1 **Functional interactomes of the Ebola virus polymerase identified by proximity proteomics** 2 **in the context of viral replication**

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19 **SUMMARY**

20 Ebola virus (EBOV) critically depends on the viral polymerase to replicate and transcribe the viral
21 RNA genome. To examine whether interactions between EBOV polymerase and cellular and viral
22 factors affect distinct viral RNA synthesis events, we applied proximity proteomics to define the
23 cellular interactome of EBOV polymerase, under conditions that recapitulate viral transcription and
24 replication. We engineered EBOV polymerase tagged with the split-biotin ligase split-TurboID,
25 which successfully biotinylated the proximal proteome while retaining polymerase activity. We
26 further analyzed the interactomes in an siRNA-based, functional screen and uncovered 35 host
27 factors, which, when depleted, affect EBOV infection. We validated one host factor, eukaryotic
28 peptide chain release factor subunit 3a (eRF3a/GSPT1), which we show physically and functionally

associates with EBOV polymerase to facilitate viral transcription termination. Our work demonstrates the utility of proximity proteomics to capture the functional host-interactome of the EBOV polymerase and to illuminate host-dependent regulations of viral RNA synthesis.

INTRODUCTION

Ebola virus (EBOV) is a member of the *Filoviridae* family in the order of *Mononegavirales*. Several ebolaviruses cause sporadic outbreaks of hemorrhagic fever disease that are unpredictable in timing and location and are associated with high mortality. The 2013-2016 epidemic emerged far from the location of any previously known EBOV outbreak, entered urban populations, and caused over 11,000 deaths. EBOV re-emerged in 2018 and again in 2020 and 2021 (WHO). Survivors of EBOV infections can experience long-term sequelae (Adekanmbi et al., 2021; Eghrari et al., 2021; Xu et al., 2019), and may harbor virus in immune-privileged sites, posing a risk of transmitting the virus months to years after recovery. Inmazeb, an EBOV-specific monoclonal antibody cocktail, is currently the only FDA-approved treatment for people infected with EBOV. There are no small molecule therapies to combat EBOV infections. Small molecule antivirals have fewer obstacles associated with cost and delivery and can suppress viral replication in immune-privileged sites.

The EBOV genome is a single-stranded, negative-sense, multicistronic RNA molecule that encodes 7 viral genes. The largest gene, L, is the catalytic subunit of the EBOV viral RNA-dependent RNA polymerase (RdRp), which is essential for replication and expression of the viral RNA genome. L and the viral polymerase cofactor VP35 together form the functional polymerase that acts on the EBOV genome. The viral RNA genome (vRNA) is coated by the viral nucleoprotein (NP), and in concert with the associated RdRp, form the viral ribonucleoprotein complex (vRNP).

Following cell entry, EBOV delivers its vRNP into the host cell cytoplasm where viral replication and transcription take place. Transcription of vRNA by the EBOV polymerase initiates at a single promoter located at the 3'-end of the genome, and discontinuously proceeds through the multicistronic viral genome, stopping at each gene end (GE) signal, which are conserved across all gene borders. At each GE, EBOV polymerase can either reinitiate transcription at the adjacent gene start (GS) signal of the downstream gene, or dissociate and return to the same 3' viral promoter element to initiate a new round of transcription. Thus, EBOV polymerase synthesizes mostly monocistronic viral mRNAs that are present in a progressively decreasing amounts relative to the distance from the promoter due to the transcription attenuation at each gene border. The resulting viral mRNAs can then be translated into viral proteins and sustain progression of the EBOV life cycle. The EBOV polymerase can also adopt a replicase mode, using vRNA as a template to produce a full-length complementary strand (complementary RNA; cRNA), which in

turn serves as a template for synthesis of large amounts of NP-coated, progeny viral genomes (Mühlberger, 2007).

The underlying mechanisms by which EBOV executes transcriptase and replicase activities are not fully understood, partly due to a lack of structural insight for the full-length EBOV polymerase. For RdRps of other negative-strand RNA viruses for which structural information is available, coordination of multiple domains with distinct enzymatic functions and conformational rearrangement of different domains can endow the viral polymerase with different functions (Te Velthuis et al., 2021). Viral trans-factors can also facilitate function switching (Fearn and Collins, 1999; Muhlberger et al., 1999). For instance, the EBOV transcription factor VP30 can recognize non-coding, cis-regulatory sequences within the viral genome and contribute to transcriptase activity of EBOV polymerase (Biedenkopf et al., 2013; Biedenkopf et al., 2016).

Here, we asked what role cellular factors might play in modulating distinct steps of viral RNA synthesis mediated by the EBOV polymerase. A previous study examined cellular factors that interact with the EBOV polymerase, but the results were based on over-expression of L in the absence of other needed viral cofactors (Takahashi et al., 2013), which may not have captured the complete functional polymerase interactome. We applied recently developed proximity labeling technologies (Reference) to characterize the cellular interactomes of EBOV polymerase in the context of viral RNA synthesis in living cells. We identified 43 high-confidence candidate interactors of EBOV polymerase in the presence of VP30, and 21 interactors that associate with EBOV polymerase in the absence of VP30. Using a high-content imaging-based siRNA screen with human hepatocyte Huh7 cells infected with authentic EBOV under BSL-4 containment, we confirmed that most of these 21 hits play a functional role in EBOV infection. We focused on one functional interactor, eukaryotic peptide chain release factor subunit 3a (eRF3a/GSPT1), and determined that it physically and functionally associates with the EBOV polymerase. Our results suggest that at early stages of EBOV infection, GSPT1 has an antiviral effect, but at later stages of infection, EBOV hijacks GSPT1 to support viral transcription termination. Our work uncovered a network of host factors that interact with EBOV polymerase and functionally participate in the EBOV life cycle. Further characterization of these host factors can provide new insights into EBOV replication and illuminate novel therapeutic targets for small molecule antiviral development or drug repurposing.

RESULTS

Generating a functional EBOV polymerase with proximity labeling activity. We selected TurboID, an engineered promiscuous biotin ligase that is optimized for biotinylation of exposed lysine residues on proteins within a ~10 nm radius (Branon et al., 2018). Since the EBOV polymerase (EBOV_pol) requires both L and VP35 proteins to function, we used the split-TurboID

system, which was engineered from full-length TurboID to produce two inactive fragments (Cho et al., 2020), sN- and sC-TurboID, that when brought together via protein-protein interactions, reconstitute full-length TurboID and its biotin ligase activity. To construct EBOV L-sNTurboID, we inserted sNTurboID into a predicted flexible loop region in EBOV L, a site that can tolerate insertion of mCherry (Hoenen et al., 2012). To construct EBOV VP35-sCTurboID, we fused the sCTurboID and a HA tag to the EBOV VP35 C terminus, thus preserving the N terminus and internal oligomerization domain, which are implicated in binding to NP and L, respectively, and which are both needed to support viral RNA synthesis (Leung et al., 2015; Moller et al., 2005). Interaction of EBOV L with VP35 upon EBOV_{pol} formation allows trans-complementation of the associated split-TurboID fragments and biotinylation of neighboring proteins. In addition, the presence of the EBOV transcription factor VP30 can promote conversion of EBOV_{pol} from a replicase to a transcriptase (Biedenkopf et al., 2013), and likely modulates the polymerase interactome (**Figure 1A**).

We confirmed that EBOV_{pol} split-TurboID fusion is functionally active using an established EBOV viral minigenome (MG) system (Jasenosky et al., 2010a) (**Figure S1**). This MG system recapitulates viral RNA synthesis using a Firefly luciferase reporter as a comprehensive measure of MG replication, transcription, and translation of the MG reporter transcript. As a control, we used wild-type VP35 (VP35-WT) and wild-type L (L-WT). We measured polymerase activity in the presence or absence of VP30, using the activity of WT EBOV_{pol} (L-VP35 WT) in the presence of VP30 as the standard for normalization. L-WT and C-terminal HA-tagged VP35 (VP35-HA) were compared to VP35-sCTurboID, which served as a control for VP35 expression levels. To increase VP35 expression, we transferred both VP35-HA and VP35-sCTurboID from the original pCEZ to the pCAGGS vector. The EBOV_{pol} split-TurboID fusion (L-VP35-sTurboID) retains 65% of WT polymerase activity (L-VP35-WT) without noticeable difference in expression levels. The split-TurboID tagged VP35 protein (VP35-sCTurboID) expressed at higher levels than the VP35-HA control. Interestingly, withdrawal of VP30 did not affect activity of either WT or split-TurboID tagged EBOV_{pol} (**Figure 1B**). This result suggests that, in contrast to other EBOV MG systems (Biedenkopf et al., 2016), the EBOV MG construct we used here permits VP30-independent transcription, a previously described non-canonical transcription mode (Weik et al., 2002). The retention of functional activity by our EBOV MG system without VP30 allows us to probe a context-dependent viral polymerase interactome in the absence of VP30, and downstream validation of VP30-dependent roles of interactors of interest.

We validated the proximity labeling activity by detecting biotinylated proteins in cells expressing EBOV_{pol} split-TurboID fusion together with components of the EBOV MG system. EBOV_{pol} split-TurboID fusion produced, in a time-dependent manner, a broad range of biotinylated proteins in the presence of exogenous biotin (**Figure 1C&S2**).

We used confocal microscopy to detect biotinylation by EBOV_pol split-TurboID fusion in fixed cell specimens. HEK 293T cells transfected with the EBOV MG system, either with the split-TurboID fusion or epitope-tagged EBOV_pol (control) were labeled with biotin for one hour at one day post-transfection. In the absence of a suitable EBOV L-specific monoclonal antibody for immunofluorescence, we used polymerase cofactor VP35 localization as a proxy for EBOV_pol localization. VP35-sCTurboID fluorescence signals were confined inside viral inclusion bodies and overlapped with biotinylation signals. The VP35-HA control was present only at the edges of inclusion bodies and no endogenous biotinylation signal was observed (**Figure 1D**). These different localization patterns of VP35 may be due to the higher expression level of VP35-sTurboID relative to VP35-HA or partitioning of other viral proteins in the EBOV inclusion body, as both phenotypes (inclusion-filling and inclusion-edge) are found in EBOV-infected cells (Nanbo et al., 2013). Most EBOV VP30 did not co-localize with VP35-positive inclusion bodies, but instead formed its own inclusions. The distinct localization is consistent with the VP30-independent EBOV MG activity.

Proximity proteomics to define EBOV polymerase interactomes. For proximity labeling-based proteomics experiments, we transfected HEK 293T cells with the EBOV MG system components, including wild-type EBOV_pol (WT_pol) or EBOV_pol split-TurboID (sTurboID_pol), followed by biotin labeling. Transfected cells were lysed, and biotinylated proteins were captured with streptavidin (SA) beads that were then subjected to on-bead trypsin digestion. Digested peptides in solution were labeled with unique tandem-mass tags (TMTs) for quantitative proteomics (**Figure 2A**).

