Article:
García-Dorival, I, Wu, W, Armstrong, SD et al. (4 more authors) (2016) Elucidation of the Cellular Interactome of Ebola Virus Nucleoprotein and Identification of Therapeutic Targets. Journal of Proteome Research, 15 (12). pp. 4290-4303. ISSN 1535-3893

https://doi.org/10.1021/acs.jproteome.6b00337

Elucidation of the cellular interactome of the Ebolavirus nucleoprotein and identification of therapeutic targets

Isabel García-Dorival[1,2], Weining Wu[1], Stuart Armstrong[1,2], John N. Barr[3], Miles W. Carroll[4,5], Roger Hewson[4,5] and Julian A. Hiscox[1,2*]

[1]Institute of Infection and Global Health, University of Liverpool, Liverpool, L3 5RF, UK.

[2]NIHR Health Protection Research Unit in Emerging and Zoonotic Infections, Liverpool, UK.

[3]School of Molecular and Cellular Biology, Faculty of Biological Science, University of Leeds, Leeds, LS2 9JT, UK.

[4]Public Health England, Porton Down, Salisbury, SP4 0JG, UK.

[5]NIHR Health Protection Research Unit in Emerging and Zoonotic Infections, Porton Down, UK.

Abstract

Ebola virus (EBOV) infection results in severe disease and in some cases lethal haemorrhagic fever. The infection is directed by seven viral genes that encode nine viral proteins. By definition viruses are obligate intracellular parasites and require aspects of host cell biology in order to replicate their genetic material, assemble new virus particles and subvert host cell anti-viral responses. Currently licenced antivirals are targeted against viral proteins to inhibit their function. However, experience with treating HIV and influenza virus demonstrates that resistant viruses are soon selected. An emerging area in virology is to transiently target host cell proteins that play critical pro-viral roles in virus biology, especially for acute infections. This has the advantage that the protein being targeted is evolutionary removed from the genome of the virus. Proteomics can aid in discovery biology and identify cellular proteins that may be utilised by the virus to facilitate infection. This work focused on defining the interactome of the EBOV nucleoprotein and identified that cellular chaperones, including HSP70, associate with this protein to promote stability. Utilisation of a mini-genome replication system based on a recent Makona isolate demonstrated that disrupting the stability of NP had an adverse effect on viral RNA synthesis.

Keywords

Ebola virus, nucleoprotein, label free proteomics, HSP70, inhibitor, interactome.

Introduction

A new variant of Ebola virus (EBOV), Ebola-Makona, has been responsible for causing a large sustained viral outbreak in West Africa (2014-2015). During the course of the crisis several experimental therapies and vaccines have been trialled, with some success, but overall this has illustrated the long lack of development of therapeutic strategies for EBOV. An emerging area in virology is the repurposing of inhibitors (normally used in human medicine) that disrupt the functioning of cellular proteins critical for the biology of such obligate intracellular parasites. These inhibitors have distinct advantages in that they may have passed regulatory challenges for application in human health. The identification of such critical cellular proteins for virus biology can be achieved through proteomic approaches to investigate viral/host protein-protein interactions. In many cases this is facilitated by the use of an EGFP trap. This technique has proved a versatile tagging strategy for immune-precipitation on a variety of different viral proteins with disparate functions, such as RNA dependent RNA polymerases[1], interferon antagonists (including EBOV VP24)[2; 3] and proteins interacting with viral RNA[4; 5].

EBOV has a negative sense RNA genome that is encapsidated by the virally encoded nucleoprotein (NP). The N-terminal region of NP is thought to be important for RNA binding via its interaction with the phosphodiester backbone of the RNA.[6] When the NP binds to the viral RNA genome it forms

a highly stable NP-RNA complex structure (the RNP). This complex along with the viral protein 30 (VP30), the viral protein 35 (VP35) and the RNA-polymerase RNA dependent (L) form the polymerase complex[7], and these four viral proteins when expressed in cells, can replicate and transcribe EBOV RNA. This has been exploited in the development of plasmid based mini-genome systems that faithfully recapitulate EBOV RNA synthesis in the absence of infectious virus.[8] Thus opening the study of the function of the four viral proteins, viral RNA and also high throughput screening at containment level 2 (CL2). This work targeted NP to determine whether it interacted with host cell proteins, what these proteins were and how these might function in the biology of NP.

In order to comprehensively define the cellular interactome of EBOV NP a high-affinity co-immunoprecipitation coupled to a label free mass spectrometry-based approach was used. Using conservative selection criteria, approximately 150 cellular proteins were identified that had a high probability of interacting with NP. These included the heat shock protein 70 (HSP70) and members of the protein chaperone family. Inhibition of HSP70 function resulted in degradation of NP suggesting a role for HSP70 in modulating the stability of the protein. This interactome of EBOV NP was also compared to the interactome of Reston virus (RESTV) NP. This virus is a member of the Ebola virus genera and reported as non-pathogenic in humans.

To assess the effect of inhibiting HSP70 on the synthesis of EBOV RNA, a mini-genome system was developed based on the EBOV Makona isolate, taken from the 2014-2015 West African outbreak. This mini-genome system recapitulated the essential stages of virus replication and transcription and expressed a luciferase reporter gene under the control of viral replication/transcription signals, and can be used to investigate viral genetics and screen inhibitory compounds to both viral (and cellular) proteins.[9] Perturbations to replication and/or transcription would result in alterations in the abundance and hence activity of luciferase, which can be easily assayed. The four viral proteins essential for encapsidation of viral RNA and replication and transcription (L, VP35, VP30 and NP) were expressed as codon optimized proteins from support plasmids. The data presented in this work indicated that amount of luciferase produced by the mini-genome system decreased in cells treated with the HSP70 inhibitor compound VER-155008 compared to untreated cells, therefore the synthesis of EBOV RNA was reduced by inhibition of HSP70. These results suggest that HSP70 may play an important role for the EBOV virus replication probably by providing stability to NP.

Experimental procedures

Construction of plasmids that express EBOV NP fused to EGFP.

A codon optimized cDNA sequence for the ORF for EBOV (NCBI reference sequence number: NP_066243.1) and RESTV (NCBI reference sequence number: NP_690580.1) were cloned into the pEGFP-C1 and pEGFP-N1 plasmids to generate NP with C-terminal EGFP tag (NP-EGFP) or N-terminal EGFP tag (EGFP-NP). Once cloned, the plasmids and the insert integrity were confirmed by sequencing.

Plasmids used for the EBOV Makona mini-genome system.

The mini-genome system for EBOV (Makona strain), expressing luciferase, used in this study, was designed following the mini-genome system model developed by Mülhgerber in 1999.[8] The EBOV isolate H.sapiens-wt/GIN/2014/Makona-Gueckedou-C07 complete genome (NCBI sequence number KJ660347.2) sequence was used for the construction of the support plasmids. Furthermore, 50 sequences of EBOV isolates, characterised during this outbreak, were also used as a point of reference. [10]

Expression of NP and EGFP tag in HEK 293T cells.

Human embryonic kidney 293 (HEK 293T) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich) and 1% penicillin-streptomycin (Sigma Aldrich) at 37℃ with 5% CO_2. In order to transfect HEK 293T cells, two 145cm^2 dishes were seed with 4x10^6 cells 24 hours prior to transfection.

Calcium phosphate transfection was performed with 25.6µg of plasmid DNA for each plasmid; EGFP, EGFP-NP and NP-EGFP. Cells were harvested 24 hr post transfection, lysed and co-immunoprecipitated using a GFP-Trap (Chromotek).

