

1 **Mutation of Ebola virus VP35 Ser129 uncouples interferon antagonist**
2 **and replication functions**

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4 Morwitzer, MJ¹, Corona A², Zinzula L³, Fanunza E², Nigri C³, Distinto S², Vornholt C¹, Kumar
5 V⁴, Tramontano E², Reid SP^{1*}

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7 ¹ Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha,
8 NE 68198, USA

9 ²Department of Life and Environmental Sciences, University of Cagliari, Italy

10 ³The Max-Planck Institute of Biochemistry, Department of Molecular Structural Biology, Am
11 Klopferspitz 18, 82152 Martinsried, Germany

12 ⁴Mass Spectrometry and Proteomics Core Facility, University of Nebraska Medical Center,
13 Omaha NE, 68198, USA

14

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16 *Corresponding author- St Patrick Reid- Patrick.reid@unmc.edu

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18 **Abstract**

19 Ebolaviruses are non-segmented, negative-sense RNA viruses (NNSVs) within the order
20 *Mononegavirales* that possess the multifunctional virion protein 35 (VP35), a major determinant
21 of virulence and pathogenesis that is indispensable for viral replication and host innate immune
22 evasion. VP35 is functionally equivalent to the phosphoprotein (P) of other mononegaviruses
23 such as rhabdoviruses and paramyxoviruses. Phosphorylation of the P protein is universally
24 regarded as functionally important however, a regulatory role(s) of phosphorylation on VP35
25 function remains unexplored. Here, we identified a highly conserved Ser129 residue near the
26 homo-oligomerization coiled coil motif, which is essential for VP35 functions. Affinity-purification
27 MS followed by post-translational modification (PTM) analysis predicted phosphorylation of
28 Ser129. Co-immunoprecipitation, cross-linking, and biochemical characterization studies
29 revealed a moderately decreased capacity of VP35-S129A to oligomerize. Functional analysis
30 showed that Ser-to-Ala substitution of Ebola virus (EBOV) VP35 did not affect IFN inhibitory
31 activity but nearly abolished EBOV minigenome activity. Further coimmunoprecipitation studies
32 demonstrated a lost interaction between VP35-S129A and the amino terminus of the viral
33 polymerase but not between viral nucleoprotein (NP) or VP35-WT. Taken together, our findings
34 provide evidence that phosphorylation modulates VP35 function, supporting VP35 as a NNSV P
35 protein and providing a potentially valuable therapeutic target.

36 **Importance**

37 Ebola virus (EBOV) can cause severe disease in humans. The 2013-2016 West African
38 epidemic and the two recent outbreaks in the Democratic Republic of the Congo underscore the
39 urgent need for effective countermeasures, which remain lacking. A better understanding of
40 EBOV biology and the modulation of multifunctional viral proteins is desperately needed to
41 develop improved therapeutics. We provide evidence here that function of virion protein 35
42 (VP35) is modulated by phosphorylation of Ser129, a conserved residue among other
43 ebolavirus species. These findings shed light on EBOV biology and present a potential target for
44 broad acting anti-ebolavirus therapeutics.

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54 Introduction

55 Ebolaviruses are zoonotic pathogens associated with severe hemorrhagic disease in humans
56 (Messaoudi et al., 2015, Nelson et al., 2017). The genus *Ebolavirus*, which is of the family
57 *Filoviridae* and order *Mononegavirales*, includes six species of filamentous, enveloped, non-
58 segmented negative-sense RNA viruses (NNSVs): Ebola (EBOV), Sudan (SUDV), Reston
59 (RESTV), Tai Forest (TAFV), Bundibugyo (BDBV), and Bombali (BOMV) viruses (Feldmann et
60 al., 2013, Kuhn, 2017, Goldstein et al., 2018). Among these, EBOV has caused the most
61 substantial outbreaks, along with SUDV and BDBV (Messaoudi et al., 2015). Once sporadic,
62 EBOV outbreaks have become increasingly frequent and large, as evidenced by the
63 unprecedented 2013-2016 West African epidemic and the 2017 and currently ongoing
64 outbreaks in the Democratic Republic of the Congo (WHO, 2016, CDC, 2018). Even with
65 several promising therapeutic candidates under investigation, there remains no licensed
66 therapeutics available to prevent or treat infection (Keshwara et al., 2017, Cross et al., 2018,
67 Fanunza et al., 2018a). Thus, continued efforts to identify and develop therapeutics, particularly
68 those with pan-filovirus efficacy, should be a priority.