To assess enrichment efficiency, biotinylated material bound to SA beads (SA-pull down) from equal amounts of starting material (Input) was eluted in SDS loading buffer and analyzed by western blot (**Figure 2B**). We saw enrichment of biotinylated proteins specific to samples with sTurboID_pol expression compared to those with WT_pol expression. The addition of VP30 to sTurboID_pol appeared to slightly suppress protein expression of both sTurboID_pol and VP30 itself, resulting in fewer biotinylated proteins (**Figure 2C**). Both components of EBOV sTurboID_pol were detected in the SA-pull down fraction, suggesting that self-biotinylation occurred. Unexpectedly, the SA-pull down contained no VP30, indicating that it may not directly associate with EBOV L or VP35, or that its spatial localization was outside the labeling radius of sTurboID_pol. Consistent with this finding, we saw no appreciable colocalization of VP30 with VP35-positive inclusion bodies (**Figure 1D**).

To analyze EBOV_pol interactomes, we assessed host proteins identified in the proximity interactome by two criteria: degree of enrichment compared to the control proteome (WT_pol), and the corresponding statistical confidence. We normalized the abundance ratio for every protein in the TurboID_pol sample to that in the WT_pol sample and determined the value in terms of fold-change. We then performed multiple comparisons across three biological replicates and

determined adjusted *p*-values (adjusted to False Discovery Rate of 0.05) to identify proteins enriched in the TurboID_{pol} interactome. High-confidence hits were selected using a threshold of adjusted *p*-value < 0.05 and log₂(fold change) > 1. We found 43 high-confidence hits for cellular proteins that interact with the EBOV polymerase in the presence of VP30 (**Figure 2C**). Another 28 high-confidence hits were found in the absence of VP30 (**Figure 2D**), 7 of which were the same as those seen in the presence of VP30.

Effect of siRNA targeting high-confidence EBOV polymerase interactors on viral infection. Next, we used an siRNA-based functional screen (Fang et al., 2018) to assess how the EBOV polymerase-interacting protein candidates affected EBOV infection in cultured cells. We transfected human hepatocyte Huh7 cells with individual siRNAs targeting each of 64 high-confidence hits and subsequently infected them with recombinant EBOV-eGFP (Towner et al., 2005). We determined the percentage of GFP-positive cells, as an indicator of EBOV infection, at 48 hours post-infection (hpi) (**Figure 3A**). We excluded targets for which depletion significantly altered the cell count, and considered as true hits those having at least two independent siRNA-KD resulting in the same infection phenotype across two experiments.

Surprisingly, most high-confidence hits are antiviral, shown by an increased percentage of EBOV-eGFP infection in the presence of siRNA-KD (**Figure 3B**). Limited (10-30% of cells) infection rates for cells transfected with non-silencing control (NSC) siRNA may have favored detection of enhanced infection over reduced infection. However, our results are strongly supported by stringent statistical metrics (two independent experiments with two MOIs, 4 independent siRNAs per target) and are guided by quantitative parameters of the resulting phenotype (i.e., Relative % infection and cell count) (**Figure S3**). We identified 35 potential EBOV-specific antiviral factors (**Figure 3C&D**, **Figure S4**). siRNA knockdown of CKAP5 or RPL18A reduced both EBOV infection and cell count, which prevented their consideration as proviral factors. In contrast, depletion of the EBOV entry receptor NPC1 (positive control) by two individual siRNAs inhibited EBOV infection by nearly 50%, without significantly affecting the cell count (**Figure 3D, top panel**).

Based these results, we highlighted functional interactors identified in the proximity proteomics experiment in an EBOV polymerase interactome network. We clustered all polymerase interactors based on their STRING-classified biological processes (Szklarczyk et al., 2019). Many interactors are involved in critical cellular pathways that could be functionally involved in the EBOV life cycle (**Figure 4A**).

GSPT1 is functionally relevant to EBOV RNA synthesis. Two functional polymerase interactors, UPF1 and GSPT1, are both key components of the cellular nonsense mRNA decay (NMD) pathway. UPF1 restricts infection by multiple RNA viruses (May and Simon, 2021), whereas GSPT1 has not been implicated in viral infections. Thus, as a proof-of-principle, we focused on GSPT1,

which is a translation termination factor that is also termed eRF3a, eukaryotic peptide chain release factor GTP-binding subunit (Zhouravleva et al., 1995).

First, we used co-immunoprecipitation (co-IP) assays to verify whether GSPT1 indeed physically interacts with EBOV polymerase (EBOV_pol). Using HEK 293T cells co-expressing an N-terminal Flag-tagged GSPT1 (long isoform, 68.7KDa) and EBOV polymerase components, either individually or in combination, we found that L-WT, in the presence of VP35-HA, consistently co-IPs with Flag-GSPT1 (**Figure 5A**). However, Flag-GSPT1 did not co-IP with the VP35-HA component of EBOV_pol (**Figure 5B**, left panel). Interestingly, we observed a pronounced degradation product of L-WT in the input cell lysate but not the co-IP fraction, suggesting that the interaction between Flag-GSPT1 and EBOV L-WT requires full-length L (or at least the presence of regions otherwise subject to degradation). In addition, EBOV VP30 associates with Flag-GSPT1 only in the presence of EBOV_pol; VP30 alone does not associate with GSPT1 (**Figure 5A&B**, right panel). L, VP35 and VP30 all bind RNA, but GSPT1 is not known to have RNA-binding activity. Therefore, the GSPT1-EBOV protein-protein interactions we detected are likely not due to indirect interactions bridged by RNA.

We next examined whether endogenous GSPT1 interacts with EBOV_pol in a cellular context that recapitulates viral RNA synthesis. In HEK 293T cells transfected with the components of the EBOV MG system, we observed a specific pattern of GSPT1 clusters in the cytoplasm that appear to contact EBOV VP35-positive inclusion bodies (**Figure 5C**, top panel). In control cells, however, endogenous GSPT1 has a mostly diffuse nucleocytoplasmic distribution, with sporadic clusters occasionally observed (**Figure 5C**, bottom panel). Accordingly, we determined the functional consequence of GSPT1 association with EBOV_pol using the EBOV MG assay. Consistent with the siRNA screen results, over-expression or GSPT1 knockdown (KD) significantly decreases and increases, respectively, EBOV MG activity. Meanwhile, changes in GSPT1 expression level did not significantly affect expression of a control eGFP-reporter (**Figure 5C&S5**). Together, our data suggest that GSPT1 is a *bona fide* EBOV polymerase interactor that may participate in EBOV viral RNA synthesis.

Hijacking of GSPT1 by EBOV to facilitate transcription termination. For the siRNA screen we measured viral infection at a single time point (48 hours post-infection). Thus, we sought to further validate the phenotype of GSPT1-KD by following the multi-step EBOV growth kinetics in Huh7 cells. We first confirmed that GSPT1-KD persisted for 7 days post-siRNA transfection, supporting that a reduction in endogenous GSPT1 would be likely sustained throughout the 4-day viral growth curve experiments (**Figure S5**). We infected GSPT1-KD cells with EBOV and measured viral titers in the cell supernatant on 4 consecutive days. We quantified viral RNA and protein levels in infected cell lysates, and imaged infected monolayers at the experiment endpoint.

Intriguingly, GSPT1-KD initially increased EBOV titers by 2- to 4-fold relative to the non-silencing control (NSC). However, at 3- and 4-days post-infection (dpi), GSPT1-KD correlated with reduced EBOV titers (**Figure 6A**). At 4 dpi, we observed an increase in EBOV NP mRNA accumulation, with a marginal decrease in EBOV vRNA accumulation (**Figure 6B&C**). Despite the increase in viral mRNA, viral protein levels, including EBOV glycoprotein subunit 2 (GP2), were reduced, and fewer EBOV-infected cells were seen with GSPT1-KD (**Figure 6C&D**). Notably, we observed that EBOV infection triggered a significant upregulation of GSPT1 mRNA levels (**Figure 6E**), and the appearance of a lower-molecular weight GSPT1 species (~40kDa) that could correspond to a cleavage product of GSPT1 (**Figure 6C**). These findings suggest virus-specific, time-dependent regulation of GSPT1 protein levels.

GSPT1 antiviral activity during the first 48 hours of EBOV infection could be linked to the role of GSPT1 in the cellular NMD pathway (Hoshino, 2012). The NMD pathway often targets viral mRNAs for degradation and thereby restricts growth of many RNA viruses, some of which evolved mechanisms to evade this host restriction (Balistreri et al., 2017). EBOV infection induced upregulation of GSPT1 and appearance of the GSPT1 cleavage product is consistent with EBOV antagonizing GSPT1-assisted, post-transcriptional elimination of viral mRNAs. However, the observed increase in EBOV mRNA levels does not translate into increased accumulation of any viral protein, suggesting that GSPT1 may play another role in the EBOV life cycle.

The concurrent increase in EBOV mRNAs and decrease in EBOV protein accumulation might reflect aberrant transcription triggered by GSPT1-KD. For the multicistronic EBOV genome, one type of aberrant transcription is read-through mRNA synthesis, which occurs when the viral polymerase reads-through the transcription termination signal of an upstream gene to continue transcribing the downstream gene unit (Brauburger et al., 2014). The outcome of readthrough mRNA synthesis can be an apparent increase in EBOV mRNA levels that is associated with fewer transcription attenuation steps and accompanied by decreased viral translation of the downstream genes in the readthrough mRNAs.

The accumulation of all EBOV mRNAs we measured was increased upon GSPT1-KD. However, the magnitude of this increase was greater for genes located on the promoter-distal, 5' end of the EBOV genome, compared to genes on the promoter-proximal, 3' end at the polymerase entry site (**Figure 6F&G**). Accordingly, in GSPT1-depleted cells, we detected higher incidence of EBOV transcription read-through in 3 of the 4 gene borders we examined that were marked by the detection of mRNAs containing gene junction sequences derived from two adjacent transcription units (**Figure 6F&H**). However, GSPT1-KD did not appear to affect transcription readthrough at the VP24/L gene border. Further, we observed varying degrees of reduction in accumulation of 6 viral proteins upon GSPT1-KD (**Figure 6I**). Together, these results indicate that at later times of viral

infection, GSPT1 can be hijacked by the EBOV polymerase to facilitate a critical step of the highly regulated viral transcription process and modulate the level of individual EBOV mRNAs.