NP-EGFP co-immunoprecipitation

NP/EGFP fusion proteins and EGFP co-immunoprecipitations were done using a GFP-Trap®_A (Chromotek) as described in previous publications.[2] Co-immunoprecipitated samples were then analysed using label free mass spectrometry. To investigate whether the interaction of NP with selected cellular proteins was mediated by RNA binding an RNAse treatment (15 units of RNAse (QIAGEN; 19101) was performed after the co-immunoprecipitations the with GFP-Trap.

Reverse co-immunoprecipitations

Reverse co-immunoprecipitations for the cellular proteins; chaperon HSP70 and its co-chaperon BAG-2 were performed using 50µl of the immobilized recombinant protein G resin (Generon) and specific antibodies against HSP70 (Abcam; ab2787) and BAG-2 (Abcam; ab47106). Reverse co-immunoprecipitation for EGFP-NP and NP-EGFP was done using an EBOV/NP Ab (IBT bioservices, 01-012); and on EGFP only as a negative control. The reverse co-immunoprecipitations were performed as described before.[2] For western blot analysis the elution step was done in 100µl of 2xSDS-sample buffer (120mM Tris/Cl pH6.8; 20% glycerol; 4%SDS, 0.04% bromophenol blue; 10% β-mercaptoethanol)

Label free Mass Spectrometry and bioinformatics analysis.

Label free proteomic analysis was performed as described previously for studying the interactome of EBOV VP24[2], the mass spectrometry analysis was performed in triplicate for EGFP-NP, NP-EGFP and EGFP. Once label free mass spectrometry results were processed, the Perseus software (MaxQuant) was used for the statistical analysis of the data. This helped differentiate the background proteins (those cellular proteins that interact with EGFP or matrix alone), from interacting proteins (those cellular proteins that interact with either EBOV or RESTV EGFP-NP or NP-EGFP). LFQ Intensity values were analysed using a T-Test analysis with a p-value of 0.01; a volcano plot graphic and tables were generated showing the statistical significant proteins, i.e. those proteins that had the highest probability of interacting with either EBOV or RESTV NP.

Western blot analysis

After the co-immunoprecipitation or reverse co-immunoprecipitation, western blot was performed as described previously. Antibodies used included EBOV/NP (IBT bioservices; 0301-012), GFP (Santa Cruz Biotechnology; sc-8334), DNAJA2 (Santa Cruz Biotechnology; sc-136515), BAG-2 (Abcam; ab47106), PCNA (Abcam; ab29), AIF (Abcam; ab32516), STAT1 (Abcam; ab3987), HSP70 (Abcam; ab2787), HSP90 (Abcam; ab1429). After three washes, blots were incubated with appropriate HRP secondary antibody diluted in blocking buffer at a 1:2000 for 1 hour at room temperature. Blots then were developed using enhanced chemiluminescent reagent (BioRad)

and detected using a BioRad Imaging system.

Luciferase assay and luciferase expression from the EBOV Makona mini-genome system in BSR-T7 cells.

BSR-T7 cells were grown in DMEM(Sigma-Aldrich) with 10% FBS(Sigma-Aldrich) plus 1X Glutamax (Invitrogen) and 600 μg/ml of G418(Life technologies) added at every second passage (stock at 100 mg/ml, add 90 μl to 15ml of medium in a T75 flask). BSR-T7 cells are derived from BHK-21 cells. These were used in this experiment for their ability to continuously express T7 polymerase. For transfection of a 24-well dish (Appleton woods), $8x10^4$ cells were seeded overnight to achieve a desired cell density of approximately 90%. Lipofectamine 2000 (Invitrogen) was used to transfect the BSR-T7 cells with the complete (+L) luciferase-based or incomplete (-L) luciferase based EBOV Makona mini-genome system. In order to do this different amounts of the mini-genome plasmids (miniG-Luc 0.5 μg, N 0.25 μg, VP35 0.125 μg, VP30 0.125 μg and L 0.125 μg or no L) plus a co-transfected "control" (plasmid expressing Renilla luciferase; Promega; pGL4.74[hRluc/TK]) reporter that was used as an internal baseline control. The transfection experiments were done using 2.0μl of Lipofectamine 2000 according to the manufacturer's instructions.

Luciferase was then visualised by western blot analysis, using an antibody against Firefly luciferase (Abcam; ab185923), and by the Dual-Luciferase® Reporter Assay System (Promega; E1910) according to the manufacturer's instructions.

Inhibition of HSP70 using VER-15508 and its effect on the replication and transcription of EBOV.

Transfection of the EBOV Makona mini-genome system using BSR-T7 cells was done as described before. Then, at 4h post-transfection, Lipofectamine was removed by changing the medium which contained different concentrations of the HSP70 inhibitor (VER-155008). VER-15508 was diluted in DMSO to make a stock solution of 10mM; then the stock solution was diluted in medium and add to the cells in different concentrations (5μM, 10μM, 20μM, 40μM, 60μM, 80μM and 100 μM). Cells were then incubating at 37C°, 5% CO_2 for 24 h before the analysis. Luciferase detection was then done using a western blot analysis and a Dual-Luciferase® Reporter Assay System (Promega; E1910).

Inhibition of HSP70 using VER-15508 and its effect on NP expression.

For NP transfection, $8x10^4$ BSR-T7 cells were seeded overnight in a 24-well multi well dish (Appleton woods), to achieve desired cell density of approximately 90%. Lipofectamine 2000 (Invitrogen) was used to transfect the BSR-T7 cells as was described before, 0.8 μg of EGFP, EGFP-NP or NP-EGFP DNA plasmid were add into 50 μl of OPTI-MEM (Invitrogen) while 2.0μl of Lipofectamine 2000 was add into another 50μl of OPTI-MEM (Invitrogen). Lipofectamine 2000 was then incubate for 5 min at room temperature in the OPTI-MEM media and then the mixture of Lipofectamine 2000 plus the EGFP, EGFP-NP or NP-EGFP plasmids was added; the mixture was then mix and incubated for 20 min at room temperature and

finally added dropwise onto cells maintained in 1 ml of growth media (DMEM with 10% FBS). At 4h post-transfection, Lipofectamine was removed by changing medium which contained different concentrations of the VER-15508 (10μM, 20μM, 40μM, 60μM and 80μM). Then, the cells were left incubating at 37C°, 5% CO_2 for 24 h before the analysis. NP-EGFP detection was done using a western blot analysis using a highly specific EGFP antibody (Santa Cruz Biotechnology; sc-8334) and an EBOV NP antibody (IBT bioservices; 0301-012).

Results

Expression of EBOV NP in 293T cells

To determine the cellular interacting partners of EBOV NP a high affinity EGFP co-immunoprecipitation (co-IP) coupled to a label-free mass spectrometry approach was used (Figure 1A). The target protein, NP, was expressed as an EGFP-fusion protein; this technique has been shown to improve the sensitivity and also to allow the discrimination between specific and non-specific interaction to the target proteins.[11; 12] The gene encoding for a codon optimized EBOV NP was cloned either at the 5' or 3' of an EGFP gene (NP-EGFP and EGFP-NP), creating a contiguous open read framing and the expression of a fusion protein. The expression of EGFP at either the N or C-terminal of NP was to mitigate for any steric hindrance caused by the presence of EGFP.