69 The ebolavirus genome is approximately 19 kb and encodes nine proteins, among which the
70 multifunctional virion protein 35 (VP35) is a major determinant of virulence and pathogenesis
71 (Leung et al., 2010b, Feldmann et al., 2013, Messaoudi et al., 2015). VP35 is a potent immune
72 antagonist, inhibiting interferon- α/β (IFN- α/β) production, activation of the IFN-inducible protein
73 kinase R (PKR) antiviral protein, and RNA interference (Basler et al., 2000, Basler et al., 2003,
74 Feng et al., 2007, Schumann et al., 2009, Haasnoot et al., 2007, Fabozzi et al., 2011). VP35
75 inhibits retinoic acid-inducible gene I (RIG-I)-like receptor signaling and IFN- α/β production by
76 several mechanisms, including sequestering immunostimulatory double-stranded RNA (dsRNA)
77 intermediates, inhibiting PACT-induced RIG-I ATPase activity, preventing kinases TANK-binding
78 kinase 1 (TBK1), and/or IB kinase epsilon (IKK ϵ) from activating interferon regulatory factors 3
79 and 7 (IRF3/IRF7), inactivating IRF7 by SUMOylation, and inhibiting TRIM6-mediated type I IFN
80 production (Cardenas et al., 2006, Bale et al., 2013, Dilley et al., 2017, Luthra et al., 2013, Prins
81 et al., 2009, Hartman et al., 2008, Chang et al., 2009, Bharaj et al., 2017). In addition to immune
82 suppression, VP35 also functions as an essential cofactor of the viral RNA-dependent RNA
83 polymerase (named L for large polymerase protein). The functional viral polymerase complex is
84 comprised of nucleoprotein (NP) and VP30 in addition to L and VP35 (Muhlberger et al., 1998,
85 Muhlberger et al., 1999, Prins et al., 2010). VP35 is thought to act as a bridge between the
86 catalytic subunit of L and the NP-associated viral RNA, thus VP35-L and VP35-NP interactions
87 are likely both essential for viral RNA synthesis (Becker et al., 1998, DiCarlo et al., 2007,
88 Trunschke et al., 2013, Kirchdoerfer et al., 2015, Leung et al., 2015). Moreover, VP35 serves as
89 a structural protein that is necessary, along with NP and VP24, for nucleocapsid formation
90 (Huang et al., 2002, Shi et al., 2008, Takamatsu et al., 2018). Furthermore, a recent study
91 identified novel NTPase and helicase-like activities of EBOV VP35, which suggests VP35 may
92 also participate in viral RNA remodeling activity (Shu et al., 2019). The N-terminus of VP35
93 contains a NP-chaperoning domain as well as a homo-oligomerization domain, and the C-
94 terminus contains a dsRNA/IFN inhibitory domain (RBD/IID) (Kirchdoerfer et al., 2015, Leung et
95 al., 2015, Reid et al., 2005, Bale et al., 2012, Bale et al., 2013, Kimberlin et al., 2010, Leung et
96 al., 2009a, Leung et al., 2009b, Leung et al., 2010c, Leung et al., 2010a, Leung et al., 2010d,

97 Ramanan et al., 2012). Homo-oligomeric organization of VP35 occurs via a predicted coiled-coil
98 motif in the homo-oligomerization domain, and is known to be required for replication and full
99 IFN antagonism (Reid et al., 2005, Moller et al., 2005). All ebolavirus VP35s form tetramers,
100 whereas only the oligomerization domain of EBOV VP35 (eVP35) is able to form trimers
101 (Zinzula et al., 2019b, Chanthamontri et al., 2019).

102 Filovirus VP35 is the functional equivalent to the phosphoprotein (P) of other members of the
103 order *Mononegavirales*, which also includes families such as *Rhabdoviridae*, *Paramyxoviridae*,
104 and *Bornaviridae*. As its name suggests, P protein is the most highly phosphorylated viral
105 protein in any infected cell or virion of mononegaviruses. Phosphorylation of P protein is
106 universally regarded as functionally important, with increasing experimental support. Thus far,
107 phosphorylation has been shown to modulate P protein function of vesicular stomatitis virus
108 (VSV) (Chattopadhyay and Banerjee, 1987, Barik and Banerjee, 1991, Barik and Banerjee,
109 1992a, Barik and Banerjee, 1992b, Gao and Lenard, 1995, Gao et al., 1996, Pattnaik et al.,
110 1997, Das and Pattnaik, 2004), respiratory syncytial virus (RSV) (Barik et al., 1995, Asenjo and
111 Villanueva, 2000, Villanueva et al., 2000, Asenjo et al., 2006, Asenjo et al., 2008, Asenjo and
112 Villanueva, 2016), Chandipura virus (CHPV) (Chattopadhyay et al., 1997, Raha et al., 1999,
113 Raha et al., 2000), Borna disease virus (BDV) (Schmid et al., 2007), parainfluenza virus 5
114 (PIV5) (Timani et al., 2008), Rabies virus (RABV) (Moseley et al., 2007), Rinderpest (RPV)
115 (Saikia et al., 2008), measles virus (MV) (Sugai et al., 2012), mumps virus (MuV) (Pickar et al.,
116 2014), and Newcastle disease virus (NDV) (Qiu et al., 2016). However, it is unknown whether
117 the function of filovirus P protein is regulated by phosphorylation. Of note, although all P
118 proteins are thought to perform similar transcriptional and replication functions, there is
119 surprisingly little sequence homology, even between the Indiana and New Jersey serotypes of
120 VSV. EBOV VP35 is known to be moderately phosphorylated (Elliott et al., 1985, Prins et al.,
121 2009). Accordingly, it might be expected that phosphorylation of VP35 is functionally important.

