind to EIF4G2 as a decoy that would interfere with stable complex formation between EIF4E and capped mRNAs. This, in turn, would release capped

mRNAs to serve as primers for LASV transcription. Additional support for this model stems from the observation that cap-binding affinity of EIF4E was reduced upon binding of LASV Z protein to EIF4E (30), further lowering the affinity barrier for a LASV encoded cap-binding protein, or another proviral factor, to capture fragments of capped cellular mRNAs for priming LASV transcription (**Figure S5**).

In addition, our siRNA-based functional screen revealed another LASV polymerase interactor, UPF1, as an antiviral factor. The ATP-dependent RNA helicase upstream frameshift 1 (UPF1) is a key player in multiple cellular RNA decay pathways, typically through binding to the 3' UTR of a target transcript (31), suggesting that UPF1 might play an antiviral role in LASV infection by mediating LASV mRNA decay. We speculate that LASV mRNAs can trigger UPF1-dependent mRNA decay through either the nonsense-mediated mRNA decay (NMD) pathway or another mechanism resembling the replication-dependent histone mRNA decay pathway (HMD) (**Figure S6**). In the cellular NMD pathway, UPF1 degrades aberrant RNA transcripts by joining the translation-termination complex (GSPT1/eRF3-eRF1) upon ribosomal recognition of a premature termination codon (PTC) on the mRNA (32). After recruitment to the termination complex, UPF1 is phosphorylated, thereby triggering a downstream cascade of nucleases and de-capping enzymes to dismantle the target transcript (33). LASV mRNAs are likely to incorporate premature termination codons owing to the presence of an upstream open reading frame (uORF), which can be acquired by viral transcripts as byproducts of the cap-snatching mechanism (34). In the HMD pathway, phosphorylation of UPF1 is associated with the recognition of 3' UTR stem-loop structure on histone mRNA by the stem-loop-binding protein (SLBP). Similar to histone mRNAs, which lack 3' poly(A) tail, LASV mRNAs are also non-poly-adenylated and harbor a stem-loop structure on the 3' end (35, 36) due to the LASV intergenic region (IGR) mediated, structure-dependent transcription termination (37, 38). Future experiments are needed to determine whether UPF1-LASV L interaction is RNA-dependent, and whether UPF1 directly targets LASV mRNAs for degradation, perhaps by detecting UPF1 phosphorylation upon intracellular exposure to LASV mRNAs. SLBP was not captured in the LASV polymerase interactome, but we cannot rule out the possibility that SLBP recognizes the 3' stem-loop on LASV mRNAs. These findings provide the first evidence of a possible participation of the host RNA quality control mechanisms on modulating mammarenavirus infection.

We also identified DIS3 and PUM1 as LASV polymerase interactors. DIS3 is the catalytic subunit of host exosome complex, which participates in the downstream RNA degradation of UPF1-dependent mRNA decays (31), and contains both 3'-to-5' exoribonuclease and endoribonuclease activities (39). PUM1 is a sequence-dependent RNA sensor protein, which targets specific mRNAs for post-transcriptional degradation (40, 41). Since none of the LASV 3' UTRs contains the PUM1 consensus sequence (5'-UGUAHAUA-3'), it is unlikely that PUM1 targets LASV mRNAs for degradation. We speculate that LASV polymerase may selectively hijack key components of cellular mRNA decay pathway to harvest 5'-capped fragments of cellular transcripts. In this context, a subset of cellular mRNAs targeted by PUM1 and undergoing PUM1-mediated mRNA destabilization can be retrieved by LASV polymerase for the 5' cap structure before RNases such as DIS3 reach the 5' end of dismantled transcripts (**Figure S7**). The identification of multiple cellular mRNA decay components as LASV L interactors opens new directions on the investigation of the molecular basis of LASV cap-snatching.

G1-to-S-phase transition 1(GSPT1)/eukaryotic peptide chain release factor GTP-binding subunit A(eRF3a) was the only L-interactor that exhibited a proviral phenotype. GSPT1 is a core component of the cellular translation termination machinery (13, 42), also participates in the cellular NMD pathway, and has been shown to regulate cell cycle progression (43) and promote apoptosis (44). As GSPT1 participates in multiple cellular pathways, it may regulate viral infection via different mechanisms in a context-dependent manner. Results from co-IP assays showed a physical association between LASV L polymerase and GSPT1, supporting the validity of proximity labeling-based proteomics to identify *bona fide* LASV L-interactors. GSPT1 knock-down resulted in significantly reduced production of LASV infectious progeny in a multi-step growth kinetics experiment. This result correlates with a role of GSPT1 supporting viral protein translation, without

significantly affecting the accumulation of viral RNA species. Whether the proviral phenotype we have observed in GSPT1 is indirectly mediated by its involvement in regulating cell cycle or apoptosis remains to be determined. Future studies are needed to assess the functional consequence of the physical association between GSPT1 and LASV polymerase and to verify whether GSPT1 contributes to any step of LASV RNA synthesis. Notably, we found that CC-90009, a cereblon E3 ligase modulating drug which specifically targets GSPT1 for proteasomal degradation, exhibited potent antiviral activity against LASV without appreciable cytotoxicity. The fact that CC-90009 is currently in phase 1 clinical development for the treatment of acute myeloid leukemia (AML) raises the interesting possibility of exploring its repurposing as an antiviral drug against LASV.