The fusion proteins were overexpressed in cells, and proteins were extracted from lysed cells and used for co-immunoprecipitation. To identify the potential interacting partners of NP, the eluate or bound fraction was then analysed by label free mass spectrometry. To reduce the false positive rate, EGFP alone was also expressed in 293T cells as a control. The 293T cells were selected for this study due to their high efficiency for calcium phosphate mediated transfection, annotation of the human protein database and because this cell line is permissive for EBOV infection.[13; 14; 15]

Protein expression was confirmed in 293T cells using immunofluorescence; the efficiency of transfections for all plasmids was approximately 70% (Figure

1B). Interacting partners for NP were then co-immunoprecipitated using an EGFP-Trap. After co-immunoprecipitation, both input and elution (or bound) samples were analysed using western blot; a protein corresponding to the molecular weight of NP-EGFP (131 kDa) was detected using and anti-EGFP Ab and an anti-NP antibody (Figure 1C). Label free mass spectrometry and quantitative proteomics was then used to distinguish between the EBOV NP-EGFP and EBOV NP-EGFP interactomes from the control (EGFP alone), which led to the identification of possible interacting partners for NP.

Identification of the potential cellular interacting partners of EBOV NP

Potential interacting partners for NP were identified by label free mass spectrometry; a statistical analysis approach was used in order to help distinguish the NP interacting partners from the background non-specific binding proteins (those cellular proteins that bind to the binding matrix or EGFP alone);[11; 12] the experiment was repeated three times. Approximately 1050 proteins were identified by mass spectrometry in the co-immunoprecipitation. After statistical analysis approximately 150 proteins were filtered to have a high probability (95% of confidence) of interacting with NP. The statistical analysis of the mass spectrometry results and all data sets were calculated using the Perseus program algorithm (Max Plank Institute). A t-Test analysis was done with a p-value <0.01. Additionally, proteins identified with a single peptide were removed. In the t-Test analysis each group of proteins detected in the MS for all the constructs that express NP was compared to the control (plasmid expressing EGFP alone).

The data was then organized in the form of volcano plots (Figure 2); in these volcano plots the logarithmic ratio of protein intensities (x-axis) was plotted against the negative logarithmic p-values of the t test (y-axis). The proteins that were significant or had a high probability of interacting with NP had a high log ratio and therefore are located in the upper right quadrant. Each dot outside the volcano represented a protein with a high probability of interacting with NP; those proteins inside the volcano are potential background binding proteins or proteins that do not interact with NP. For EGFP-NP, 124 cellular proteins were identified as interacting partners with statistical significance while for NP-EGFP, 151 cellular proteins were identified as interacting partners. Of these, 109 cellular proteins were common to EGFP-NP and NP-EGFP, suggesting a similar interactome for both constructs. Unique proteins were also identified that bound only to either EGFP-NP (15 cellular proteins) or to NP-EGFP (42 cellular proteins (Tables 2A and 2B). The cellular proteins that were unique to each construct had generally lower fold enrichment compared to those that interacted with both constructs. One exception was the stress-induced phosphoprotein 1 (STIP1). These unique proteins may be reflective of forming association with NP proximal to the position of the EGFP-tag, which may sterically hinder the interaction. Selected proteins with a higher probability of interacting with NP were selected for follow on studies (Tables 1A and 1B). A complete list of the possible interacting partners for EGFP-NP and NP-EGFP are shown in Tables S1 and S2 in the supplementary data section.

Validation of EBOV NP interactions.

Selected cellular targets identified as possible interacting partners of NP were then investigated by western blot analysis on repeat pull downs using the EGFP trap. Reverse immunoprecipitations were also performed to confirm the interaction between selected cellular proteins and the NP/EGFP fusion proteins. The selected cellular targets investigated in the immune-precipitations of NP were HSP70, HSP90, DNAJA2, PCNA, AIF and BAG2. These targets were selected due to their common functions (the protein chaperones), probability of interacting with NP (proteins with high binding ratio), and the availability of small molecules inhibitors. The presence of these proteins was confirmed by western blot (Figure 3A). Whole cell lysate (input) and eluate (bound) sample from co-immunoprecipitations analysis (GFP-Trap) were separated by 1D-SDS PAGE, proteins were then transferred in western blot and detected using a primary antibody against each selected protein. The data indicated that HSP70, HSP90, DNAJA2, PCNA, AIF and BAG2 associated with EGFP-NP and NP-EGFP. The cellular protein STAT1 was used as a negative control for the pull downs as it was not identified by LC-MS/MS, but was present in the input fractions (Figure 3A).

NP is a RNA binding protein, therefore to investigate whether these interactions were protein-protein rather than protein-RNA mediated, input samples were treated with RNAse before co-immunoprecipitation (Figure 3B). After immunoprecipitation of the treated input samples, the bound samples were analysed again by western blot using specific antibodies against selected proteins HSP70, BAG2 and DNAJA2 (Figure 3C). The

selected target proteins were found in the bound fraction after the RNAse treatment, which indicated that the interactions were not potentially mediated by RNA. However, DNAJA2 showed a reduction in the amount of protein in the pull down after the RNAse treatment (Figure 3C), indicating its interaction with NP was (part) RNA-mediated.

To further validate the mass spectrometry results and the potential interacting partners of NP, reverse co-immunoprecipitation was performed against selected cellular targets where antibody combinations allowed. These targets were NP (as a positive control), HSP70 and BAG2. NP was overexpressed in 293T cells and then cellular proteins were extracted. Immunoprecipitations were then performed using protein G beads and specific monoclonal antibodies against HSP70 or BAG2. The bound samples obtained from the reverse co-immunoprecipitations were then analysed by western blot, which confirmed the presence of NP (Figure 4A) and the interaction with HSP70 and BAG2 (Figure 4B and 4C, respectively). As a result of these interactions we hypothesized that HSP70 and other chaperones were important for viral RNA synthesis by promoting the stability of the N protein.

Interaction with HSP70 maintains the stability of NP.

To test the hypothesis that the function of HSP70 was critical for maintaining the stability of NP, a small molecular inhibitor compound was selected that prevents the interaction of HSP70 with the target protein,[16; 17]. This compound was used to treat cells expressing EBOV NP. The small molecule

has been used previously to study the interaction between HSP70 and other viral proteins.[1; 18; 19]. Therefore, increasing concentrations of VER-155008 was added to cells expressing either EGFP-NP, NP-EGFP or EGFP. Western blot was used to evaluate the abundance of EGFP, EGFP-NP or NP-EGFP in the presence of increasing concentrations of VER-155008 (Figure 5). The data indicated that as the concentration of VER-155008 increased, the abundance of the NP fusion protein decreased in comparison to GAPDH and EGFP (note equal amounts of protein were compared from each treatment condition). Only the highest concentration of VER-155008 had a negative impact on EGFP alone. Given the known activity of VER-155008 in disrupting the chaperone function of HSP70, we postulate that HSP70 is involved in maintaining the stability of NP (when expressed as an EGFP fusion protein).