122 In this study, we aimed to further elucidate modulation of VP35 function by evaluating it as a
123 NNSV P protein. We used affinity purification-mass spectrometry (AP-MS) to identify putative
124 post-translational modifications (PTMs) of VP35. Phosphorylation was predicted for a highly
125 conserved Ser129 residue proximal to the homo-oligomerization domain. Oligomerization
126 studies indicated that an alanine mutant VP35-S129A had moderately reduced ability to homo-
127 oligomerize. Further functional studies revealed that while the mutant retained IFN antagonist
128 activity, minigenome activity was nearly abolished. VP35-NP and VP35-L interactions were
129 interrogated by co-immunoprecipitation (co-IP) and immunofluorescence (IF) experiments.
130 While VP35-NP interaction remained intact, VP35-L was abrogated, indicating that
131 phosphorylation of VP35 at S129 is crucial to the ability of VP35 to interact with L. These
132 results support VP35 as a NNSV P protein and further elucidate the regulation of VP35
133 functions, potentially offering a novel pan-filovirus therapeutic target.

134 **Methods**

135 *Antibodies*

136 Mouse anti-HA (H3663), rabbit anti-HA (H6908), mouse anti-FLAG (F3165), rabbit anti-FLAG
137 (F7425), rabbit anti-ISG56 (SAB1410690), and mouse anti-Calnexin (C7617) antisera were

138 purchased from Sigma-Aldrich (ST. Louis, MO, USA). Rabbit anti-VP35 (GTX134032) was
139 purchased from GeneTex (Irvine, CA, USA). Mouse anti-His (TA150088) was purchased from
140 OriGene (Rockville, MD, USA). Rabbit anti-L was purchased from IBT BioServices (Rockville,
141 MD, USA). Goat anti-rabbit HRP (65-6120), goat anti-mouse HRP (32430), goat anti-mouse
142 Alexa Fluor 488 (A32723), goat anti-rabbit Alexa Fluor 568 (A11011), and Hoechst 33342
143 (H3570) were purchased from ThermoFisher Scientific (Grand Island, NY, USA).

144 *Cell lines and viral RNA production*

145 HeLa, HEK293T, and A549 cells were grown in Dulbecco's modified Eagle's medium (Gibco)
146 supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Sigma).
147 Cells were maintained at 37°C in a humidified 5% CO₂ incubator. Viral RNA (vRNA) was
148 produced in A549 cells, infected with IAV/Puerto-Rico/8/34 (IAV PR8) strain at a multiplicity of
149 infection of 5. Five hours after infection, total RNA was isolated using the RNeasy® Kit from
150 Qiagen (Hilden, Germany). TRIzol™ Reagent was purchased from Thermo Fisher Scientific.

151 *Plasmids and Reagents*

152 Plasmid pGL-IFN-β-luc was kindly provided by Professor Stephan Ludwig, Institute of Molecular
153 Virology, University of Münster, Germany). pRL-TK was purchased from Promega (Promega
154 Italia S.r.l. Milan, Italy). pcDNA3-EBOV VP35, carrying EBOV VP35 gene from Zaire species
155 (1976 Yambuku-Mayinga strain) was constructed as previously reported (Cannas et al., 2015).
156 The VP35-S129A substitution was introduced in the pcDNA3-EBOV VP35 plasmid using the
157 QuikChange mutagenesis kit by following the manufacturer's instructions (Agilent Technologies
158 Inc., Santa Clara, CA). Primers' sequences: VP35-S129A Forward: 5'-
159 GATATGGCAAAAACAATCGCCTCATTGAACAGGGTTTG-3'; VP35-S129A reverse: 5'-
160 CAAACCCTGTTCAATGAGGCGATTGTTTTTGGCATATC-3'. pCAGGS-HA-EBOV-NP was
161 purchased from BEI (NR-49343). pCAGGS-NP-V5, pCAGGS-V5-VP30, and pcDNA3 vectors
162 were generated at the USAMRIID. pCAGGS-FLAG-VP35 and pCAGGS-L_1-505 were a kind
163 gift from Dr. Christopher F. Basler (Georgia State University). pCAGGS_L_EBOV and
164 pCAGGS_3E5E_luciferase were gifts from Dr. Elke Mühlberger (Addgene plasmid # 103052; #
165 103055) (Nelson et al., 2017).