In summary, we have presented here the first comprehensive proximity interactome of the LASV polymerase in the context of viral replication. For the proximity proteomics experiment, we used a cell-based LASV MG system that recreates the molecular events associated with viral RNA synthesis during *bona fide* LASV infection. We validated the protein-protein interaction networks using an siRNA-based, functional screen with authentic LASV infection. As a proof-of-concept, we characterized the mechanisms of action by which GSPT1, a novel host factor identified here for the first time as a proviral factor required for LASV infection. Further work is required to explore the role of these functional interactors in other primary target cells including macrophage and dendritic cells and in suitable animal models. Likewise, the antiviral effect of CC-90009, a pharmacological compound targeting GSPT1, should be examined in other related arenaviruses. Together, our findings illuminate new mechanisms of host regulation of LASV replication and reveal a landscape of novel targets for host-directed antiviral drug development.

Materials and Methods

Plasmids, siRNAs, antibodies, primers: see SI Appendix.

Cell cultures and viruses: see SI Appendix.

Compound: CC-90009 (GSPT1-targeting compound) was purchased from MedKoo Biosciences (Cat# 207005).

LASV minigenome (MG) assay: HEK 293T cells (2×10^5 per well) were seeded in 24-well plates one day prior to transfection with the following plasmids: 300 ng pCAGGS-T7 polymerase, 300 ng pCAGGS-LASV MG encoding a ZsGreen fluorescent reporter, 300 ng pCAGGS-LASV-L, 150 ng pCAGGS-LASV-NP. In some cases: pCAGGS-LASV-L was replaced with a tagged variant. A control was included in every assay in which the pCAGGS-L plasmid was omitted to verify active MG RNA synthesis mediated by LASV L. Plasmids were transfected with Lipofectamine 3000 transfection reagent (1.5 μ l/well). At 72 hours post-transfection, cell lysates were collected and measured for green fluorescence intensity.

Biotinylation with polymerase-TurboID fusion protein: See SI Appendix for experimental details.

Immunofluorescent analysis (IFA) using confocal microscopy: Acetone cleaned, glass coverslips (1.5 mm thickness) placed inside 24-wells were treated with human fibronectin (50 mg/ml) for 20 min at 37 °C incubator, prior to HEK 293T cells seeding (4×10^4 /well). Twenty-four hours later, the monolayer was transfected with the LASV minigenome system using Trans-IT LT1 as described. For in-cell biotinylation experiment, biotin (500 μ M final concentration) was added to the culture media allowing for one hour in-cell biotinylation of cells transfected with the LASV MG components. Labeling was stopped by washing cells with cold DPBS. For all IFA specimens, cells were fixed in 4% paraformaldehyde for 15 mins at room temperature, permeabilized with 0.1% Triton-X100 for 10 mins, and blocked in 1% normal goat serum for at least one hour. Primary antibody incubations were performed at room temperature for two hours or at 4 °C overnight.

Specimens were washed three times with PBS-0.1% Tween-20 and incubated with secondary antibodies for one hour at room temperature. Nuclei were counterstained by Hoechst. Confocal images were acquired on Zeiss LSM880-airyscan system under super-resolution mode, using a 63x/NA1.4 oil objective. Please see *SI Appendix* for full description of antibodies used in IFA experiments.

Proximity labeling-based proteomics (sample preparation, quality control, proteomic experiment, and data analysis): See *SI Appendix* for details.

siRNA functional screening: Huh7 cells (5×10^3 per well) were seeded in clear-bottom, black 96-wells plate, leaving edge wells filled with DPBS. Twenty-four hours, Huh7 monolayers were transfected with 10 nM of individual siRNAs using Lipofectamine RNAiMAX (0.1 μ l per well). Knock down (KD) of each target was performed using four individual siRNA transfections, each one performed in triplicate. Each plate includes two sets of triplicated internal control wells transfected with 10 nM All Star negative control (NSC) to account for plate-to-plate variations. Forty-eight hours later, plates were transferred to BSL4 for infection with LASV-eGFP at indicated MOIs. Infected monolayers were fixed and inactivated by 10% formalin at two days post infection and removed from BSL4 facility. Percentage of LASV-eGFP infected Huh7 cells (% infection) with different siRNA treatment were quantified by high-content imager CX5 using a 10x objective. Cell count in each well was determined by the number of Hoechst-stained nuclei. Data analysis of siRNA screen described in *SI Appendix*.