The stability of wild type NP is dependent on HSP70 and disruption negatively impacts viral RNA synthesis

We hypothesized that disruption of HSP70 would negatively impact wild type NP and have a concomitant effect on viral RNA synthesis. To dissect this precise function and recapitulate this safely at CL-2, a mini-genome system for EBOV was developed (based on EBOV sequence from the recent West African outbreak). This was designed following the methodology developed to study other variants of EBOV.[8] The EBOV mini-genome system used in this study consisted of four support plasmids that expressed codon optimized L, VP30, VP35 and NP with a mini-genome plasmid expressing an artificial viral genomic RNA that contained the leader and trailer sequences from

EBOV with the luciferase (luc) gene as a reporter gene (Figure 6A). Expression of viral mRNAs were under the control of a T7 promoter and translation of the resulting proteins facilitated by an IRES. The amount of each plasmid used for the mini-genome system to produce luciferase was optimized (Table 3), as well as the time for the expression of luciferase to obtain the maximum signal-to-noise ratio. Validation of the system was confirmed by examining the expression of luciferase in the absence and presence of the L protein, where we predicted that absence of this protein would result in no luciferase. This was confirmed by comparing luciferase activity (Figure 6B) and the amount of luciferase by western blot (Figure 6C) between transfected cells with the three other support plasmids but either without and with the L protein. Thus luciferase protein/activity is dependent on synthesis of its mRNA by the viral components of the mini-genome system. Disruption of these components would negatively impact luciferase production which can then be quantified.

We hypothesized that disruption of HSP70 with VER-155008 in the context of the mini-genome system would result in the decreased abundance of NP and a reduction in the amount of luciferase, due to the involvement of NP in viral RNA synthesis. To test this, cells expressing the mini-genome were treated with increasing concentrations of VER-155008 which would impact the stability/abundance of the EGFP-tagged NPs. The amount of NP, luciferase and GAPDH was compared for each treatment condition and to a control. These were untreated cells either not expressing or expressing the L with the rest of the mini-genome components (Figure 7A). The activity of luciferase in

these different treatment conditions was also measured (Figure 7B). Similar to the observations with the EGFP-tagged NPs, increasing concentrations of VER-155008 negatively impacted the abundance of native NP and also luciferase expression but had no effect on GAPDH. Also, the activity of luciferase was decreased in the mini-genome system in the presence of increasing concentrations of VER-155008. Taken together this data indicated that the stability of NP was dependent on functioning HSP70. When NP was disrupted, synthesis of the luciferase mRNA in the mini-genome system was adversely effected.

The interaction with protein chaperones is conserved in other members of the Ebola virus family.

We hypothesized that if protein chaperones are important for the biology of EBOV NP then association with these proteins would be conserved in other members of the Ebola virus family. In terms of pathogenic outcome in humans, RESTV is most different from EBOV, in that no disease has been reported in humans that have been found to been potentially infected with the virus.[7] EBOV and RESTV NPs share 68% identity at the amino acid level. To investigate the interactome of RESTV NP, the protein was expressed in cells as either a N or C-terminal EGFP fusion protein. After mass spectrometry and data processing 113 cellular proteins were found to interact with RESTV EGFP-NP and 134 proteins were found to interact with RESTV NP-EGFP (Tables S3 and S4). From those, 92 were common for both constructs while 21 cellular proteins interacted with EGFP RESTV-NP and 42 proteins with RESTV NP-EGFP (Tables S5 and S6). Examination of the

resulting volcano plots (Figure 8) indicated that RESTV NP also interacted with same protein chaperones as EBOV NP. Western blot was also used to independently validate the interactions between selected cellular proteins and RESTV NP (Figure 9).

Discussion

There is little information available regarding the interactions of EBOV NP with cellular proteins. Elucidation of these interactions will provide a better understanding of the function of NP in virus biology and will also indicate potential cellular targets for potential novel antivirals that target the function of host proteins crucial for virus biology.[1; 2; 3; 4] To elucidate the cellular interactome of EBOV NP, a pull down approach coupled to mass spectrometry was used. This identified ~150 cellular proteins that potentially associated with EBOV NP and included protein chaperones. Interactions were confirmed with both repeat forward and reverse pulldowns. Some of these proteins, such as HSP90, have previously been proven to be important for the Ebolavirus biology.[20] Several novel interactions were also found, including; HSP70, BAG2, DNAJA2, AIF and PCNA. These cellular factors may be common to NPs encoded by other negative strand RNA viruses that belong, as does EBOV, to the order Mononegavirales. For example, chaperones have been shown to associate with the NP of Rabies virus and Measles virus[21; 22], thus indicating a general pattern. Certainly, the cellular interactome of the NP from another Ebola virus (RESTV) was similar to that of EBOV (Table S1, S2, S3, S4).

Due to its emerging importance in viral biology as illustrated by in depth work on Dengue virus[18], HSP70 was selected for further functional analysis using a specific small molecule inhibitor. HSP70 is involved in several cellular

processes including protein folding, regulation of the stress response, and control of the activity of regulation proteins.[23] Due to its multi-functional nature, HSP70 may also play an important role in viral survival strategies[24, 25; 26] such as avoiding degradation of certain viral proteins[21; 27], that may be expressed in large quantities or have complex folding; viral envelope protein/folding maturation[27] among others. These factors make chaperones essential for the stability and function of viral proteins and also can provide new possible drug target therapy against viruses.[20; 29; 30] In this study, VER-155008, a small molecule inhibitor against HSP70, was used to inhibit the function of HSP70 and its concomitant effects on EBOV biology in the context of a mini-genome system was evaluated. The targeted inhibition of HSP70 may concomitantly affect the stability of NP, but not necessarily the interaction between HSP70 and NP.

Conclusions

Treatment of cells expressing NP with VER-155008 showed a reduction in the abundance NP both in the context of the fusion proteins used for IP but also in the context of the mini-genome system. Suggesting that the chaperone activity of HSP70 was responsible for maintaining the stability of NP. In this latter system, the abundance and activity of luciferase in the EBOV mini-genome system decreased with increasing concentrations of the HSP70 inhibitor, suggesting a negative effect on the replication and/transcription of EBOV RNA. Similar results were found for HRSV (another member of the Mononegavirales family); where treatment with VER-155008 showed a reduction in the replication of HRSV[1] and also for Dengue virus where small doses of VER-155008 were demonstrated to affect virus

replication and therefore progeny virus production.[18] Overall, the data provides further evidence that protein chaperones play important roles in positively contributing to virus biology, and that this can be disrupted using a small molecule inhibitor.

Acknowledgements

The research was funded by the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Emerging and Zoonotic Infections at the University of Liverpool in partnership with Public Health England (PHE) and Liverpool School of Tropical Medicine (LSTM). The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, the Department of Health or Public Health England.

Author Information

Corresponding author.

Julian A. Hiscox (julian.hiscox@liverpool.ac.uk) (0151 795 0222).

Authors' contributions

IGD, WW and SA performed the study. IGD, WW, RH, MWC and JAH conceived the study and together with SA, MWC designed the experiments. IGD and WW conducted the interactome analysis and validation with SA performing the LC-MS/MS and informatics.. IGD, JNB and WW designed and perform the experiments with the mini genome system. IGD and JAH wrote the manuscript, with all authors contributing.

Notes

The authors declare no competing financial interest.

Supporting Information

Table S1 and S2: Complete list of cellular proteins that have a higher probability of forming protein-protein interactions with EBOV/NP. Shown are candidate proteins identified using the (S1) EGFP-EBOV/NP, (S2) EBOV/NP-EGFP fusion proteins and identified using label free quantitative proteomics.

Table S3 and S4: Complete list of cellular proteins that have a higher probability of forming protein-protein interactions with RESTV/NP. Shown are candidate proteins identified using the (S1) EGFP-RESTV/NP, (S2) RESTV/NP-EGFP fusion proteins and identified using label free quantitative proteomics.