166 jetPRIME transfection reagent was purchased from Polyplus-transfection (S.A., Illkirch,
167 France). T-Pro P-Fect Transfection Reagent was purchased from T-Pro Biotechnology (New
168 Taipei County, Taiwan). Lipofectamine 2000 and Lipofectamine 3000 were purchased from
169 Thermo Fisher Scientific. D-luciferin and Coelenterazine were purchased from Gold
170 Biotechnology (U.S. Registration No 3,257,927). Primers were purchased from (Metabion,
171 Planegg, Germany). The Dual-Glo Luciferase Assay System (E2920) was purchased from
172 Promega (Madison, WI, USA) and used per manufacturer's instructions.

173 *Viral sequences*

174 The following filovirus sequences were used for sequence comparison and/or cloning where
175 indicated: EBOV (*Zaire ebolavirus* isolate Ebola virus/H.sapiens-tc/COD/1976/Yambuku-
176 Mayinga, GenBank: NC_002549.1), SUDV (*Sudan ebolavirus* isolate Sudan virus/H.sapiens-

177 tc/UGA/2000/Gulu-808892, GenBank: NC_006432.1), RESTV (*Reston ebolavirus* isolate
178 Reston virus/M.fascicularis-tc/USA/1989/Philippines89-Pennsylvania, GenBank: NC_004161.1),
179 TAFV (*Tai Forest ebolavirus* isolate Tai Forest virus/H.sapiens-tc/CIV/1994/Pauleoula-CI,
180 GenBank: NC_014372.1), BDBV (*Bundibugyo ebolavirus* isolate Bundibugyo virus/H.sapiens-
181 tc/UGA/2007/Butalya-811250, GenBank: NC_014373.1), BOMV (*Bombali ebolavirus* isolate
182 Bombali ebolavirus/Mops condylurus/SLE/2016/PREDICT_SLAB000156, GenBank:
183 NC_039345.1). Multiple sequence alignment was made by Clustal Omega (Sievers and
184 Higgins, 2018).

185 *Mass spectrometry analysis*

186 Affinity-tagged VP35 was overexpressed in HeLa cells using Lipofectamine 2000 as per
187 manufacturer's instruction. Affinity purified material was resolved by SDS-PAGE and stained
188 with Coomassie (ThermoFisher Scientific). The stained protein band corresponding to protein of
189 interest was excised and processed for in-gel digestion at the UNMC proteomics facility using
190 the protocol of Shevchenko and colleagues (Shevchenko et al., 2006). Extracted peptides were
191 re-suspended in 2% acetonitrile (ACN) and 0.1% formic acid (FA) and loaded onto trap column
192 Acclaim PepMap 100 75µm x 2 cm C18 LC Columns (Thermo Scientific™) at flow rate of 4
193 µl/min then separated with a Thermo RSLC Ultimate 3000 (Thermo Scientific™) on a Thermo
194 Easy-Spray PepMap RSLC C18 75µm x 50cm C-18 2 µm column (Thermo Scientific™) with a
195 step gradient of 4–25% solvent B (0.1% FA in 80 % ACN) from 10-57 min and 25–45% solvent
196 B for 57–62 min at 300 nL/min and 50°C with a 90 min total run time. Eluted peptides were
197 analyzed by a Thermo Orbitrap Fusion Lumos Tribrid (Thermo Scientific™) mass spectrometer
198 in a data dependent acquisition mode. A survey full scan MS (from m/z 350–1800) was
199 acquired in the Orbitrap with a resolution of 120,000. The AGC target for MS1 was set as
200 4×10^5 and ion filling time set as 100 ms. The most intense ions with charge state 2-6 were
201 isolated in 3 s cycle and fragmented using HCD fragmentation with 40 % normalized collision
202 energy and detected at a mass resolution of 30,000 at 200 m/z. The AGC target for MS/MS was
203 set as 5×10^4 and ion filling time set 60 ms dynamic exclusion was set for 30 s with a 10 ppm
204 mass window. Protein identification was performed by searching MS/MS data against the swiss-
205 prot human protein database downloaded on Aug 20, 2018. The search was set up for full
206 tryptic peptides with a maximum of two missed cleavage sites. Acetylation of protein N-terminus
207 and oxidized methionine were included as variable modifications and carbamidomethylation of
208 cysteine was set as fixed modification. The precursor mass tolerance threshold was set 10 ppm
209 for and maximum fragment mass error was 0.02 Da. Qualitative analysis was performed using
210 PEAKS 8.5 software. The significance threshold of the ion score was calculated based on a
211 false discovery rate of $\leq 1\%$.