Viral growth kinetics and quantification of viral proteins and RNAs: Huh7 cells (5×10^4 per well) were seeded in 24-well plates and transfected with 10 nM siRNA (NSC or GSPT1 si8, experimentally validated) the next day as described above or incubated with 0.1 μ M GSPT1 inhibitor (CC-90009) or DMSO. Forty-eight hours after transfection, Huh7 monolayers were transferred into BSL-4 facilities and infected with LASV-eGFP at MOI of 0.5 (PFU/ml) for one hour. Infection of the cells was followed by removal of the inoculum and replenishing with 2% FBS containing fresh media. For virus titration, aliquots of supernatant were harvested at indicated time points from infected monolayers and used to infect Vero-E6 cells for foci-quantification. At indicated time points, infected Huh7 monolayers were collected directly in Trizol or 4x Laemmli buffer for inactivation and downstream RNA/protein quantifications. Equal volume of Laemmli lysates were loaded on SDS-PAGE gel for western blot analysis. Equal amount of total RNA in each sample was used for strand-specific RT-qPCR.

Co-immunoprecipitation: Full description in *SI Appendix*.

Cell viability quantification by Cell-titer Glo assay: Full description in *SI Appendix*.

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Figures and figure legends

Figure 1. Generation of a functional LASV polymerase with proximity-labeling activity.

(A) Expression construct of LASV L-TurboID fusion polymerase and a schematic describing proximity biotinylation of LASV L interacting host factors. (B) Polymerase activity and expression of LASV L-TurboID compared to wild-type L and HA-tagged L in cells transfected with the LASV minigenome (MG) components. ZsGreen fluorescence signal was quantified in each sample and normalized to the L-WT control. Representative western blots showing expression of LASV L variants and β -actin in HEK293T lysates. LASV MG assay was repeated in three independent replicates with triplicated wells for each condition. Bar graphs containing nine individual data points for each condition are displayed, with means plus standard error of the means/SEM (error bars). Two-tailed, unpaired t tests were performed comparing activity of different polymerase variants to the wild-type controls. Values significantly different from the controls are indicated by asterisks as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) Streptavidin blot demonstrating biotin ligase activity of LASV L-TurboID in cells transfected with the LASV MG components. (D) Confocal

564 immunofluorescence images of LASV L-TurboID mediated proximity biotinylation in cells co-
565 expressing the LASV MG components. LASV L-HA and L-TurboID were detected by using an anti-
566 HA antibody. Biotinylated proteins were detected by using the streptavidin-AF594. Scale bar: 5 μ m.
567 Representative images acquired by Zeiss-LSM880 Airyscan from two independent experiments
568 are shown. Each experiment includes at least three different fields of view.

569

570 **Figure 2. Proximity proteomics of LASV polymerase in living cells.**

571 (A) Proximity proteomic sample preparation workflow. (B) Validation of streptavidin enrichment of
572 LASV L-TurboID mediated proximity biotinylation. Total lysates are indicated as “Input”, biotinylated
573 proteins enriched by streptavidin magnetic beads and indicated as “SA-pull down”. Representative
574 blots and a silver-stained gel of three biological replicates are presented. (C) A scatter plot showing
575 LASV polymerase interacting proteins in HEK 293T cells determined by quantitative proteomic
576 analysis. For each protein, its degree of enrichment indicated by fold change and statistical
577 confidence of enrichment, which is indicated by adjusted p-value, are numerated and log
578 transformed. High-confidence hits are highlighted by red rectangular box.

Figure 3. Identification of functional polymerase interactors by siRNA screen in LASV-infected cells. (A) Workflow of siRNA screen by high-content imaging of virus infected Huh7 cells. Raw percentage of infection and cell count of each siRNA treatment were normalized to that of non-silencing controls (NSC), respectively. (B) Normalized values are displayed in heat maps of relative percentage of infection and relative cell count for LASV. Each value is the mean of technical triplicates. Multiple unpaired t tests were performed to determine the statistical significance of siRNA-mediated changes on percentage of infection compared to that of NSC. Determined *p*-values were log-transformed and displayed as the size of each data point on a scatter plot for LASV (C). Data points in the heatmap with a *p*-value > 0.05 were eliminated from the scatter plot. Genes for which multiple siRNAs significantly affected infection are labeled on the corresponding data points; one label is in red for display purpose. Representative fluorescent images of Huh7 monolayer at 48 hours post infection are shown with LASV-eGFP infected cells in green and nuclei in blue for NSC and selected siRNA treatment. One representative result from two rounds of siRNA screen using different MOIs is shown.