BIBLIOGRAPHY

1. Munday, D. C.; Wu, W.; Smith, N.; Fix, J.; Noton, S. L.; Galloux, M.; Touzelet, O.; Armstrong, S. D.; Dawson, J. M.; Aljabr, W.; et al. Interactome Analysis of the Human Respiratory Syncytial Virus RNA Polymerase Complex Identifies Protein Chaperones as Important Cofactors That Promote L-Protein Stability and RNA Synthesis. J. Virol. **2015**, 89 (2), 917–930.

2. García-Dorival, I.; Wu, W.; Dowall, S.; Armstrong, S.; Touzelet, O.; Wastling, J.; Barr, J. N.; Matthews, D.; Carroll, M.; Hewson, R.; et al. Elucidation of the Ebola Virus VP24 Cellular Interactome and Disruption of Virus Biology through Targeted Inhibition of Host-Cell Protein Function. J. Proteome Res. **2014**, 13 (11), 5120–5135.

3. Wu, W.; Tran, K. C.; Teng, M. N.; Heesom, K. J.; Matthews, D. A.; Barr, J. N.; Hiscox, J. A. The Interactome of the Human Respiratory Syncytial Virus NS1 Protein Highlights Multiple Effects on Host Cell Biology. J. Virol. **2012**, 86 (15), 7777–7789.

4. Emmott, E.; Munday, D.; Bickerton, E.; Britton, P.; Rodgers, M. a; Whitehouse, A.; Zhou, E.-M.; Hiscox, J. a. The Cellular Interactome of the Coronavirus Infectious Bronchitis Virus Nucleocapsid Protein and Functional Implications for Virus Biology. J. Virol. **2013**, 87 (17), 9486–9500.

5. Liu, L.; Lear, Z.; Hughes, D. J.; Wu, W.; Zhou, E.; Whitehouse, A.; Chen, H.; Hiscox, J. a. Resolution of the Cellular Proteome of the Nucleocapsid Protein from a Highly Pathogenic Isolate of Porcine

Reproductive and Respiratory Syndrome Virus Identifies PARP-1 as a Cellular Target Whose Interaction Is Critical for Virus Biology. Vet. Microbiol. **2015**, 176 (1-2), 109–119.

6. Noda, T.; Hagiwara, K.; Sagara, H.; Kawaoka, Y. Characterization of the Ebola Virus Nucleoprotein-RNA Complex. J. Gen. Virol. **2010**, 91 (Pt 6), 1478–1483.

7. Feldmann, H., Sanchez, A., & Geisbert, T. W.. Filoviridae: marburg and ebola viruses. Fields virology. 6th ed. Philadelphia: Lippincott Williams & Wilkins, 923-56. **2013**.

8. Mühlberger, E. M.; Weik, M.; Volchkov, V. E.; Klenk, H.-D.; Becker, S. Comparison of the Transcription and Replication Strategies of Marburg Virus and Ebola Virus by Using Artificial Replication Systems. J. Virol. **1999**, 73 (3), 2333–2342.

9. Hoenen, T.; Groseth, A.; Callison, J.; Takada, A.; Feldmann, H. A Novel Ebola Virus Expressing Luciferase Allows for Rapid and Quantitative Testing of Antivirals. Antiviral Res. **2013**, 99 (3), 207–213.

10. Carroll, M. W.; Matthews, D. A.; Hiscox, J. A.; Elmore, M. J.; Pollakis, G.; Rambaut, A.; Hewson, R.; Garcia-Dorival, I.; Bore, J. A.; Koundouno, R.; et al. Temporal and Spatial Analysis of the 2014-2015 Ebola Virus Outbreak in West Africa. Nature **2015**, 524 (7563), 97–101.

11. Trinkle-Mulcahy, L.; Boulon, S.; Lam, Y. W.; Urcia, R.; Boisvert, F.-M.; Vandermoere, F.; Morrice, N. a; Swift, S.; Rothbauer, U.; Leonhardt, H.; et al. Identifying Specific Protein Interaction Partners Using Quantitative Mass Spectrometry and Bead Proteomes. J. Cell Biol. **2008**, 183 (2), 223–239.

12. Trinkle-Mulcahy, L. Resolving Protein Interactions and Complexes by Affinity Purification Followed by Label-Based Quantitative Mass Spectrometry. Proteomics **2012**, 12 (10), 1623–1638.

13. Khan, K. H. Gene Expression in Mammalian Cells and Its Applications. Adv. Pharm. Bull. **2013**, 3 (2), 257–263.

14. Jordan, M.; Schallhorn, A.; Wurm, F. M. Transfecting Mammalian Cells: Optimization of Critical Parameters Affecting Calcium-Phosphate Precipitate Formation. Nucleic Acids Res. **1996**, 24 (4), 596–601.

15. Kuhn, J., & Calisher, C. H. Filoviruses: a compendium of 40 years of epidemiological, clinical, and laboratory studies (Vol. 20). Springer Science & Business Media, **2008**.

16. Wen, W.; Liu, W.; Shao, Y.; Chen, L. VER-155008, a Small Molecule Inhibitor of HSP70 with Potent Anti-Cancer Activity on Lung Cancer Cell Lines. Exp. Biol. Med. (Maywood). **2014**, 239 (5), 638–645.

17. Williamson, D. S.; Borgognoni, J.; Clay, A.; Daniels, Z.; Dokurno, P.; Drysdale, M. J.; Foloppe, N.; Francis, G. L.; Graham, C. J.; Howes, R.; et al. Novel Adenosine-Derived Inhibitors of 70 kDa Heat Shock Protein, Discovered through Structure-Based Design. J. Med. Chem. **2009**, 52 (6), 1510–1513.

18. Taguwa, S.; Maringer, K.; Li, X.; Bernal-Rubio, D.; Rauch, J. N.; Gestwicki, J. E.; Andino, R.; Fernandez-Sesma, A.; Frydman, J. Defining Hsp70 Subnetworks in Dengue Virus Replication Reveals Key Vulnerability in Flavivirus Infection. Cell **2015**, 163 (5), 1108–1123.

19. Manzoor, R.; Kuroda, K.; Yoshida, R.; Tsuda, Y.; Fujikura, D.; Miyamoto, H.; Kajihara, M.; Kida, H.; Takada, A. Heat Shock Protein 70 Modulates Influenza A Virus Polymerase Activity.; **2014**; Vol. 289.

20. Smith, D. R.; McCarthy, S.; Chrovian, A.; Olinger, G.; Stossel, A.; Geisbert, T. W.; Hensley, L. E.; Connor, J. H. Inhibition of Heat-Shock Protein 90 Reduces Ebola Virus Replication. Antiviral Res. **2010**, 87 (2), 187–194.

21. Lahaye, X.; Vidy, a.; Fouquet, B.; Blondel, D. Hsp70 Protein Positively Regulates Rabies Virus Infection. J. Virol. **2012**, 86 (9), 4743–4751.

22. Zhang, X.; Bourhis, J. M.; Longhi, S.; Carsillo, T.; Buccellato, M.; Morin, B.; Canard, B.; Oglesbee, M. Hsp72 Recognizes a P Binding Motif in the Measles Virus N Protein C-Terminus. Virology **2005**, 337 (1), 162–174.

23. Mayer, M. P.; Bukau, B. Hsp70 Chaperones: Cellular Functions and Molecular Mechanism. Cell. Mol. Life Sci. **2005**, 62 (6), 670–684.

24. Mayer, M. P. Recruitment of Hsp70 Chaperones: A Crucial Part of Viral Survival Strategies. Rev. Physiol. Biochem. Pharmacol. **2005**, 153, 1–46.