212 *Co-immunoprecipitations (co-IPs)*

213 HeLa cells (1×10^6 cells) were transfected with the indicated plasmids using 3 µL of the
214 transfection reagent jetPRIME (Polyplus) per 1 µg DNA per manufacturer's instructions. The
215 total amount of DNA for each transfection was kept constant in each experiment by
216 complementing with empty vector. Twenty-four h post-transfection, cells were lysed in a
217 modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM EDTA, 1% NP-40, 0.25%

218 Na-deoxycholate) containing protease and a phosphatase inhibitor cocktail (Thermo Scientific,
219 Waltham, MA, USA). Approximately 10% WCL was reserved for IB analysis before performing
220 co-IPs. To immunoprecipitate indicated proteins, lysates were incubated with EZview Red ANTI-
221 FLAG M2 Affinity Gel (Sigma) or EZview Red Anti-HA Affinity Gel (Sigma) for 1 h at 4 °C. Beads
222 were washed 4 to 5 times with TBS, re-suspended in 2X Lane Marker Reducing Sample Buffer
223 (Thermo Scientific), and then subjected to SDS-PAGE and immunoblotting, as indicated below.

224 *Immunoblotting (IB)*

225 Cell lysates were subjected to reducing SDS-PAGE and proteins were transferred onto PVDF
226 membranes. Blots were probed with indicated primary antibodies either 1–2 h at RT or overnight
227 at 4°C. Secondary incubations were performed for 1–2 h at RT using either goat anti-rabbit HRP
228 or goat anti-mouse HRP. Radiance chemiluminescence substrate (Azure Biosystems; Dublin,
229 CA, USA) was used to visualize protein on an Azure c600 imaging system.

230 *DSP Crosslinking*

231 HeLa cells were transfected with VP35-WT or VP35-S129A plasmids as previously indicated.
232 Twenty-four h post-transfection the cells were incubated in a PBS reaction buffer with or without
233 the Dithiobis(succinimidylpropionate) DSP cross-linker (Thermo Scientific) at final concentration
234 of 1 mM for 30 min at RT. The reaction was quenched by the addition of a stop solution (1M
235 Tris-HCl pH 7.5) for 15 min. The cells were subsequently lysed in the modified RIPA buffer and
236 resuspended in a non-reducing sample buffer (Thermo Scientific) and subjected to SDS-PAGE
237 and immunoblot analysis.

238 *Molecular cloning, expression and purification of recombinant proteins*

239 Constructs encoding for the recombinant EBOV (Zaire ebolavirus, Yambuku-Mayinga,
240 GenBank: NC_002549.1) WT and S129A VP35 oligomerization domains (residues 83-175)
241 were subcloned into pET21b vectors (Novagen) from synthetic DNA preparations (BioCat), then
242 expressed in *E.coli* BL21 (DE3) cells and purified by affinity and size-exclusion chromatography
243 as previously described (Zinzula et al 2019). Briefly, heat-shock transformed bacterial cells were
244 grown in LB medium (Carl-Roth) at 37 °C and 200 rpm up to 0.8 optical density at 600 nm, then
245 induced at 20 °C with 0.5 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) (Carl-Roth) for 16
246 hours. Proteins were purified by affinity on a 1 mL complete HisTag Purification Column (Roche)
247 and by size-exclusion on a Superose 12 10/300 GL (GE Healthcare) column, respectively.

248 *Miniaturized differential scanning fluorimetry (nanoDSF)*

249 NanoDSF was performed by using a Prometheus NT.48 nanoDSF instrument (NanoTemper
250 Technologies). Recombinant EBOV VP35 WT and S129A oligomerization domains were diluted
251 at 2.5 mg/mL in 25 mM tris-HCl pH 7.4, 150 mM NaCl, 2 mM MgCl₂ and loaded on 10 µL
252 standard UV capillaries. Measurement of the intrinsic protein fluorescence at 330 and 350 nm
253 wavelength was performed over a 20-95 °C thermal gradient run at 0.5 °C/min rate. Melting
254 temperature (T_m) values were determined as the first derivative maxima of the intrinsic
255 fluorescence 330/350 nm ratio functions. Data were averaged from at least three independent
256 experiments.

257 *Circular dichroism (CD) spectroscopy and secondary structure content analysis*

258 CD spectroscopy was performed on a J-715 spectropolarimeter (Jasco) flushed with N₂ and
259 equipped with a PDF 3505 Peltier thermostat (Jasco). Purified recombinant EBOV VP35 WT
260 and S129A oligomerization domains were diluted at 0.1 mg/mL in 25 mM tris-HCl pH 7.4, 150
261 mM NaCl, 2 mM MgCl₂ and transferred to a high-precision 1-mm pathlength quartz cuvette
262 (Helma Analytics). Wavelength scans were recorded between 190 and 250 nm, at 4 °C, 23 °C
263 and 37 °C, at 50 nm/min scanning speed with 1 s response time and 0.1 nm data pitch, each
264 spectrum being the average of four accumulations. Mean molar residue ellipticity (MRE) values
265 were calculated as $MRE = [\theta] = 3300 m\Delta A/lcn$, where m is the molecular mass in Daltons, A is
266 the absorbance, l is the path length in centimeters, c is the protein concentration expressed in
267 milligrams per milliliter and n is the number of amino acid residues. Data processing for
268 secondary structure content determination was performed by using the CONTINLL
269 deconvolution method and the SMP56 reference set implemented in the Spectra Manager
270 software package.