DISCUSSION

Here we present the first systematic analysis of the EBOV polymerase-cellular interactome in the context of viral RNA synthesis. We used proximity-labeling to characterize the EBOV polymerase interactomes *in situ*, and performed an siRNA screen in human hepatocytes infected with EBOV to identify functionally important interactors with EBOV polymerase during EBOV infection. We discovered 64 high-confidence EBOV polymerase interactors, 31 of which were validated by our siRNA screen as functional hits. Most polymerase-interactors we identified are antiviral rather than proviral, suggesting that the viral polymerase, or viral RNA products, or both, are targets of host innate defense mechanisms. As a proof-of-principle, we chose one functional hit, GSPT1, to further dissect the interplay between EBOV polymerase and this host factor. GSPT1 appeared to play antiviral role in concert with another host factor, UPF1, in mediating RNA decay. However, our data indicated that EBOV can in turn recruit GSPT1 to assist in highly regulated viral transcription.

We identified 12 EBOV polymerase interactors that were previously shown by various approaches, most involving traditional affinity-purification mass spectrometry (AP-MS), to be components of the EBOV-host interactome (**Figure 4B**) (Fan et al., 2020; Garcia-Dorival et al., 2016; Morwitzer et al., 2019; Spurgers et al., 2010; Takahashi et al., 2013). To avoid using cells infected with authentic EBOV, which requires Biosafety level-4 (BSL4) containment, these proteomic experiments typically used cells overexpressing a single viral bait protein. Such bait proteins and protein-protein interactions analyzed in AP-MS experiments must be stable to facilitate affinity-purification and analysis. However, the EBOV polymerase can be unstable and interactions between EBOV polymerase and other cellular or viral factors can be dynamic. To capture both stable and dynamic protein interactions, here we used a proximity-labeling approach involving split-TurboID with an EBOV minigenome (MG) system that incorporates all the molecular components relevant to the EBOV polymerase activity and recapitulates EBOV RNA synthesis events. This system also allowed us to compare the cellular interactome of EBOV polymerase in the absence or presence of the EBOV VP30, which is essential viral cofactor for transcription, but not replication, of the viral genome.

In the absence of VP30, we identified eight functional hits that interacted with EBOV polymerase, of which three are implicated in other viral infections. ZC3H7A interacts with SARS-CoV2 polymerase and negatively regulates pan-coronavirus infection (Hoffmann et al., 2021); HNRNPA1 interacts with Hepatitis C virus (HCV) polymerase to regulate HCV replication (Kim et al., 2007; Rios-Marco et al., 2016); and MRE11 acts as a cytosolic dsDNA sensor that activates the STING pathway during Herpes simplex virus infection (Kondo et al., 2013). Our siRNA screen indicated

that these same host factors can also suppress EBOV infection, suggesting that there may be a conserved panel of host restriction factors that respond to infection with various viral pathogens. Among the eight hits, we also identified the dsRNA sensor EIF2AK2/PKR, which was previously shown to be antagonized by EBOV VP35 (Feng et al., 2007; Schumann et al., 2009).

In the presence of VP30, we found 19 functional hits specific to EBOV polymerase. These hits are functionally enriched in four biological processes, including cytoskeleton organization (DOCK7, CTTN, CORO7, CNN3, AMOT, FLNA), chaperone-mediated protein folding (UNC45A and CCT8), negative regulation of translation (EIF4E2 and GIGYF2) and RNA catabolic processes (UPF1 and GSPT1). Certain cytoskeleton re-organizations might be associated with growth of EBOV inclusion bodies, which can consume a significant portion of the cytoplasmic space as infection progresses (Hoenen et al., 2012; Nanbo et al., 2013). Future work is needed to address whether these host factors restrict expansion of EBOV inclusion bodies to suppress viral infection. Interestingly, EIF4E2 and GIGYF2, a translation repression complex, can both suppress cap-dependent translation of specific mRNAs (Peter et al., 2017), and interact with SARS-CoV2 NSP2 to enhance SARS-CoV2 infection (Hoffmann et al., 2021). In contrast, we found that EIF4E2 and GIGYF2 antagonize EBOV infection, suggesting that viral mRNA features unique to each virus might account for the differential regulation by EIF4E2-GIGYF2 complex during viral infection.

Here we discovered two critical players in the cellular nonsense-mediated decay (NMD) pathway, UPF1 and GSPT1, which can both regulate EBOV infection. Although the role of NMD decay in virus infection has been examined for positive-strand and double-strand RNA viruses, retroviruses and influenza (Balistreri et al., 2017; Declercq et al., 2020; Popp et al., 2020; Tran et al., 2021), how NMD affects negative-strand RNA viruses like EBOV that replicate in the cytoplasm remains unclear. In the cellular NMD pathway, UPF1 degrades aberrant mRNAs by binding to the translation-termination complex (GSPT1/eRF3-eRF1) upon ribosomal recognition of a premature termination codon (PTC) in the mRNA (Kurosaki and Maquat, 2016). After recruitment to the termination complex, UPF1 is phosphorylated by SMG1, which facilitates recruitment of other cellular factors to dismantle the target transcript (Kurosaki et al., 2019). EBOV mRNAs likely incorporate PTCs owing to the presence of upstream open reading frames (uORF). Indeed, the 5' untranslated region (UTR) of 4 of the 7 EBOV mRNAs contain upstream AUGs (Shabman et al., 2013) that can lead to uORF expression and triggering of UPF1-mediated mRNA decay. Our siRNA screen indicated that UPF1 can restrict EBOV infection, but whether it directly targets EBOV mRNAs for degradation, whether it is a conserved host antiviral defense mechanism to limit negative-strand RNA virus infection, and/or whether EBOV can evade host responses by the UPF1-assisted NMD pathway remain to be determined.

GSPT1 is a core component of translation termination machinery (Frolova et al., 1996; Zhouravleva et al., 1995) and triggers the NMD pathway through interactions with UPF1. GSPT1

also regulates cell cycle progression (Chauvin et al., 2007) and promotes apoptosis (Hegde et al., 2003). This participation in multiple cellular pathways suggests that GSPT1 may regulate viral infection in different ways in a context-dependent manner.

We observed an antiviral role for GSPT1 in EBOV infection in our siRNA screen. We confirmed this antiviral role of GSPT1 in a monocistronic EBOV MG system, as well as in production of infectious progeny 1- to 2- days after viral infection. Together with our finding that EBOV mRNA accumulation increased upon GSPT1-KD, EBOV mRNAs could be negatively regulated by the GSPT1-dependent NMD pathway that may also involve UPF1. However, further studies are needed to determine the interplay between GSPT1-dependent mRNA decay and EBOV mRNA accumulation.

Multiple lines of evidence demonstrated that at later times of infection, EBOV can counteract GSPT1-mediated restrictions and hijack GSPT1 to facilitate viral infection. The physical interaction of GSPT1 with EBOV polymerase and VP30 suggests that GSPT1 may participate in EBOV transcription, a process that follows a “stop-start” model, in which the viral polymerase starts transcription at the 3' promoter region and proceeds discontinuously through the viral genome to synthesize a gradient of viral mRNAs (Hume and Muhlberger, 2019). EBOV transcription is regulated by the conserved gene start (GS) and gene end (GE) sequences flanking each ORF, as well as, in some cases, intergenic regions (IGRs) separating two adjacent genes. Failure to recognize GEs can lead to read-through mRNAs that may not be correctly translated by the host ribosome (Brauburger et al., 2014). Our results support an enhanced-read-through phenotype in that we saw both a disproportionate increase in viral mRNAs and concomitant increased occurrence of readthrough mRNAs with GSPT1-KD. Interestingly, we detected transcriptional readthrough occurring at the VP24/L gene border, although at a lower frequency compared to other gene borders, but this readthrough is not affected by GSPT1-KD. This observation might correlate with the two consecutive VP24 GE signals located in the VP24/L gene border and the reinforced inhibitory effect on transcription read-through.

As a result, translation efficiency of ORFs located downstream within read-through viral mRNAs is decreased, leading to fewer copies of viral proteins and in turn attenuated virus particle production (**Figure 6 A, I & H**). The monocistronic EBOV minigenome (MG) system we used cannot recapitulate readthrough events, and therefore the decreased EBOV MG activity when GSPT1 is overexpressed could reflect increased NMD suppression (**Figure 5C**). Future studies using a multicistronic EBOV MG system that recapitulates EBOV transcription read-through may clarify the supporting role that GSPT1 may play in EBOV transcription termination. As with other mononegaviruses including vesicular stomatitis virus (VSV) (Barr et al., 2002) and respiratory syncytial virus (RSV) (Fearn and Collins, 1999), EBOV transcription is regulated by *cis*-acting elements in the viral genome, including 3' transcription promoter elements, GSs, GEs and IGRs

(Biedenkopf et al., 2016; Brauburger et al., 2014; Muhlberger et al., 1999; Weik et al., 2002). Studies examining EBOV transcription regulation have largely focused on the virus-encoded, *trans*-acting factor VP30 and its role in activating EBOV transcription initiation. Our study, for the first time, revealed a possible mechanism for EBOV transcription regulation that involves a host *trans*-acting factor in EBOV transcription termination. How the GSPT1-assisted transcription termination coordinates with the *cis*-acting elements in the EBOV genome remains unclear. Based on the similarity of transcription regulation shared with other mononegaviruses, GSPT1 might also support transcription termination of other related viruses and thus GPST1-assisted transcription termination could be a pharmacological target to abrogate viral infection. Combined with the observation that EBOV infection can modulate endogenous levels of GSPT1 and its protein integrity, EBOV polymerase may engage GSPT1-mediated regulation of synthesis of individual EBOV mRNAs to fine-tune viral infection.

In summary, we present a comprehensive proximity interactome of EBOV polymerase attained in the context of a reconstituted, functional vRNP replication unit and validated the functional role of individual EBOV polymerase interactors in an siRNA-screen with authentic EBOV infection. As a proof-of-concept, we characterized the mechanisms of action for one novel host factor, GSPT1, in the transcriptional and post-transcriptional regulation of EBOV mRNA synthesis. Further work is needed to explore the role of other functional interactors in EBOV infection that we identified and to confirm their functional importance in other primary target cells including macrophage and dendritic cells. Together, our findings illuminate new mechanisms of host regulation of EBOV replication and reveal a landscape of novel targets for host-derived antiviral development.

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AUTHOR CONTRIBUTIONS

J.F., J.C.T., and E.O.S. designed research and wrote the paper; J.F. performed all non-BSL4 experiments and analyzed data; C.P. performed all BSL-4 experiments; A.B. provided supervision

for BSL-4 research; G.T. and G.C performed proteomic experiments and analyses; K.F.C. and A.Y.T provided a critical resource; all authors edited and approved the paper.

DECLARATION OF INTERESTS

The authors declare no competing interest.