25. Xiao, A.; Wong, J.; Luo, H. Viral Interaction with Molecular Chaperones: Role in Regulating Viral Infection. Arch. Virol. **2010**, 155 (7), 1021–1031.

26. Kim, M. Y.; Oglesbee, M. Virus-Heat Shock Protein Interaction and a Novel Axis for Innate Antiviral Immunity. Cells **2012**, 1 (3), 646–666.

27. Katoh, H.; Kubota, T.; Kita, S.; Nakatsu, Y.; Aoki, N.; Mori, Y.; Maenaka, K.; Takeda, M.; Kidokoro, M. Heat Shock Protein 70 Regulates Degradation of the Mumps Virus Phosphoprotein via the Ubiquitin-Proteasome Pathway. J. Virol. **2015**, 89 (6), 3188–3199.

28. De Silva, A. M.; Balch, W. E.; Helenius, A. Quality Control in the Endoplasmic Reticulum: Folding and Misfolding of Vesicular Stomatitis

Virus G Protein in Cells and in Vitro. J. Cell Biol. **1990**, 111 (3), 857–866.

29. Geller, R.; Taguwa, S.; Frydman, J. Broad Action of Hsp90 as a Host Chaperone Required for Viral Replication. Biochim. Biophys. Acta - Mol. Cell Res. **2012**, 1823 (3), 698–706.

30. Neckers, L.; Tatu, U. Molecular Chaperones in Pathogen Virulence: Emerging New Targets for Therapy. Cell Host Microbe **2008**, 4 (6), 519–527.

31. Uebelhoer, L. S.; Albariño, C. G.; McMullan, L. K.; Chakrabarti, A. K.; Vincent, J. P.; Nichol, S. T.; Towner, J. S. High-Throughput, Luciferase-Based Reverse Genetics Systems for Identifying Inhibitors of Marburg and Ebola Viruses. Antiviral Res. **2014**, 106 (1), 86–94.

Figure 1. Expression of EBOV NP in 293T cells. (A) Schematic representation of the methodology used in this study. 293T cells were grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37℃ with 5% CO_2. Two 145cm^2 dishes were seeded with 4x10^6 cells 24 hours prior to calcium phosphate transfection with 25.6 µg of plasmid DNA encoding EGFP, EGFP-NP and NP-EGFP respectively. 24 hours post transfection the cells were harvested, lysed and immunoprecipitated using a GFP-Trap (Chromotek). Label free Mass Spectrometry analysis on the eluted samples was then carried out. (B) Expression of EGFP-NP, NP-EGFP and the control EGFP in 293T cells was confirmed by immunofluorescence using confocal microscopy; the panels show that expression of the four constructs were similar, however EGFP-NP showed a higher and more uniform expression in cells than NP-EGFP. (C) Analysis of the pull down products using a western blot confirmed the presence of EGFP-NP, NP-EGFP and the control EGFP with the expected molecular weight.

Figure 2. Volcano plots representing results of the MS and the statistical analysis for EBOV NP. The pull down and label free mass spectrometry was done in triplicate. In these volcano plots the dots outside the volcano represents the potential protein interacting partners for (A) EGFP-NP and (B) NP-EGFP. For any potential protein interaction partner with NP the value of its abundance co-immunoprecipitated with any of EBOV NP was compared to the value of co-immunoprecipitated with the control (EGFP alone).

Figure 3. Validation of possible interacting partners of EBOV NP. (A) The presence of selected cellular proteins in the pulldowns identified in the label free mass spectrometry analysis was confirmed by western blot analysis. This used both whole cell lysate or input samples (I) and the eluate samples (E) for the three different constructs. Specific monoclonal antibodies against HSP90, HSP70, AIF, DNAJA2, PCNA, BAG2 and STAT-1 were used to detect their respective proteins. (B) RNase treatment was confirmed by using agarose gel electrophoresis on samples that had been either left untreated or treated with RNase (as indicated by – or +, respectively). (C) Invesitgation whether EBOV NP interactions were RNA mediated using western blot on RNAase untreated and treated extracts. Specific monoclonal antibodies against HSP70, DnaJA2 and BAG2 were used to detected the target proteins.

Figure 4. Validation of EBOV NP interactions using reverse immunoprecipitation. To further validate the interaction results, reverse immunoprecipitations were preformed against selected cellular proteins identified by the label free mass spectrometry and subsequent statistical analysis. Specific antibodies against EBOV NP (as a positive control), HSP70 and BAG2 were used for the reverse pull down. Then, the presence of the protein complex NP-EGFP was confirmed using western blot analysis and a specific antibody against EGFP (for reverse pull down for EBOV-NP); and an EBOV/NP antibody in for the reverse pull down for HSP70 and BAG2.

Figure 5. Effect of VER-155008 on the abundance of EBOV NP in BSR-T7 cells. Different concentrations of the HSP70 inhibitor (VER-155008) were tested to determine the effect of different concentrations of the drug on the abundance of NP. Decreased expression of NP was observed when the cells were treated with VER-155008**.**

Figure 6 Optimization of amount of plasmids for the mini-genome system. (A) Schematic representation of the mini-genome system. The EBOV (Makona) mini-genome system consists of five plasmids: four support plasmids that express the NP, VP30, VP35 and L; and the mini-genome plasmid which contains the leader and trailer sequences of EBOV (Makona strain) and has a sequences that encode luciferase as a reporter gene. **(B)** Measurement of luciferase expression using the Dual Luciferase assay (Promega). **(C)** Western blot of luciferase expressed by the mini-genome system at 24 and 48 hours; for the western blot analysis an antibody against Firefly Luciferase was used.

Figure 7. Effect of the HSP70 inhibitors in the replication and transcription of EBOV. A mini-genome system for Ebola virus that expressed luciferase was used to measure the effect of VER-155008 to determine the effect of this compound on the replication-transcription of EBOV RNA. **(A)** For the experiment with the mini-gnome system, seven different concentrations of VER-15508 were used for the inhibition experiment. The results of this experiment was analysed by western blot analysis using an antibody against Firefly Luciferase .**(B)** The Dual Luciferase Assay (Promega) was also used to measure the amount of Luciferase produced by the mini-genome system. The x-axis indicates the different concentration of compound used in the experiment; in the y-axis, the fold change of Luciferase expression compared to the control. The results of these experiments were confirmed the western blot analysis.

Figure 8. Volcano plot representing results of the MS and the statistical analysis for RESTV NP. The pull down and label free mass spectrometry was performed in triplicate. In these volcano plots the dots outside the volcano represents the potential protein interacting partners for **(A) RESTV RESTV-NP and (B) RESTV NP-EGFP.** For any potential protein interaction partner with NP the value of its abundance co-immunoprecipitated with any of RESTV NP was compared to the value of co-immunoprecipitated with the control (EGFP alone).

Figure 9. Validation of possible interacting partners of RESTV NP by western blot analysis. Confirmation of proteins detected in the label free mass spectrometry analysis by western blot analysis was done in the whole cell lysate or Input samples (I) and in the eluate sample (E) for the three different constructs. Specific monoclonal antibodies against HSP90, HSP70, AIF, DNAJA2, PCNA, BAG2 and STAT-1 were used to detect the respecitve proteins. An anti-EGFP antibody was used as a control to show the presence of the constructs RESTV EGFP-NP and RESTV NP-EGFP in the input and in the elution samples.