271 *Size exclusion chromatography (SEC) and multi-angle light scattering (MALS)*

272 SEC-MALS was performed on a Superdex 200 10/300 GL gel filtration column (GE Healthcare),
273 connected to a 1100 HPLC system with variable UV absorbance detector set at 280 nm (Agilent
274 Technologies) and coupled in line with a mini DAWN TREOS MALS detector and an Optilab
275 rEX refractive-index detector (Wyatt Technology, 690 nm laser). Purified recombinant EBOV
276 VP35 WT and S129A oligomerization domains (~50 to 100 µg) were loaded by autoinjection
277 and run at 23 °C and 0.5 mL/min flow rate in 25 mM tris-HCl pH 7.4, 150 mM NaCl, 2 mM
278 MgCl₂. Protein absolute molecular masses were calculated with the ASTRA 6 software (Wyatt
279 Technology) with the dn/dc value set to 0.185 mL/g. Bovine serum albumin (ThermoFisher) was
280 used as calibration standard. Data were averaged from at least two independent experiments.

281 *Computational modeling*

282 The coordinates for the oligomerization domain of VP35 from Ebola virus were taken from the
283 RCSB Protein Data Bank (Berman et al., 2000) as reported in the entry 6GBO.(Zinzula et al.,
284 2019a) The structure processing and optimization were carried out by means of Maestro Protein
285 Preparation Wizard default setting (2018). Original water molecules were removed. After
286 preparation, the structure was refined to optimize the hydrogen bond network using OPLS_2005
287 force field.(Kaminski et al., 2001). S129A mutation was generated starting from VP35-WT.
288 Therefore, the mutant geometry was optimized with a full minimization protocol considering
289 OPLS_2005 force field (Kaminski et al., 2001) and GB/SA implicit water, setting 10000 steps
290 interactions analysis with Polak-Ribier Coniugate Gradient (PRCG) method and a convergence
291 criterion of 0.1 kcal/(molÅ).

292 *Luciferase reporter gene assay for IFN promoter activation*

293 The luciferase reporter gene assay for IFN promoter activation was adapted from (Fanunza et
294 al., 2018b). HEK293T cells (1.5×10^4 cells/well) were seeded in 96-well plates 24 h before
295 transfection. Cells were transfected using T-Pro P-Fect Transfection Reagent, according to the

296 manufacturer's protocol. Plasmids pGL-IFN- β -luc (60 ng), pRL-TK (10 ng), and various dilutions
297 (100, 10, 1.0, 0.1, 0.01 ng) of pcDNA3 vector control (VC), pcDNA3_VP35-WT, or
298 pcDNA3_VP35-S129A were mixed with the transfection reagent in reduced serum medium
299 Optimem (Gibco) and incubated 20 min at RT. Transfection complexes were then gently added
300 into individual wells of the 96-well plate. Twenty-four hours after transfection, cells were
301 stimulated with influenza A virus (IAV)-RNA, pre-mixed with the transfection reagent in reduced
302 serum medium Optimem, and incubated for 24 h at 37 °C in 5% CO₂. Cells were harvested with
303 lysis buffer (50 mM Na-MES [pH 7.8], 50 mM Tris-HCl [pH 7.8], 1 mM dithiothreitol, and 0.2%
304 Triton X-100). To lysates were added luciferase assay buffer (125 mM Na-MES [pH 7.8], 125
305 mM Tris-HCl [pH 7.8], 25 mM magnesium acetate, and 2.5 mg/mL ATP). Immediately after
306 addition of 50 μ l of Luciferin buffer (25 mM D-luciferin, 5 mM KH₂PO₄) the luminescence was
307 measured in Victor3 luminometer (Perkin Elmer), and again after addition of 50 μ l of
308 Coelenterazine assay buffer (125 mM Na-MES [pH 7.8], 125 mM Tris-HCl [pH 7.8], 25 mM
309 magnesium acetate, 5 mM KH₂PO₄ and 10 μ M Coelenterazine). Relative light units (RLU) of
310 firefly luciferase signal were normalized to *Renilla* luciferase, with percentage of IFN induction
311 values calculated relative to unstimulated controls (indicated as 100%). Each assay was
312 performed in triplicate. Mean \pm standard deviation (SD) values and paired, two-tailed t tests
313 were calculated using GraphPad Prism 6.01 software (GraphPad Software, Inc.; San Diego,
314 CA, USA).