REFERENCES

- Adekanmbi, O., Ilesanmi, O., and Lakoh, S. (2021). Ebola: A review and focus on neurologic manifestations. *J Neurol Sci* 421, 117311.
- Balistreri, G., Bognanni, C., and Muhlemann, O. (2017). Virus Escape and Manipulation of Cellular Nonsense-Mediated mRNA Decay. *Viruses* 9.
- Barr, J.N., Whelan, S.P., and Wertz, G.W. (2002). Transcriptional control of the RNA-dependent RNA polymerase of vesicular stomatitis virus. *Biochim Biophys Acta* 1577, 337-353.
- Biedenkopf, N., Hartlieb, B., Hoenen, T., and Becker, S. (2013). Phosphorylation of Ebola virus VP30 influences the composition of the viral nucleocapsid complex: impact on viral transcription and replication. *J Biol Chem* 288, 11165-11174.
- Biedenkopf, N., Schlereth, J., Grunweller, A., Becker, S., and Hartmann, R.K. (2016). RNA Binding of Ebola Virus VP30 Is Essential for Activating Viral Transcription. *J Virol* 90, 7481-7496.
- Branon, T.C., Bosch, J.A., Sanchez, A.D., Udeshi, N.D., Svinkina, T., Carr, S.A., Feldman, J.L., Perrimon, N., and Ting, A.Y. (2018). Efficient proximity labeling in living cells and organisms with TurboID. *Nat Biotechnol* 36, 880-887.
- Brauburger, K., Boehmann, Y., Tsuda, Y., Hoenen, T., Olejnik, J., Schumann, M., Ebihara, H., and Muhlberger, E. (2014). Analysis of the highly diverse gene borders in Ebola virus reveals a distinct mechanism of transcriptional regulation. *J Virol* 88, 12558-12571.
- Chauvin, C., Salhi, S., and Jean-Jean, O. (2007). Human eukaryotic release factor 3a depletion causes cell cycle arrest at G1 phase through inhibition of the mTOR pathway. *Mol Cell Biol* 27, 5619-5629.
- Cho, K.F., Branon, T.C., Rajeev, S., Svinkina, T., Udeshi, N.D., Thoudam, T., Kwak, C., Rhee, H.W., Lee, I.K., Carr, S.A., *et al.* (2020). Split-TurboID enables contact-dependent proximity labeling in cells. *Proc Natl Acad Sci U S A* 117, 12143-12154.

450 Declercq, M., Biquand, E., Karim, M., Pietrosemoli, N., Jacob, Y., Demeret, C., Barbezange, C.,
451 and van der Werf, S. (2020). Influenza A virus co-opts ERI1 exonuclease bound to histone mRNA
452 to promote viral transcription. *Nucleic Acids Res* 48, 10428-10440.

453 Eghrari, A.O., Bishop, R.J., Ross, R.D., Davis, B., Larbelee, J., Amegashie, F., Dolo, R.F.,
454 Prkalapakorn, S.G., Gaisie, C., Gargu, C., *et al.* (2021). Characterization of Ebola Virus-
455 Associated Eye Disease. *JAMA Netw Open* 4, e2032216.

456 Fan, J., Liu, X., Mao, F., Yue, X., Lee, I., and Xu, Y. (2020). Proximity proteomics identifies novel
457 function of Rab14 in trafficking of Ebola virus matrix protein VP40. *Biochem Biophys Res*
458 *Commun* 527, 387-392.

459 Fang, J., Pietzsch, C., Ramanathan, P., Santos, R.I., Illykh, P.A., Garcia-Blanco, M.A., Bukreyev,
460 A., and Bradrick, S.S. (2018). Staufen1 Interacts with Multiple Components of the Ebola Virus
461 Ribonucleoprotein and Enhances Viral RNA Synthesis. *mBio* 9.

462 Fatscher, T., Boehm, V., Weiche, B., and Gehring, N.H. (2014). The interaction of cytoplasmic
463 poly(A)-binding protein with eukaryotic initiation factor 4G suppresses nonsense-mediated
464 mRNA decay. *RNA* 20, 1579-1592.

465 Fearn, R., and Collins, P.L. (1999). Role of the M2-1 transcription antitermination protein of
466 respiratory syncytial virus in sequential transcription. *J Virol* 73, 5852-5864.

467 Feng, Z., Cervený, M., Yan, Z., and He, B. (2007). The VP35 protein of Ebola virus inhibits the
468 antiviral effect mediated by double-stranded RNA-dependent protein kinase PKR. *J Virol* 81, 182-
469 192.

470 Fatscher, T., Boehm, V., Weiche, B., and Gehring, N.H. (2014). The interaction of cytoplasmic
471 poly(A)-binding protein with eukaryotic initiation factor 4G suppresses nonsense-mediated
472 mRNA decay. *RNA* 20, 1579-1592.

473 Flyak, A.I., Kuzmina, N., Murin, C.D., Bryan, C., Davidson, E., Gilchuk, P., Gulka, C.P., Illykh, P.A.,
474 Shen, X., Huang, K., *et al.* (2018). Broadly neutralizing antibodies from human survivors target a
475 conserved site in the Ebola virus glycoprotein HR2-MPER region. *Nat Microbiol* 3, 670-677.

476 Frolova, L., Le Goff, X., Zhouravleva, G., Davydova, E., Philippe, M., and Kisselev, L. (1996).
477 Eukaryotic polypeptide chain release factor eRF3 is an eRF1- and ribosome-dependent
478 guanosine triphosphatase. *RNA* 2, 334-341.

479 Garcia-Dorival, I., Wu, W., Armstrong, S.D., Barr, J.N., Carroll, M.W., Hewson, R., and Hiscox, J.A.
480 (2016). Elucidation of the Cellular Interactome of Ebola Virus Nucleoprotein and Identification of
481 Therapeutic Targets. *J Proteome Res* 15, 4290-4303.

482 Hegde, R., Srinivasula, S.M., Datta, P., Madesh, M., Wassell, R., Zhang, Z., Cheong, N., Nejme, J.,
483 Fernandes-Alnemri, T., Hoshino, S., *et al.* (2003). The polypeptide chain-releasing factor
484 GSPT1/eRF3 is proteolytically processed into an IAP-binding protein. *J Biol Chem* 278, 38699-
485 38706.

486 Hoenen, T., Shabman, R.S., Groseth, A., Herwig, A., Weber, M., Schudt, G., Dolnik, O., Basler,
487 C.F., Becker, S., and Feldmann, H. (2012). Inclusion bodies are a site of ebolavirus replication. *J*
488 *Virol* 86, 11779-11788.

489 Hoffmann, H.H., Sanchez-Rivera, F.J., Schneider, W.M., Luna, J.M., Soto-Feliciano, Y.M.,
490 Ashbrook, A.W., Le Pen, J., Leal, A.A., Ricardo-Lax, I., Michailidis, E., *et al.* (2021). Functional
491 interrogation of a SARS-CoV-2 host protein interactome identifies unique and shared
492 coronavirus host factors. *Cell Host Microbe* 29, 267-280 e265.

493 Hoshino, S. (2012). Mechanism of the initiation of mRNA decay: role of eRF3 family G proteins.
494 *Wiley Interdiscip Rev RNA* 3, 743-757.

495 Hume, A.J., and Muhlberger, E. (2019). Distinct Genome Replication and Transcription Strategies
496 within the Growing Filovirus Family. *J Mol Biol* 431, 4290-4320.

497 Jasenosky, L.D., Neumann, G., and Kawaoka, Y. (2010a). Minigenome-based reporter system
498 suitable for high-throughput screening of compounds able to inhibit Ebolavirus replication
499 and/or transcription. *Antimicrob Agents Chemother* 54, 3007-3010.

500 Jasenosky, L.D., Neumann, G., and Kawaoka, Y. (2010b). Minigenome-based reporter system
501 suitable for high-throughput screening of compounds able to inhibit Ebolavirus replication
502 and/or transcription. *Antimicrob Agents Chemother* 54, 3007-3010.

503 Kim, C.S., Seol, S.K., Song, O.K., Park, J.H., and Jang, S.K. (2007). An RNA-binding protein, hnRNP
504 A1, and a scaffold protein, septin 6, facilitate hepatitis C virus replication. *J Virol* 81, 3852-3865.

505 Kondo, T., Kobayashi, J., Saitoh, T., Maruyama, K., Ishii, K.J., Barber, G.N., Komatsu, K., Akira, S.,
506 and Kawai, T. (2013). DNA damage sensor MRE11 recognizes cytosolic double-stranded DNA and
507 induces type I interferon by regulating STING trafficking. *Proc Natl Acad Sci U S A* 110, 2969-
508 2974.

509 Kurosaki, T., and Maquat, L.E. (2016). Nonsense-mediated mRNA decay in humans at a glance. *J*
510 *Cell Sci* **129**, 461-467.

511 Kurosaki, T., Popp, M.W., and Maquat, L.E. (2019). Quality and quantity control of gene
512 expression by nonsense-mediated mRNA decay. *Nat Rev Mol Cell Biol* **20**, 406-420.

513 Leung, D.W., Borek, D., Luthra, P., Binning, J.M., Anantpadma, M., Liu, G., Harvey, I.B., Su, Z.,
514 Endlich-Frazier, A., Pan, J., *et al.* (2015). An Intrinsically Disordered Peptide from Ebola Virus
515 VP35 Controls Viral RNA Synthesis by Modulating Nucleoprotein-RNA Interactions. *Cell Rep* **11**,
516 376-389.

517 May, J.P., and Simon, A.E. (2021). Targeting of viral RNAs by Upf1-mediated RNA decay
518 pathways. *Curr Opin Virol* **47**, 1-8.

519 Moller, P., Pariente, N., Klenk, H.D., and Becker, S. (2005). Homo-oligomerization of
520 Marburgvirus VP35 is essential for its function in replication and transcription. *J Virol* **79**, 14876-
521 14886.

522 Morwitzer, M.J., Tritsch, S.R., Cazares, L.H., Ward, M.D., Nuss, J.E., Bavari, S., and Reid, S.P.
523 (2019). Identification of RUVBL1 and RUVBL2 as Novel Cellular Interactors of the Ebola Virus
524 Nucleoprotein. *Viruses* **11**.

525 Mühlberger, E. (2007). Filovirus replication and transcription. *2*, 205-215.

526 Muhlberger, E., Weik, M., Volchkov, V.E., Klenk, H.D., and Becker, S. (1999). Comparison of the
527 transcription and replication strategies of marburg virus and Ebola virus by using artificial
528 replication systems. *J Virol* **73**, 2333-2342.

529 Nanbo, A., Watanabe, S., Halfmann, P., and Kawaoka, Y. (2013). The spatio-temporal distribution
530 dynamics of Ebola virus proteins and RNA in infected cells. *Sci Rep* **3**, 1206.