Table 1: Cellular proteins that have a higher probability of forming protein-protein interactions with EBOV/NP. Shown are candidate proteins identified using the (A) EBOV EGFP-NP, (B) EBOV NP-EGFP fusion proteins and identified using label free quantitative proteomics. Protein identifier, protein name and gene names are indicated, total and unique peptides used to identify the protein are indicated. The –LogP value is a comparison of the cellular protein between the EBOV/NP pull down and EGFP, where the higher the number means the higher probability of interacting, and a threshold above 2.0 has been selected. The t-test difference is the difference of the means of the intensities of the cellular proteins in the EBOV-NP pull down and EGFP. The percentage of sequence coverage of the protein identified using the peptides is indicated SC%. The abundance (ppm) of the protein in an average human cell is listed – data taken from the PaxDb: Protein Abundance Across Organisms database

Table.1A: Significant proteins that interact with EGFP-EBOV/NP

	Protein IDs	Protein names	Gene names	Peptides	Unique peptides	-Log t-test P value	T-test Difference (EGFP-EBOV/NP vs EGFP)	SC [%]	Abundance (PPM)
1	Q5JP53;P07437	Tubulin beta chain	TUBB	42	8	1.3	7.3	78.4	1041 ppm
2	Q13825;B4DYI6	Methylglutaconyl-CoA hydratase, mitochondrial	AUH	8	8	4.7	6.9	28.0	27.3 ppm
3	Q16342;F5H4V9	Programmed cell death protein 2	PDCD2	11	11	5.7	6.7	39.0	NA
4	Q3ZCM7;F5H0I4	Tubulin beta-8 chain	TUBB8	13	2	4.3	6.1	25.2	175 ppm
5	P31689;B7Z5C0	DnaJ homolog subfamily A member 1	DNAJA1	25	25	3.2	5.9	63.5	15.2 ppm
6	Q96EY1;I3L1T6	DnaJ homolog subfamily A member 3, mitochondrial	DNAJA3	10	1	4.8	5.5	26.9	1.19 ppm
7	P49411;H3BNU3	Elongation factor Tu, mitochondrial	TUFM	23	23	3.0	5.4	59.7	991 ppm
8	O43175;Q5SZU1	D-3-phosphoglycerate dehydrogenase	PHGDH	33	33	2.8	5.4	64.4	100 ppm
9	Q14257;F8WCY5	Reticulocalbin-2	RCN2	12	12	6.1	5.3	50.2	112 ppm
10	Q9BUF5;K7ESM5	Tubulin beta-6 chain	TUBB6	23	10	2.7	5.3	67.0	183 ppm

11	Q9BVA1	Tubulin beta-2B chain	TUBB2B	36	1	4.4	5.2	73.9	543 ppm
12	P30153;F5H3X9	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	PPP2R1A	14	14	2.9	5.2	36.8	184 ppm
13	P17987;E7ERF2	T-complex protein 1 subunit alpha	TCP1	26	26	5.3	5.2	62.2	114 ppm
14	Q9H7B4;B0QZ88	Histone-lysine N-methyltransferase SMYD3	SMYD3	7	7	4.4	5.2	17.5	NA
15	Q10567;P63010	AP-1 complex subunit beta-1	AP1B1	13	13	2.7	5.0	13.8	127 ppm
16	O95831;E9PMA0	Apoptosis-inducing factor 1, mitochondrial	AIFM1	13	13	3.8	4.9	32.6	688 ppm
17	K7EJL1;B4DDG7	AP-1 complex subunit mu-1	AP1M1	8	8	5.9	4.9	26.2	3.03 ppm
18	H0YEN5;P15880	40S ribosomal protein S2	RPS2	10	10	2.7	4.7	49.7	131 ppm
19	Q9BQE3;F5H5D3	Tubulin alpha-1C chain	TUBA1C	37	2	2.4	4.5	76.8	691 ppm
20	F5GZS6;J3KPF3	4F2 cell-surface antigen heavy chain	SLC3A2	8	8	3.3	4.5	17.9	147 ppm
21	P68363;A8MUB1	Tubulin alpha-1B chain;Tubulin alpha-4A chain	TUBA1B	39	4	1.8	4.4	76.5	749 ppm
22	Q9Y230;B3KQ59	RuvB-like 2	RUVBL2	28	28	2.4	4.3	74.7	23.4 ppm
23	P56192;A6NC17	Methionine--tRNA ligase, cytoplasmic	MARS	12	12	4.2	4.3	20.0	6.11 ppm
24	P04843;B7Z4L4	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	RPN1	10	10	1.5	4.3	21.7	179 ppm
25	Q9GZT9	Egl nine homolog 1	EGLN1	3	3	4.3	4.2	14.6	NA
26	P85037;E9PM37	Forkhead box protein K1	FOXK1	8	6	3.1	4.1	11.5	NA
27	O60884;H3BMW5	DnaJ homolog subfamily A member 2	DNAJA2	10	10	3.1	4.1	29.1	3.39 ppm
28	P27824;B4DGP8	Calnexin	CANX	17	17	1.1	4.1	34.3	1213 ppm
29	B7Z9I1;Q5T4U5	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADM	8	8	3.8	4.1	29.1	828 ppm
30	P35613;R4GN83	Basigin	BSG	3	3	5.6	4.1	12.5	21.7 ppm
31	P11177;C9J634	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	PDHB	9	9	2.7	4.1	27.9	393 ppm
32	H7C4H2;Q9Y5M8	Signal recognition particle receptor subunit beta	SRPRB	6	6	2.6	4.0	46.2	17.5 ppm
33	P08238;Q58FF7	Heat shock protein HSP 90-beta	HSP90AB1	35	26	1.7	4.0	49.0	693 ppm
34	Q9Y5J9;G3XAN8	Mitochondrial import inner membrane translocase subunit Tim8 B	TIMM8B	6	6	4.6	3.9	60.2	NA
35	Q9BSD7;Q5TDF0	Cancer-related nucleoside-triphosphatase	NTPCR	7	7	4.4	3.9	56.8	4.71 ppm

Table.1B: Significant proteins that interact with EBOV/NP-EGFP**.**

	Protein IDs	Protein names	Gene names	Peptides	Unique peptides	-Log P value	t-test Difference (EBOV/NP-EGFP vs EGFP)	SC [%]	Abundance (PPM)
1	P31948;G3XAD8	Stress-induced-phosphoprotein 1	STIP1	58	58	4.2	8.1	74	120 ppm
2	Q14257;F8WCY5	Reticulocalbin-2	RCN2	12	12	5.3	7.4	50.2	112 ppm
3	Q5JP53;P07437	Tubulin beta chain	TUBB	42	8	1.4	6.9	78.4	1041 ppm
4	B4E2W0;P55084	Trifunctional enzyme subunit beta, mitochondrial;3-ketoacyl-CoA thiolase	HADHB	21	21	3.9	6.9	51.5	777 ppm
5	P31689;B7Z5C0	DnaJ homolog subfamily A member 1	DNAJA1	25	25	2.9	6.7	63.5	15.2 ppm
6	O95816;B4DXE2	BAG family molecular chaperone regulator 2	BAG2	15	15	3.7	6.7	77.3	5.24 ppm
7	P08238;Q58FF7	Heat shock protein HSP 90-beta	HSP90AB1	35	26	2.4	6.6	49	693 ppm
8	Q3ZCM7;F5H0I4	Tubulin beta-8 chain	TUBB8	13	2	5.3	6.5	25.2	175 ppm
9	P40939;B4DYP2	Trifunctional enzyme subunit alpha, mitochondrial;Long-chain enoyl-CoA hydratase	HADHA	16	16	4.8	6.3	30.9	1175 ppm
10	P49411;H3BNU3	Elongation factor Tu, mitochondrial	TUFM	23	23	3.9	6.2	59.7	991 ppm
11	P17066;P48741	Heat shock 70 kDa protein 6	HSPA6	19	2	5.5	6.2	20.5	104 ppm
12	Q9BUF5;K7ESM5	Tubulin beta-6 chain	TUBB6	23	10	3.9	6.0	67	183 ppm
13	Q9BVA1	Tubulin beta-2B chain	TUBB2B	36	1	5.4	5.8	73.9	543 ppm
14	Q16342;F5H4V9	Programmed cell death protein 2	PDCD2	11	11	4.1	5.8	39	NA
15	P17987;E7ERF2	T-complex protein 1 subunit alpha	TCP1	26	26	3.9	5.7	62.2	114 ppm