315 *RT-PCR assay for ISG expression*

316 HEK293T cells (3 \times 10⁵ cells/well) resuspended Dulbecco's modified Eagle's medium
317 supplemented with 10% fetal bovine serum were seeded in 6-well plates, pre-treated with 500 μ l
318 of Poly-D-lysine hydrobromide 100 μ g/ml for 1 h. After 24 h cells were transfected with 2500 ng
319 of plasmid (pcDNA3 vector control (VC), pcDNA3_VP35-WT, or pcDNA3_VP35-S129A) using
320 Lipofectamine 3000 transfection reagent, as per manufacturer's instruction. Twenty-four h after
321 transfection, cells were stimulated with 2500 ng of IAV-RNA, pre-mixed with the transfection
322 reagent in reduced serum medium Optimem, and incubated for 24 hours at 37 °C in 5% CO₂.
323 Total RNA was extracted from transfected cells with TRIzol® Reagent (Invitrogen). RNA was
324 then reverse transcribed and amplified using Luna Universal One-Step RT- qPCR kit (New
325 England Biolabs; Ipswich, MA, USA). Quantitative real-time PCR (RT-qPCR) experiments were
326 performed in triplicate in a CFX-96 Real-Time system (BioRad; Hercules, CA, USA). Primers
327 used: GAPDH Forward 5'-GAGTCAACGGATTTTGGTCGT-3', GAPDH Reverse 5'-
328 TTGATTTTGGAGGGATCTCG-3'; ISG15 Forward 5'-TCCTGGTGAGGAATAACAAGGG-3';
329 ISG15 Reverse 5'-CTCAGCCAGAACAGGTCTGTC-3'; 2'5'OAS Forward 5'-
330 AGCTTCATGGAGAGGGGCA-3'; 2'5'OAS Reverse 5'-AGGCCTGGCTGAATTACCCAT-3'.
331 Data were analyzed with Opticon Monitor 3.1. mRNA expression levels were normalized to the
332 level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), with percentage of gene
333 expression over the VC (indicated as 100%). Mean \pm standard deviation values of two replicates
334 and paired, two-tailed t tests were calculated using GraphPad Prism 6.01 software.

335 *Immunoblot for ISG56 expression*

336 HeLa cells (1×10^6 cells) were transfected with the indicated plasmids using 2 μ L of the
337 transfection reagent Lipofectamine 2000 (Thermo Fisher Scientific) per 1 μ g DNA per
338 manufacturer's instructions. Twenty four h post-transfection the cells were mock treated or
339 treated with 10 μ g/mL of poly I:C (InvivoGen). Twenty-four h post-treatment cells were
340 harvested, lysed in modified RIPA buffer and subjected to SDS-PAGE and immunoblot as
341 aforementioned with the indicated antibodies.

342 *Minigenome Assay*

343 A RNA polymerase II-driven EBOV minigenome was used as previously described (Nelson et
344 al., 2017). Briefly, HeLa cells (3.0×10^5) were seeded in 12-well plates 24 h before transfection.
345 Cells were transfected with minigenome components (125 ng pCAGGS-HA-NP, 125 ng
346 pCAGGS-FLAG-VP35, 50 ng pCAGGS-V5-VP30, 500 ng pCAGGS-L, and 750 ng of pCAGGS-
347 3E5E-luciferase) along with 50 ng pRL-TK (for transfection efficiency control) using jetPRIME
348 reagent as per manufacturer's recommendation. For the no L control, total DNA levels were
349 kept constant by complementing transfections with empty-vector pcDNA3. Reporter activity was
350 measured 48 h post-transfection using the Dual-Glo Luciferase Assay System and a Tecan
351 Spark microplate luminometer (Tecan Trading AG, Switzerland). Whole cell lysate (WCL) was
352 reserved from a representative replicate and subjected to immunoblotting as described below.
353 To account for potential differences in transfection efficiency, firefly luciferase activity was
354 normalized to *Renilla* luciferase values and plotted as fold activity calculated relative to the no L
355 control. Mean \pm standard error of the mean (SEM) values and paired, two-tailed t tests were
356 calculated using GraphPad Prism 7.05 software.

357 **Results**

358 *EBOV VP35 homo-oligomerization domain contains a putative regulatory serine* 359 *phosphorylation site*

360 Given that VP35 is analogous to the phosphoprotein of rhabdoviruses and paramyxoviruses, we
361 first sought to examine EBOV VP35 for sites of phosphorylation by mass spectrometry (MS).
362 Affinity-tagged VP35 was over-expressed in HeLa cells and isolated by immunoaffinity
363 purification. Among predicted phosphorylation sites identified by MS analysis, Ser129 was
364 chosen to evaluate for regulatory phosphorylation function due to its position, which lies just
365 outside the coiled-coil region responsible for VP35 homo-oligomerization (Figure 1A and C).
366 Moreover, multiple sequence alignment of all ebolavirus species revealed conservation among
367 all ebolavirus species except BOMV (Figure 1B). BOMV is a newly identified ebolavirus species
368 and has not yet been shown to be pathogenic in humans. All other ebolaviruses except RESTV
369 are pathogenic in humans, thus we reasoned the conservation of Ser129 may be functionally
370 important. Accordingly, it was hypothesized that Ser129 plays an important regulatory role for
371 VP35 as a NNSV P protein.