531 Peter, D., Weber, R., Sandmeir, F., Wohlbold, L., Helms, S., Bawankar, P., Valkov, E., Igreja, C.,
532 and Izaurralde, E. (2017). GIGYF1/2 proteins use auxiliary sequences to selectively bind to 4EHP
533 and repress target mRNA expression. *Genes Dev* **31**, 1147-1161.

534 Popp, M.W., Cho, H., and Maquat, L.E. (2020). Viral subversion of nonsense-mediated mRNA
535 decay. *RNA* **26**, 1509-1518.

536 Rios-Marco, P., Romero-Lopez, C., and Berzal-Herranz, A. (2016). The cis-acting replication
537 element of the Hepatitis C virus genome recruits host factors that influence viral replication and
538 translation. *Sci Rep* **6**, 25729.

539 Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S.,
540 Rueden, C., Saalfeld, S., Schmid, B., *et al.* (2012). Fiji: an open-source platform for biological-
541 image analysis. *Nat Methods* 9, 676-682.

542 Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S.,
543 Rueden, C., Saalfeld, S., Schmid, B., *et al.* (2012). Fiji: an open-source platform for biological-
544 image analysis. *Nat Methods* 9, 676-682.

545 Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image
546 analysis. *Nat Methods* 9, 671-675.

547 Schumann, M., Gantke, T., and Muhlberger, E. (2009). Ebola virus VP35 antagonizes PKR activity
548 through its C-terminal interferon inhibitory domain. *J Virol* 83, 8993-8997.

549 Shabman, R.S., Hoenen, T., Groseth, A., Jabado, O., Binning, J.M., Amarasinghe, G.K., Feldmann,
550 H., and Basler, C.F. (2013). An upstream open reading frame modulates ebola virus polymerase
551 translation and virus replication. *PLoS Pathog* 9, e1003147.

552 Spurgers, K.B., Alefantis, T., Peyser, B.D., Ruthel, G.T., Bergeron, A.A., Costantino, J.A., Enterlein,
553 S., Kota, K.P., Boltz, R.C., Aman, M.J., *et al.* (2010). Identification of essential filovirion-associated
554 host factors by serial proteomic analysis and RNAi screen. *Mol Cell Proteomics* 9, 2690-2703.

555 Szklarczyk, D., Gable, A.L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M.,
556 Doncheva, N.T., Morris, J.H., Bork, P., *et al.* (2019). STRING v11: protein-protein association
557 networks with increased coverage, supporting functional discovery in genome-wide
558 experimental datasets. *Nucleic Acids Res* 47, D607-D613.

559 Takahashi, K., Halfmann, P., Oyama, M., Kozuka-Hata, H., Noda, T., and Kawaoka, Y. (2013). DNA
560 topoisomerase 1 facilitates the transcription and replication of the Ebola virus genome. *J Virol*
561 87, 8862-8869.

562 Te Velthuis, A.J.W., Grimes, J.M., and Fodor, E. (2021). Structural insights into RNA polymerases
563 of negative-sense RNA viruses. *Nat Rev Microbiol* 19, 303-318.

564 Ting, L., Rad, R., Gygi, S.P., and Haas, W. (2011). MS3 eliminates ratio distortion in isobaric
565 multiplexed quantitative proteomics. *Nat Methods* 8, 937-940.

566 Towner, J.S., Paragas, J., Dover, J.E., Gupta, M., Goldsmith, C.S., Huggins, J.W., and Nichol, S.T.
567 (2005). Generation of eGFP expressing recombinant Zaire ebolavirus for analysis of early
568 pathogenesis events and high-throughput antiviral drug screening. *Virology* 332, 20-27.

Tran, G.V.Q., Kleinehr, J., Preugschas, H.F., Anhlan, D., Mohamed, F.F., Ehrhardt, C., Ludwig, S.,
and Hrincius, E.R. (2021). Nonsense-mediated mRNA decay does not restrict influenza A virus
propagation. *Cell Microbiol* 23, e13323.

Weik, M., Modrof, J., Klenk, H.D., Becker, S., and Muhlberger, E. (2002). Ebola virus VP30-
mediated transcription is regulated by RNA secondary structure formation. *J Virol* 76, 8532-
8539.

Xu, M.J., Stanford-Moore, G., and Czechowicz, J.A. (2019). Association of Ebola Virus Infection
With Hearing Loss in Regions Where Ebola Virus Infection Is Endemic: A Systematic Review.
JAMA Otolaryngol Head Neck Surg 145, 669-675.

Zhouravleva, G., Frolova, L., Le Goff, X., Le Guellec, R., Inge-Vechtomov, S., Kisselev, L., and
Philippe, M. (1995). Termination of translation in eukaryotes is governed by two interacting
polypeptide chain release factors, eRF1 and eRF3. *EMBO J* 14, 4065-4072.

FIGURE TITLES AND LEGENDS

Figure 1. Generating a functional EBOV polymerase with proximity-labeling activity.

(A) Schematics for proximity biotinylation mediated by the EBOV polymerase (EBOV_{pol}) split-TurboID fusion in the presence or absence of VP30. **(B)** Polymerase activity and expression of EBOV_{pol} split-TurboID fusion (L-VP35 sTurboID) compared to controls (L-VP35 WT and L-WT VP35-HA) measured in EBOV minigenome (MG) assay. The percentage of polymerase activity is determined by normalizing luciferase activity in each sample to that of L-VP35 WT control.

Background of MG activity is controlled by withdrawing L from the MG system (no L). Results from three independent experiments with triplicate samples were analyzed by using unpaired, two-tailed t test under each condition compared to controls (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Values are means $\pm$ SEM. Representative western blot showing expression of EBOV L-variants, VP35-variants, VP30 and β -actin in HEK 293T lysates. The blot was sliced into two parts to sequentially probe for different target proteins. **(C)** Streptavidin blot demonstrating biotin ligase activity of EBOV_pol split-TurboID fusion in the context of the EBOV MG system. **(D)** Confocal immunofluorescence images of EBOV_pol split-TurboID mediated proximity biotinylation in cells co-expressing the EBOV MG system. V35-HA and VP35-sNTurboID were all detected by anti-HA. EBOV VP30 was detected by anti-VP30. Biotinylated proteins were detected by streptavidin-AF488. Scale bar: 5 μ m. Representative images from two independent experiments. Zoomed region of interest is outlined with a white rectangle in the original image. See also Figure S1 and S2.

Figure 2. Proximity proteomics to define the EBOV polymerase interactomes.

(A) Proximity proteomic sample preparation workflow. **(B)** Validation of streptavidin (SA) enrichment of EBOV_{pol} split-TurboID (sTurboID_{pol}) mediated proximity biotinylation. Total lysates are indicated as “Input”, biotinylated proteins enriched by SA-beads are indicated as “SA-pull down”. Representative blots and gels from three biological replicates are shown. All samples were split into three parts that were loaded onto individual gels that were used for streptavidin-HRP blot, to probe EBOV proteins, and for silver staining. The blot used to probe EBOV proteins was

excluded. **(C)**. Individual genes for which siRNAs significantly affected infection are shown as data points in a bubble plot; data point colors and sizes correspond to the siRNA tested and log-transformed *p-value*, respectively. Data points with multiple siRNAs significantly modulating EBOV infection are considered as true hits and are outlined in white with the corresponding gene also labeled in white. One label of each true hit is shown in red for display purposes. NPC1 used as a positive control is labeled in yellow. **(D)** Representative images of selected Huh7 monolayers at 48 hours post-infection. One representative result from two rounds of siRNA screens using different MOIs is shown. See also Figure S3 and S4.

Figure 4. Proximity interactomes of EBOV polymerase in the presence and absence of EBOV VP30. (A) EBOV polymerase interactomes identified by proximity proteomics. Node: high-confidence proteomic hit. Edge: identified protein-protein interaction. Nodes having two edges are shared by multiple interactomes. Black text inside nodes denotes genes that are functionally validated by siRNA screens. Selected nodes are clustered based on enriched biological processes using STRING. **(B)** A Venn diagram comparing EBOV polymerase interactors with reported interactors of EBOV proteins or virions.

Figure 5. GSPT1 is functionally relevant to EBOV RNA synthesis. (A) co-IP analysis on EBOV polymerase, the accessory proteins (VP30 and VP35) and FLAG-GSPT1. Representative results from three experiments are shown. Each blot was sliced into several parts to probe for different target proteins and imaged separately. **(B)** Confocal immuno-fluorescent analysis of GSPT1 and EBOV_pol (by VP35-HA) localization in the context of the EBOV MG system in HEK 293T cells. Arrowhead: sites where endogenous GSPT1 localize next to viral proteins. The bottom panel shows the subcellular distribution of GSPT1 alone as a negative control. Representative images from two experiments were shown. **(C)** Relative EBOV MG activity and a control GFP reporter activity in

HEK 293T cells overexpressing GSPT1. MG and reporter activity of the GSPT1-overexpressing cells are normalized to that of cells transfected with a control plasmid. Raw luciferase activities and GFP intensities were first normalized to the corresponding amount of total protein in each sample to account for loading bias. Results from three independent experiments with triplicate samples were analyzed by using unpaired, two-tailed t test under each condition compared to controls (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). Values are means $\pm$ SEM. Representative western blot showing the expression level of GSPT1 (long isoform) in different experimental conditions. See also Figure S5.

Figure 6. EBOV can subvert GSPT1-restriction and hijack GSPT1 to facilitate viral transcription termination. (A) Effect of GSPT1-KD on the EBOV viral growth kinetics in Huh7 cells. Results of two independent experiments with technical triplicates are plotted as mean $\pm$ SD. Two-way ANOVA analysis of log-transformed viral titers was performed to determine the statistical significance of the effect of GSPT1 depletion on viral growth kinetics. (B) Effect of GSPT1-KD on EBOV vRNA and NP mRNA accumulation at 4 days post-infection (dpi). (C) Western blot analysis of GSPT1 protein and EBOV GP2 levels in lysates from EBOV-infected Huh7 cells harvested at 4 dpi. Representative blots from one experiment are shown. Equal volumes of inactivated lysate were loaded onto two gels to probe cellular or viral proteins separately due to differences in expression levels. (D) Representative images of EBOV-infected Huh7 monolayer at 4 dpi are shown. (E) Effect

of EBOV infection on endogenous GSPT1 expression in Huh7 cells, compared to no infection. **(F)** Schematic of the EBOV-eGFP genome used in the viral growth kinetics experiment. Orange bars: non-coding terminus and intergenic regions. Pink box: gene unit with the coding sequence outlined in a different color. **(G)** Effect of GSPT1-KD on individual EBOV mRNA accumulation. The same data for EBOV NP mRNA levels in GSPT1-depleted cells are as shown in (B), and repeated here to allow comparison with other EBOV mRNAs. **(H)** Effect of GSPT1-KD on the accumulation of EBOV readthrough mRNAs. Locations of each readthrough sequence on the viral genome are marked as red bars in (F). For all bar graphs, results from two independent experiments with triplicate samples were analyzed by using unpaired, two-tailed t test under each condition compared to controls (*, $p < 0.05$; **, $p < 0.01$). Values are means $\pm$ SEM. **(I)** Western blot analysis of the effect of GSPT1-KD on accumulations of individual EBOV proteins in Huh7 cells. The same lysates as shown in (C) were used. Equal volumes of inactivated lysate were loaded onto three gels to probe different target proteins. Due to the low expression level of L protein, we could not detect full-length L in inactivated lysate. See also Figure S6.