16	P34932	Heat shock 70 kDa protein 4	HSPA4	23	21	4.9	5.7	37.5	46.3 ppm
17	O95831;E9PMA0	Apoptosis-inducing factor 1, mitochondrial	AIFM1	13	13	5.5	5.7	32.6	688 ppm
18	P50502;H7C3I1	Hsc70-interacting protein;Putative protein FAM10A5	ST13	14	14	5.0	5.6	34.4	179 ppm
19	O60884;H3BMW5	DnaJ homolog subfamily A member 2	DNAJA2	10	10	4.3	5.5	29.1	3.39 ppm
20	Q9Y5J9;G3XAN8	Mitochondrial import inner membrane translocase subunit Tim8 B	TIMM8B	6	6	3.6	5.2	60.2	NA
21	P11142;E9PKE3	Heat shock cognate 71 kDa protein	HSPA8	68	24	3.1	5.2	73.1	1014 ppm
22	O43175;Q5SZU1	D-3-phosphoglycerate dehydrogenase	PHGDH	33	33	2.7	5.1	64.4	100 ppm
23	Q9BQE3;F5H5D3	Tubulin alpha-1C chain	TUBA1C	37	2	2.5	5.1	76.8	691 ppm
24	Q96EY1;I3L1T6	DnaJ homolog subfamily A member 3, mitochondrial	DNAJA3	10	1	3.7	5.0	26.9	1.19 ppm
25	Q9UNE7;H3BS86	E3 ubiquitin-protein ligase CHIP	STUB1	16	16	2.5	5.0	71	3.78 ppm
26	P68363;A8MUB1	Tubulin alpha-1B chain;Tubulin alpha-4A chain	TUBA1B	39	4	2.0	4.9	76.5	749 ppm
27	P08107;E7EP94	Heat shock 70 kDa protein 1A/1B	HSPA1A	74	45	3.2	4.9	84.9	389 ppm
28	P62081;B5MCP9	40S ribosomal protein S7	RPS7	5	5	3.3	4.8	30.9	288 ppm
29	P78527;E7EUY0	DNA-dependent protein kinase catalytic subunit	PRKDC	19	19	3.7	4.7	6	12.6 ppm
30	P07900;Q86U12	Heat shock protein HSP 90-alpha	HSP90AA1	21	13	3.8	4.6	36.3	841 ppm
31	P11177;C9J634	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	PDHB	9	9	5.2	4.6	27.9	393 ppm
32	Q9Y230;B3KQ59	RuvB-like 2	RUVBL2	28	28	3.5	4.6	74.7	23.4 ppm
33	B4DX52;P25685	DnaJ homolog subfamily B member 1	DNAJB1	8	8	4.0	4.5	46.7	10.7 ppm

Table 2: Unique cellular proteins that form protein-protein interactions with EBOV EGFP-NP (A) or EBOV NP-EGFP (B). Shown are unique candidate proteins identified using the (A) EBOV EBOV-NP, (B) EBOV NP-EGFP fusion proteins and identified using label free quantitative proteomics The –LogP value is a comparison of the cellular protein between the EBOV/NP pull down and EGFP, where the higher the number means the higher probability of interacting, and a threshold above 2.0 has been selected. The t-test Difference is the difference of the means of the intensities of the cellular proteins in the EBOV/NP pull down and EGFP.

Table 2A

EGFP-EBOVNP	Gene names	Log t-test P value	T-test Difference (EGFP-EBOV/NP vs EGFP)
1	TUBA1C	2.4	4.5
2	EGLN1	4.3	4.2
3	MTA2	2.2	3.5
4	AP1G1	3.7	3.4
5	PGRMC1	1.5	3.2
6	C1QBP	1.0	3.2
7	SSBP1	2.0	3.2
8	RBBP4	1.8	2.9
9	EIF4A2	2.2	2.6
10	TFRC	1.8	2.5
11	SERPINH1	1.2	2.5
12	TKT	4.2	2.3
13	PPP2CA	3.0	2.2
14	BCAP31	2.2	2.1
15	VAT1	2.1	2.1

Table 2B

EBOV/NP EGFP	Gene names	Log P value	t-test Difference (EBOV/NP-EGFP vs EGFP)
1	STIP1	4.2	8.1
2	HSPA6	5.5	6.2
3	HSPA4	4.9	5.7
4	ST13	5.0	5.6
5	TUBA1C	2.5	5.1
6	STUB1	2.5	5.0
7	HSP90AA1	3.8	4.6
8	DNAJB1	4.0	4.5
9	DYNC1I2	3.7	4.2
10	DNAJC7	4.0	4.0
11	UBL4A	2.9	3.5
12	HNRNPH1	2.4	3.3
13	CALU	3.4	3.3
14	PSMC3	3.2	3.3
15	GLG1	4.0	3.2
16	TIMM8A	4.4	3.1
17	BAG6	1.7	3.0
18	RPS3	1.4	3.0
19	RPS24	1.3	2.9
20	RPS10	3.5	2.8
21	SGTA	4.8	2.8
22	RPL13A	3.3	2.8
23	UQCRC2	3.0	2.8
24	ATP1B3	4.6	2.7
25	B2M	3.2	2.7
26	SDF2L1	1.5	2.7
27	SLC25A3	2.8	2.6
28	RPS5	1.2	2.6
29	RPL37A	3.5	2.6
30	RPL18A	3.9	2.5
31	RPS15A	1.1	2.5
32	TUBB4A	3.2	2.5
33	GNB2	2.4	2.4
34	XPO1	2.7	2.3
35	SAMHD1	1.8	2.2
36	SRSF6	3.5	2.2
37	EMD	2.3	2.2
38	DNAJB6	2.3	2.2
39	PSMC6	3.8	2.1
40	HSPA5	2.7	2.0
41	SPTLC1	2.7	2.0
42	YTHDF2	3.6	2.0

Table 3. Optimization of amount of plasmids for the minigenome system. : Table showing the different concentrations used in order to optimize the expression of Luciferase for the miningenome system; several controls were used (columns 1 to 4) and also different concentration of plasmids were tried for the optimization; the condition number 9 was the best and therefore selected to the following experiments.

EBOV Makona Minigenome											
(ug)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Mini G	0	0.5	0.5	0	0.25	0.25	0.5	0.5	0.5	0.5	0.5
N	0	0.25	0.25	0.25	0.125	0.25	0.125	0.25	0.25	0.25	0.25
VP35	0	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
VP30	0	0.125	0	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
L	0	0	0.125	0.125	0.125	0.125	0.125	0.0625	0.125	0.25	0.5