Figure 4. The proximity interactome of LASV polymerase. (A) Network display of the LASV polymerase interactome identified by proximity proteomics. Each node represents one high-confidence proteomic hit. Each edge represents identified protein-protein interaction. Nodes that are functionally validated by siRNA screen are highlighted in pink. Nodes shown in heptagons have been previously reported for other virus-host interactomes. Selected nodes are clustered based on enriched biological processes or protein domains by functional enrichment analyses via STRING. (B) A Venn diagram comparing cellular interactors of LASV polymerase with previously reported interactors of LCMV proteins.

Figure 5. GSPT1 physically and functionally associates with LASV polymerase. (A) Western blot analyses of LASV L-HA co-immunoprecipitated with Flag-GSPT1. Representative result of three biological replicates is shown. (B) LASV MG activity and expression level of a control GFP reporter in GSPT1-depleted HEK 293T cells. GSPT1 depletion by siRNA was validated by western blot analysis. MG activity and expression level of the control GFP reporter in GSPT1si8 transfected cells were normalized to that of NSC. The LASV MG experiment was repeated in three independent replicates with triplicated wells for each condition. Bar graphs containing nine individual data points for each condition are displayed, with means plus SEM (error bars). Two-tailed, unpaired t tests were performed to determine whether depleting or overexpressing GSPT1 significantly changed activities of viral minigenome and the control reporter. Values that are significantly different from the controls are indicated by asterisks as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Confocal immuno-fluorescent analysis of the subcellular localization of endogenous GSPT1 by itself (C) or with transiently expressed LASV L-HA together with the LASV minigenome system in HEK 293T cells (D). Arrowheads in the zoomed region indicate sites where endogenous GSPT1 localize next to viral proteins. Representative images acquired by Zeiss-LSM880 Airyscan from two independent experiments are shown, each experiment includes at least three different fields of view.

Figure 6. GSPT1 is a druggable host factor required for LASV multiplication. (A) Effect of GSPT1 depletion on LASV viral growth kinetics in Huh7 cells. Representative fluorescent images of Huh7 monolayer at 72 hours post-infection (h.p.i.) are shown with virus infected cells (green) and nuclei (blue). Results of two independent experiments with technical triplicates are plotted as mean and standard deviations (error bars). Two-way ANOVA analysis on log transformed viral titers was performed to determine the statistical significance of the effect of GSPT1 depletion on viral titers. (B) Effect of GSPT1 depletion on LASV vRNA and c/mRNA accumulation at 6 h.p.i.. Six individual data points from two independent experiments with technical triplicates are displayed indicated as mean and SEM (error bars). Values that are significantly different from the controls were determined by two-tailed, unpaired t-test and indicated by asterisks as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (C) Western blot analysis of GSPT1 protein and LASV GP2 levels in lysates from LASV-infected Huh7 cells harvested at 72 h.p.i.. Lysates from triplicate wells in the growth kinetic experiments were pooled and analyzed. Representative blots from one experiment are shown. (D) Schematics of the mode of action for CC-90009 mediated GSPT1-targeted degradation. (E) Effect of CC-90009 on LASV growth kinetics in Huh7 cells. Results of one experiment with technical triplicates are plotted as mean and standard deviations (error bars). N.D.: not detectable.

Two-way ANOVA analyses on viral titers with Tukey's multiple comparisons were performed to determine the statistical significance of the effect of CC-90009 treatments on LASV titer compared to DMSO treatment. (****, $P < 0.0001$) (F) Effect of CC-90009 treatment on LASV vRNA and c/mRNA accumulation in Huh7 cells at 6 h.p.i.. Six individual data points from two independent experiments with technical triplicates are displayed indicated as mean and SEM (error bars). Values significantly different from the controls were determined by two-tailed, unpaired t-test and indicated by asterisks as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (D) Western blot analysis of GSPT1 protein and LASV GP2 levels in LASV-infected Huh7 lysates harvested at 72 h.p.i.. Lysates from triplicate wells in the growth kinetic experiments were pooled and analyzed. (H) Cell viability of CC-90009 treated Huh7 cells in the absence of viral infection was determined by Cell titer Glo 2.0. Raw values of cell viability in drug treated samples are normalized to DMSO control. Six individual data points from two independent experiments with technical triplicates are combined and indicated as mean and SEM (error bars). Values that are significantly different from the controls are determined by two-way ANOVA and indicated by asterisks as follows: *, $P < 0.05$.