684 **STAR METHODS**

685 **KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Streptavidin-HRP	Thermofisher, Invitrogen	Cat#SA10001
Mouse, anti-HA, 16B12 (monoclonal)	Biolegend Cat#901513	Cat#901513
Human, anti-EBOV NP, KZ51 (monoclonal)	Absolute antibody	Cat#Ab00692-10.0
Rabbit, anti-VP30 (#1-1)	A kind gift from Peter Halfmann and Yoshihiro Kawaoka	N/A
Mouse, anti-EBOV VP24_21.5.2.5 (monoclonal)	A kind gift from Peter Halfmann and Yoshihiro Kawaoka	N/A
Mouse, anti-EBOV VP40	Customized antibody by Zalgen labs/ Autoimmune technology	N/A
Human, BDBV223 (Cross-reactive) Anti-EBOV GP2	A kind gift from James E. Crowe (Flyak et al., 2018)	N/A
Rabbit, polyclonal anti-EBOV L	IBT Bioservices	Cat#0301-045
Rabbit, polyclonal anti-GSPT1/eRF3	Abcam	Cat#ab49878
Streptavidin-AlexaFluor488	Thermofisher/Invitrogen	Cat#s11223
Goat, anti-mouse-HRP	Thermofisher/Invitrogen	Cat#31437
Goat, anti-rabbit-HRP	Southern Biotech	Cat#4050-05
Goat, anti-human-HRP	Thermofisher/Invitrogen	Cat#A18811
Goat, anti-mouse-AlexaFluor568	Thermofisher/Invitrogen	Cat#A11004
Goat, anti-mouse-AlexaFluor647	Thermofisher/Invitrogen	Cat#A21236
Goat, anti-rabbit-AlexaFluor488	Thermofisher/Invitrogen	Cat#A11008
Goat, anti-rabbit-AlexaFluor647	Thermofisher/Invitrogen	Cat#A27040

Streptavidin-magnetic beads	ThermoFisher	Cat#88817
Anti-FLAG M2 affinity Gel	Sigma-Aldrich	Cat#A2220-5ML
Bacterial and virus strains		
EBOV-eGFP (strain Mayinga)	Bukreyev Lab	Initially provided by Jonathan Towner (CDC) and Stuart Nichol (CDC) (Towner et al., 2005)
Chemicals, peptides, and recombinant proteins		
Biotin	Sigma-Aldrich	Cat#B4501
Lipofectamine™ RNAiMAX Transfection Reagent	ThermoFisher/Invitrogen	Cat#13778150
TransIT®-LT1 Transfection Reagent	Mirus	Cat#MIR2306
Sequencing Grade Modified Trypsin	Promega	Cat#V5111
NEBBuilder HiFi DNA Assembly Master Mix	NEB	
In-Fusion® HD Cloning Plus	Takara	Cat# 638920
16% Paraformaldehyde Aqueous Solution, EM Grade, Ampoule 10ML	Electron Microscopy Sciences	Cat#15710
ProLong™ Gold Antifade Mountant	ThermoFisher/Invitrogen	Cat#P36934
Normal Goat Serum	ThermoFisher/Invitrogen	Cat#PCN5000
Hoechst 33342, Trihydrochloride, Trihydrate, 100 mg	ThermoFisher/Invitrogen	Cat#H1399
RIPA Buffer (10X)	Cell Signaling Technology	Cat#9806S
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Roche	SKU# 11873580001
Benzonase® Nuclease, Purity > 90%	Millipore/Sigma	Cat#70746
NuPage Sample reducing agent (10X)	ThermoFisher	Cat#NP0009
NuPage LDS sample buffer (4X) (Laemmli)	ThermoFisher	Cat#NP0008

TRIzol™ Reagent	ThermoFisher/Invitrogen	Cat# 15596018
DEPC-Treated Water	ThermoFisher/Invitrogen	Cat#AM9920
Iodoacetamide (IAA)	Sigma-Aldrich	SKU#11149-5G
Critical commercial assays		
Pierce™ BCA Protein Assay Kit	Thermo Scientific	Cat#23225
Pierce™ Silver Stain for Mass Spectrometry	Thermo Scientific	Cat#24600
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	Thermo Scientific	Cat#34578
Luciferase Assay System	Promega	Cat#E4550
CellTiter-Glo® 2.0 Cell Viability Assay	Promega	Cat#G9241
High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor	ThermoFisher scientific (Applied Biosystems)	Cat#4374966
SYBR™ Select Master Mix	ThermoFisher scientific (Applied Biosystems)	Cat#4472908
Direct-zol RNA Miniprep Plus	Zymo research	Cat#R2071
ZipTip with 0.2 µL C18 resin	Millipore	Cat# ZTC18M096
TMT10plex™ Isobaric Label Reagent Set, 1 x 0.8 mg	Thermo Scientific	Cat# 90110
Deposited data		
Raw proximity proteomic data of EBOV polymerase interactome	This study	Table S1
Experimental models: Cell lines		
HEK 293T	ATCC	Cat#CRL-3216
VERO C1008 [Vero 76, clone E6, Vero E6]	ATCC	Cat# CRL-1586
Huh7 used in BSL4	A kind gift from Mariano Garcia-Blanco	

Huh7 used in BSL2	A kind gift from Luke Wiseman	
Oligonucleotides		
Primers for RT-qPCRs, see Table S2	This paper	N/A
siRNAs for knockdowns, see Table S3	This paper	N/A
AllStars Negative Control siRNA	Qiagen	Cat#1027281
Oligo dT(20) Primer	Thermofisher/invitrogen	Cat#18418020
Recombinant DNA		
Plasmid: pCEZ-NP	(Jasenosky et al., 2010b)	N/A
Plasmid: pCEZ-VP35	(Jasenosky et al., 2010b)	N/A
Plasmid: pCDNA5-VP35-HA	(Fang et al., 2018)	N/A
Plasmid: pCAGGS-VP35-HA	This study	N/A
Plasmid: pCAGGS-VP35-sNTurboID	This study	N/A
Plasmid: pCEZ-VP30	(Jasenosky et al., 2010b)	N/A
Plasmid: pCEZ-L	(Jasenosky et al., 2010b)	N/A
Plasmid: pCEZ-L-sCTurboID	This study	N/A
Plasmid: pHH21-3E5E-Fluc	(Jasenosky et al., 2010b)	N/A
Plasmid: pCI-empty	This study	N/A
Plasmid: pCI-MS2V5-eRF3a F76a	(Fatscher et al., 2014)	Plasmid #65811
Plasmid: pCI-FLAG-GSPT1	This study	N/A
Plasmid: pCAGGS-eGFP	This study	N/A
Plasmid: pCAGGS-empty	This study	N/A
Plasmid: pLX304 CMV HA-HaloTag-FRB-sTurboID (C)	(Cho et al., 2020)	Plasmid: #153003

Plasmid: pLX304 CMV FKBP-V5-sTurboID (N)	(Cho et al., 2020)	Plasmid: #153002
Plasmid: pFastBac™ Dual Expression Vector	ThermoFisher/Gibco	Cat#10712024
Software and algorithms		
GraphPad Prism 9	GraphPad	N/A
Cytoscape v3.8.2	Cytoscape	https://cytoscape.org/
Image Lab Software	Bio-Rad	https://www.bio-rad.com/en-us/product/image-lab-software
SparkControl™	Tecan	
BioRender	BioRender	https://biorender.com/
Thermo Scientific™ HCS Studio Cell Analysis Software	Thermo Scientific	CX51110
ZEISS ZEN 3.4 Blue edition	ZEISS	https://www.zeiss.com/
FIJI	(Schindelin et al., 2012)	https://imagej.net/software/fiji/
ImageJ	(Schneider et al., 2012)	https://imagej.nih.gov/ij/
SnapGene Viewer	SnapGene	https://www.snapgene.com/snapgene-viewer/
STRING	(Szklarczyk et al., 2019)	https://string-db.org/
Proteome Discover v 2.3	Thermo Scientific	
SEQUEST	Yates Laboratory	http://proteomicswiki.com/wiki/index.php/SEQUEST
Scaffold 4	Proteome Software	

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687 **Resource availability**

688 **Lead contact**

689 Further information and requests for resources and reagents should be directed to and will be
690 fulfilled by the Lead Contact, Erica Ollmann Saphire (erica@lji.org).

Materials availability

All requests for unique reagents generated in this study should be directed to and will be fulfilled by the Lead Contact author. Materials will be made available through the authors upon execution of a Material Transfer Agreement.

Data and code availability

Raw proteomic data reported in this study are reported in Table S1.

Experimental model and subject details

Cell cultures

Human hepatocytes Huh7 and human embryonic kidney cells HEK 293T were maintained in Dulbecco's modified Eagle medium (DMEM-GlutaMAX) supplemented with 4.5 g/L D-Glucose, 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml). For cells used in viral infection experiment, Huh7 were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 50 µg/ml Gentamicin Sulfate; Vero-E6 cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% FBS, 1% MEM Non-essential Amino Acid Solution, 1% Sodium pyruvate solution, and 50 µg/ml Gentamicin Sulfate. All cells were grown at 37°C and 5% CO₂.

Virus stock

EBOV strain Mayinga expressing Emerald green fluorescent protein (eGFP) (1) was propagated in Vero-E6 cells with 500,000 plaque-forming unit (PFU) per T225 flask (at 90% confluency). EBOV-eGFP was harvested 5 days post-infection. All experiments using infectious EBOV were performed under biosafety level 4 (BSL-4) conditions at the Galveston National Laboratory. Virus inactivation was performed according to standard operating procedures.

METHOD DETAILS

Plasmids and cloning

To generate pCEZ-L-sCTurboID, a fragment of L coding sequence was first subcloned into a pFastbacDual intermediate plasmid using the natural restriction sites Pac1 and Hpa1. From there, a fragment of sequence encoding the sC-TurboID and a FLAG-tag sequence, flanked by two flexible linkers was internally inserted at the position 1705/1706 (TTIP/Q). This insertion was PCR amplified from pLX304 CMV HA-HaloTag-FRB-sTurboID (C), and inserted to the intermediate plasmid using NEBuilder HiFi DNA Assembly Master Mix (NEB). The L-sNTurboID fragment was ligated back to the pCEZ-L backbone using the same restriction sites Pac1 and Hpa1 by NEB T4 ligase.

To generate pCAGGS-VP35-HA, the coding sequence of VP35-HA was subcloned to the plasmid pCAGGS-empty using the restriction sites Kpn1 and Xho1 by standard ligation method. To generate pCAGGS-VP35-sNTurboID, pcDNA5-VP35-HA (gift from Mariano Garcia-Blanco) was linearized. The fragment of sNTurboID was PCR amplified from pLX304 CMV FKBP-V5-sTurboID (N) and cloned into the pcDNA5-VP35-HA using NEBuilder HiFi DNA Assembly Master Mix (NEB). The fragment of VP35-sNTurboID, including the C-terminal HA tag, was subcloned to pCAGGS plasmid using restriction sites Kpn1 and Nhe1 and standard ligation method.

To generate pCI-empty, the coding sequence of MS2V5-eRF3a-F76a was removed from pCI-MS2V5-eRF3a F76a plasmid (a gift from Niels Gebring) by PCR and re-circularized using NEBuilder HiFi DNA Assembly Master Mix (NEB). To generate pCI-FLAG-GSPT1, the coding sequence of MS2V5 was removed from pCI-MS2V5-eRF3a F76a plasmid and an N-terminal FLAG tag was inserted to the same plasmid by PCR and re-circularized using NEBuilder HiFi DNA Assembly Master Mix (NEB). The F76a mutation was mutated back to wildtype using In-fusion cloning (Clontech).

The sequence of all engineered plasmid DNA was confirmed by Sanger sequencing.

EBOV minigenome assay

HEK 293T cells (2×10^5 per well) were seeded in 24-well plates one day prior to transfection with the following plasmids: 100 ng pCEZ-NP, 100 ng pCEZ-VP35, 75 ng pCEZ-VP30, 750 ng pCEZ-L, 62.5 ng EBOV minigenome encoding a firefly luciferase reporter (pEE21). In some cases: pCEZ-VP30 was replaced with equal mass of control pCAGGS-empty plasmid; pCEZ-L and/or pCEZ-VP35 were replaced with a tagged variant. A negative control was included in every assay in which the pCEZ-L plasmid was replaced with equal mass of control pCAGGS-empty plasmid to verify minigenome activity mediated by EBOV polymerase. Plasmids were transfected with TransIT-LT1 transfection reagent (3 μ l/ μ g plasmid). At 48 hours post-transfection, cells were lysed and subjected to the luciferase reporter assay (Promega).

To measure the effect of GSPT1 knockdown on EBOV minigenome activity, we modified the assay as below. HEK 293T cells (1×10^5 per well) were seeded in 24-well plates one day prior to siRNA or plasmid. To knockdown GSPT1, cells were transfected with 10 nM of the siRNA, GSPT1si8, using Lipofectamine RNAiMAX. In control wells, cells were transfected with 10 nM of the Allstar negative control siRNA (NSC) using Lipofectamine RNAiMAX. To overexpress GSPT1, cells were transfected with 100/200/400 ng of pCI-FLAG-GSPT1 (per well) using TransIT-LT1. In control wells, cells were transfected with 200 ng of pCI-empty control vector using TransIT-LT1. At 24 hours post-transfection, cells were transfected with the EBOV MG system described earlier. In the control

plate, cells were transfected with 100 ng of pCAGGS-eGFP (per well) using TransIT-LT1 to evaluate the effect of GSPT1 modulation on a control reporter expression. Two days after the second transfection, cell lysate was harvested in 1X passive lysis buffer for luciferase reporter assay or in GFP lysis buffer (50mM Tris pH7.4, 1mM EDTA, 0.5% NP-40, 150mM NaCl) to quantify the fluorescence intensity. A fraction of each lysate was used to quantify the total protein by BCA assay. Raw luciferase intensity or GFP intensity of each sample was first normalized to the BCA quantified protein amount. The resulting relative luciferase reporter activity or GFP reporter activity was further normalized to that of the control: NSC for the GSPT1 knockdown experiment, pCI-empty for the GSPT1 overexpression experiment.

Biotinylation with polymerase-TurboID fusion protein

HEK 293T cells seeded in 24-well plates were transfected with EBOV minigenome system including the wild-type polymerase or the polymerase-TurboID fusion. Biotin (500 μ M) was added to cell culture media 2-3 days post-transfection. Multiple labeling time points were sampled for optimization. The reaction was stopped by placing cells on ice, the excess biotin was removed by DPBS washes, and soluble whole cell lysates were collected for western blot analysis using streptavidin-HRP.

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 EBOV minigenome system using Trans-IT LT1 as described earlier. For in-cell biotinylation experiment: at the same day of transfection, biotin (500 μ M) was added to the culture media allowing for 18 hours in-cell biotinylation in EBOV minigenome transfected cells. Labeling was stopped by washing cells with cold DPBS for three times. For all IFA specimens, cells were fixed in 4% paraformaldehyde for 15 mins, quenched with 20mM Glycine-DPBS for 5 mins, permeabilized with 0.1% Triton-X100-DPBS for 10 mins, and blocked in 1% normal goat serum for at least one hour, all at room temperature. 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.

For samples shown in the Figure 1D, mouse anti-HA (1:500) and rabbit anti-VP30 (1:500) antibody were used as the primary antibodies, goat anti-mouse AlexaFluor647 (1:500) and goat anti-rabbit AlexaFluor 568 (1:500) were used as the secondary antibodies. Together these combinations were

used to probe HA-tagged EBOV VP35 (VP35-HA and VP35-sNTurboID) and the wildtype VP30. Streptavidin-AlexaFluor488 (1:500) was added along with secondary antibodies to probe biotinylated proteins.

For samples shown in Figure 5B, mouse anti-HA (1:500) and rabbit anti-GSPT1 (1:500) antibody were used as the primary antibodies, goat anti-mouse AlexaFluor568 (1:500) and goat anti-rabbit AlexaFluor 488 (1:500) were used as the secondary antibodies. Together these combinations were used to probe EBOV VP35-HA and the endogenous GSPT1 protein.

Sample preparation for in-cell biotinylation and streptavidin enrichment

Protocol adapted from a previous study (Branon et al., 2018). HEK 293T cells were seeded in 6-wells plate (1×10^6 cells per well) and transiently transfected using Trans-IT LT1 a day after, with EBOV MG plasmids including plasmid expressing wild-type or the TurboID-fused viral polymerase. To enable in-cell biotinylation, at two days post-transfection, monolayers expressing EBOV MG are incubated with 500 μ M biotin containing complete media for indicated times. Biotinylation was stopped by washing monolayers with cold DPBS twice before collecting the cell pellets by centrifugation at 1500 rpm for 3 mins at 4 °C. Cell pellets (1V) were lysed in 1V of 1X RIPA buffer containing cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche) and Benzonase® Nuclease (Millipore) followed by incubation on ice for 15-20 mins. Soluble lysates were clarified by centrifugation at 13000 rpm for 5 mins at 4 °C.

For streptavidin enrichment, streptavidin-magnetic beads (80 μ l slurry/mg of total protein) were washed with 1X RIPA buffer twice and incubated with 2-3 mg of soluble cell lysate for one hour at room temperature. The beads were subsequently washed twice with 1 ml of 1X RIPA buffer, once with 1 ml of 1M KCL, once with 1 ml of 0.1M Na₂CO₃, once with 1 ml of 2M Urea in 10 mM Tris-HCl (pH 8.0), and with twice with 1 ml 1X RIPA buffer. Washed beads were then either eluted in 50 μ l of NuPage LDS sample buffer (4X) supplemented with 20 mM DTT and 2 mM biotin for quality check or processed to on beads digestion. Quality checks for successful streptavidin enrichment include silver staining and western blotting of SDS-PAGE-separated input and streptavidin-enriched samples side-by-side.

For on-bead digestion, proteins bound to beads were further washed twice with 50 mM Tris HCL (pH 7.5) and two washes of 2 M urea/50 mM Tris buffer (pH 7.5). A final volume of 80 μ l of 2 M urea/50 mM Tris containing 1 mM DTT and 0.4 μ g trypsin was added to washed beads for overnight digestion at 37 °C shaker. Digested supernatants containing biotinylated peptides were transferred to a new tube. The streptavidin beads were washed twice with 60 μ l of 2 M urea/50 mM Tris buffer (pH 7.5) and the rests were combined with the on-bead digest supernatant. The eluate was reduced

with 4 mM DTT for 30 mins at room temperature with shaking, alkylated with 10 mM IAA (iodoacetamide) for 45 mins in the dark at 25 °C with shaking. An additional 0.5 µg of trypsin was added to the sample and the digestion was completed overnight at 37 °C shaker. After final digestion, samples were acidified by adding formic acid to a final concentration of 1 % and stored in - 80 °C freezer before sending to Scripps Research (FL) Proteomic core for further processing, TMT labeling, and mass-spectrometry analysis.

Proximity proteomics (with tandem-mass-tag labeling): Following on-bead trypsin digestion of biotinylated proteins, LASSA (WT and TurboID) and EBOLA (WT, TurboID MINUS and TurboID PLUS) samples were acidified by the addition of 1% formic acid and desalted with 2 µg-capacity C18 ZipTips, respectively, and dried using vacuum. Peptides were resuspended in 100mM TEAB and labelled with TMT labels (10-plex, with one TMT label not used) according to the manufacturer's instructions and pooled.

The labelling scheme for three biological replicates of each condition was as follows:

EBOLA sample	WT_pol+VP30_01	WT_pol+VP30_02	WT_pol+VP30_03
TMT 10-plexs label	126	127C	128C
EBOLA sample	TurboID_pol-VP30_01	TurboID_pol-VP30_02	TurboID_pol-VP30_03
TMT 10-plexs label	127N	128N	131
EBOLA sample	TurboID_pol+VP30_01	TurboID_pol+VP30_02	TurboID_pol+VP30_03
TMT 10-plexs label	129N	130N	129N

The pooled and multiplexed samples were dried under vacuum, re-solubilized in 1% TFA and then desalted using 2 µg-capacity C18 ZipTips (Millipore, Billerica, MA), and then dried once again using vacuum. For mass spectrometry, dried TMT-labelled peptides were reconstituted in 5 µL of 0.1% TFA, vortexed briefly, and then sonicated for 15 mins. The peptides were subsequently on-line eluted into a Fusion Tribrid mass spectrometer (ThermoFisher Scientific, San Jose, CA) from an Acclaim PepMapTM RSLC C18 nano Viper analytical column (2 mm, 100 Å, 75-µm ID × 50 cm, Thermo Scientific, San Jose, CA) using a gradient of 5-25% solvent B (80/20 acetonitrile/water, 0.1% formic acid) in 180 mins, followed by 25-44% solvent B in 60 mins, 44-80% solvent B in 0.1 min, a 5-mins hold of 80% solvent B, a return to 5% solvent B in 0.1 min, and finally a 20-mins hold of solvent B. All flow rates were 300 nl/min delivered using a nEasy-LC1000 nano liquid chromatography system (Thermo Fisher Scientific, San Jose, CA). Solvent A consisted of water

and 0.1% formic acid. Ions were created at 2.1-2.5 kV using the EASY-SprayTM ion source held at 50 °C (Thermo Fisher Scientific, San Jose, CA). A synchronous precursor selection (SPS)-MS3 mass spectrometry method was used based on published protocol (Ting et al., 2011), scanning between 380-2000 m/z at a resolution of 120,000 for MS1 in the Orbitrap mass analyzer, and performing CID at top speed in the linear ion trap of peptide monoisotopic ions with charge 2-8, using a quadrupole isolation of 0.7 m/z and a CID energy of 35%. The top 10 MS2 ions in the ion trap between 400-1200 m/z were then chosen for HCD at 65% energy and detection in the Orbitrap at a resolution of 60,000 and an AGC target of 1E5 and an injection time of 120 msec (MS3). Three technical replicates were performed for each sample.

Bioinformatic analysis of mass spectrometry data: The raw data technical replicates were added as “fractions” into Proteome Discover v 2.3 (Thermo Fisher Scientific, San Jose, CA). As it is the default option for this software, peptide spectrum match raw values of both technical replicates from the same biological sample were summed for the same peptide group discovery. Quantitative analysis of the TMT experiments was performed simultaneously to protein identification. The precursor and fragment ion mass tolerances were set to 10 ppm, 0.6 Da, respectively), enzyme was Trypsin with a maximum of 2 missed cleavages. Steptavidin FASTA and Uniprot Human proteome FASTA file with added sequences specific for EBOV proteins and TurboID fusion proteins, were used in SEQUEST searches. The impurity correction factors obtained from Thermo Fisher Scientific for each kit was included in the search and quantification. The following settings were used to search the streptavidin enriched data; dynamic modifications; Oxidation / +15.995Da (M), Deamidated / +0.984 Da (N, Q), TMT6plex / +229.163 Da (K) ,Biotin / +226.078 Da (K) and static modifications of TMT6plex / +229.163 Da (N-Terminus), Carbamidomethyl +57.021 (C). Only unique+ Razor peptides were considered for quantification purposes. Target Decoy feature of Proteome Discoverer 2.3 was used to set a false discovery rate (FDR) of 0.01. Total peptides quantified were normalized to *Streptavidin* to adjust for loading bias and Protein Abundance Based method was used to calculate the protein level ratios. Low Abundance Resampling method was used to impute missing data and co-isolation threshold and SPS Mass Matches threshold were set to 50 and 65, respectively. ANOVA (Individual Proteins) was performed using Proteome Discoverer 2.3 workflow and FDR of 0.05 was chosen as the cut off to identify the top tier proteins that are differentially expressed across WT and TurboID samples.

Sequences added to the Uniprot Human proteome database for this experiment:

- Ebola VP30
- Ebola NP
- Ebola VP35_wildtype

- Ebola L_wildtype
- Ebola VP35_sCTurboID fusion protein
- Ebola L_sNTurboID fusion protein

Thresholding and network analysis of viral polymerase-proximity interactome: To curate a list of high confident hits for both viral polymerase-proximity interactome, we applied a threshold of > 1 for $\log_2$ transformed relative (rel.) abundance ratio (TurboID_pol/WT_pol) and a threshold of < 0.05 for the adjusted *p-value* for each abundance ratio. To analyze the differential interactome of EBOV polymerase with and without VP30, since the experimental set up only included the wild-type EBOV polymerase with VP30 as the control, abundance ratios of proteins identified in the TurboID polymerase interactome with or without VP30 were first normalized to the WT pol +VP30 control to get the $\log_2$ (fold change) value. Subsequently, the difference of the two $\log_2$ (fold change) values, $\Delta\log_2$ (fold change), was determined as a measure of the differential interactome of EBOV polymerase with and without VP30. To curate a list of high confidence hits, we retained the same threshold of “ < 0.05 ” for the adjusted *p-value* for corresponding abundance ratio of (TurboID_pol - VP30/WT_pol) and further applied a threshold of $\Delta\log_2$ (fold change) > 1 . Above mentioned lists including critical parameters used in thresholding were presented in **Table S1**.

High-confidence hits in both EBOV polymerase interactomes in the presence and absence of VP30 were displayed as protein-protein networks using Cytoscape. The web-based bioinformatic analyzer STRING was used to perform functional enrichment analysis to cluster nodes involved in the same biological process (FDR $< 1\%$).

siRNA screen with authentic EBOV infected cells

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 AllStar negative control (NSC) to account for plate-to-plate variations. Forty-eight hours later, plates were transferred to BSL4 for infection with EBOV-eGFP at indicated MOIs. Infected monolayers were fixed and inactivated by 10% formalin at two days post-infection and removed from BSL4 facility. Fixed monolayers were stained with Hoechst (1 μ g/ml in DPBS) for 15 mins at room temperature prior to imaging. Percentage of EBOV-eGFP infected Huh7 cells (% infection) and cell count with different siRNA treatment were quantified by CellInsight™ CX5 High Content Screening (HCS) Platform (ThermoFisher) using a 10x objective.

Relative % virus infection and relative cell count were calculated by normalizing raw percent viral infection rate and cell count of individual siRNA treatment to corresponding control wells in each plate. Heat maps showing relative %virus infection and relative cell count were generated by GraphPad Prism 9 using the normalized value of each individual siRNA treatment. The mean of numerical values from each triplicated well are used for plotting. To quantitatively analyze the impact of siRNA treatment on viral infection and cell count, multiple t-tests were performed, comparing normalized % infection of each siRNA treatment to corresponding NSC control. Bubble plots were generated by plotting numerical values of normalized cell count of each siRNA treatment against their value of normalized % infection if their *p-value* (represented by the size of each data point) was < 0.05. Outliers with cell count significantly different from the average by one standard deviation were ruled out because the effect of siRNA treatment on %infection in these cases can be confounded by a marked alteration in the total number of cells. siRNAs used for the screen were from the Genome-wide ON TARGET-Plus (OTP) Human siRNA library from Dharmacon/Thermo Scientific. Sequence information of each siRNA been used is curated in **Table S2**.

Co-Immunoprecipitations (co-IP)

Co-IP reactions were performed as previously described(Fang et al., 2018). HEK 293T cells (1×10^6) were seeded in 6-wells plate. Twenty-four hours later, cells in each well were transfected with 500 ng of pCI-empty or pCI-FLAG-GSPT1 plasmid combined with 3 μ g of pCEZ-L and 400 ng of pCAGGS-VP35-HA, plus and minus 300 ng of pCEZ-VP30, using TransIT LT1 transfection reagent. For experiments probing interactions between GSPT1 and EBOV VP35 or VP30, 1 μ g of pCI-empty or pCI-FLAG-GSPT1 plasmid combined with 1 μ g of pCAGGS-VP35-HA or pCEZ-VP30 (per well) were added to the co-transfection mix using the Lipofectamine 3000 transfection reagent. Three days later, transfected cells were washed with DPBS and lysed in 1X RIPA buffer including protease inhibitor cocktail (cOmplete™, EDTA-free Protease Inhibitor Cocktail), on ice for 15 mins. Soluble whole cell lysates were clarified by centrifugation at 12,000X rpm for 10 mins. FLAG-M2 affinity beads (40 μ l per reaction) were washed with DPBS for once, NT2 buffer for twice before blocking with BSA in NT2 buffer (0.5 mg/ml) for 0.5 – 1 hour at room temperature. Lysates containing equal amounts of total protein (1-1.5 mg determined by BCA assay) were incubated with BSA-blocked FLAG-M2 affinity beads and rotate overnight at 4 °C. Supernatant containing unbound proteins was removed by spin at 5000x g for 30 s, followed by four washes in 1 ml NT2 buffer. Immunoprecipitated proteins were eluted in 4X-SDS-loading buffer containing reducing agent with 10 mins incubation at 95 °C and analyzed by western blotting.

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). Forty-eight hours after transfection, Huh7 monolayers were transferred into BSL-4 facilities and infected with EBOV-eGFP at an MOI of 0.003 (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 four days post-infection, infected Huh7 monolayers were either stained with Hoechst and imaged by Olympus IX73 epi-fluorescent microscope using a 10X objective or collected directly in Trizol or 4x Laemmli buffer for inactivation and downstream RNA/protein quantifications. Inactivation procedures described here follow the approved standard operating procedures. 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. Fluorescent image acquired using Blue/Green Excitation for the GFP signal and Ultraviolet Excitation for the Hoechst stained nucleus. Images from the same field of view but different channels were merge using ImageJ/FIJI.

Strand-specific RT-qPCR

Total RNA was extracted from each Trizol-inactivated sample (per well) using Direct-zol RNA Miniprep Plus according to the manufacturer's protocol, eluted in 50 μ l DEPC-treated water and quantified by nanodrop. 200 ng of total RNA from each sample was used as the template for reverse transcription (RT) using High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol. Strand-specific RT primers were used to amplify vRNA/genome. Total mRNAs, including EBOV mRNAs, were amplified using an Oligo-dT primer due to the presence of poly-A tail. The resulting cDNAs were 1:100 diluted in DEPC-treated water and used in the subsequent quantitative real-time PCR on a Bio-Rad CFX Real-Time System. SYBR select master mix was used to mix with diluted cDNA template and target specific qPCR primer pairs in a 20 μ l reaction. Each reaction was performed in triplicated wells. PCR condition was following the Standard Cycling Mode (Primer $T_m \geq 60$ °C) according to manufacturer's protocol. A default dissociation curve was performed immediately after the real-time PCR run to obtain the T_m (melting temperature) of each target. All qPCR primers specifically target EBOV sequence were confirmed by detecting no signal in MOCK infected control sample. The T_m of samples using the same qPCR primer pairs were confirmed are the same. Amplification plots have baseline subtracted, and the relative quantification ($\Delta\Delta C_T$) method was used to analyze results. GAPDH was used as the house-keeping gene for data normalization. In Figure 6B, 6G and 6H, samples from NSC were used as control to calculate the relative fold-change of the target amplicon in GSPT1-knockdown

980 (GSPT1si8) samples. In Figure 6E, samples from MOCK infected cells were used as control to
981 calculate the relative fold-change of the target amplicon in EBOV infected cells.

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