372 *VP35-S129A has moderately diminished oligomerization capacity*

373 Homo-oligomerization of VP35 is known to be required for both replication and contributes to
374 anti-IFN functions (Reid et al., 2005, Moller et al., 2005). Thus, we sought to determine whether

375 Ser129 impacts the ability of VP35 to oligomerize. Using a non-phosphorylated mimic of VP35
376 generated by alanine substitution at Ser129 (VP35-S129A), we first evaluated the ability of
377 VP35-S129A to interact with VP35-WT. HeLa cells were transfected with HA-VP35-WT or His-
378 VP35-S129A alone and in combination with FLAG-VP35-WT. After 24 h, proteins were
379 extracted and immunoprecipitations (IPs) using anti-FLAG beads were performed. IP samples
380 and whole cell lysates (WCLs) were then subjected to SDS-PAGE and products were analyzed
381 by immunoblot. As expected, HA-VP35-WT was shown to be pulled down only in the presence
382 of FLAG-VP35-WT, demonstrating specificity of binding (Figure 2A, top panels). Similarly, His-
383 VP35-S129A was also pulled down in the presence of FLAG-VP35-WT, indicating that the
384 VP35-S129A mutant retains the ability to self-associate. Appropriate protein expression was
385 confirmed by WCL analysis (Figure 2A, bottom panels).

386 To next evaluate VP35-S129A oligomerization ability, we performed cross-linking experiments,
387 along with a series of biochemical studies. VP35-WT and VP35-S129A were separately over-
388 expressed in HeLa cells for 24 h. Following cross-linking with Dithiobis(succinimidylpropionate)
389 (DSP), the oligomeric forms of VP35 were analyzed by immunoblot under non-reducing,
390 unboiled conditions. After cross-linking with DSP, VP35-WT had a marked increase in higher
391 oligomeric forms, as well as a modest decrease in monomeric form, relative to that of the non-
392 DSP treated VP35-WT isolate (Figure 2B). Notably though, cross-linking of VP35-S129A
393 resulted in a modest reduction in the formation of higher oligomeric forms compared to VP35-
394 WT. This indicates that while decreased, VP35-S129A can still oligomerize.

395 Full length VP35 has been shown to form both trimers and tetramers in solution (Reid et al.,
396 2005, Zinzula et al., 2009, Luthra et al., 2015, Bruhn et al., 2017, Ramaswamy et al., 2018), with
397 the homo-tetrameric state being the dominant one among ebolavirus species (Zinzula et al.,
398 2019b, Chanthamontri et al., 2019). Given that our VP35-S129A mutant displayed reduced
399 capability to undergo oligomerization under cross-linking experiments, we next wanted to
400 assess if the introduced mutation was affecting the quaternary structure of the oligomerization
401 domain. To this aim, we performed size-exclusion chromatography coupled with multi-angle
402 light scattering (SEC-MALS) on a truncated VP35 construct bordering the N-terminal coiled-coil
403 (residues 75-185). WT and S129A VP35 oligomerization domains both eluted in one distinct
404 peak with an average molecular mass of 51.8 and 51.6 kDa, respectively (Figure 2C).
405 Corresponding to roughly 3.9 folds in stoichiometry compared to their monomeric molecular
406 weight, these masses are consistent with the presence of a homo-tetramer in solution for both
407 WT and S129A VP35. We then wondered if the defective oligomerization phenotype observed
408 by the cross-linking immunoblot was due to any difference in stability between the homo-
409 tetrameric oligomerization domain of the WT and the S129A mutant. To address this question,
410 we performed thermal stability analysis by miniaturized differential scanning fluorimetry
411 (nanoDSF). As shown by their thermal denaturation curves, WT and S129 VP35 oligomerization
412 domains displayed melting temperature (T_m) values of 72.1°C and 70.6°C, respectively (Figure
413 2D). Although both these inflection points are reminiscent of a stable tertiary structure, the lower
414 T_m displayed by the S129A mutant suggests that a higher degree of flexibility exists at the level
415 of the VP35 oligomerization domain.

416 Therefore, in order to assess if the S129A mutation could cause structural perturbations
417 sufficient to locally affect the oligomerization domain folding, we tested it by circular dichroism
418 (CD) spectroscopy. In agreement to what has been previously observed for the WT VP35
419 oligomerization domain (Luthra et al., 2015; Zinzula et al., 2019), the CD spectra of the VP35
420 S129A mutant was typical to that of a protein mainly consisting of α -helices. Moreover, the
421 value of the 222/208 nm ellipticity ratio was close to 1 for both proteins at all three temperature
422 values tested (4°C, 23°C and 37°C), indicating that the coiled-coil motif - through which the α -
423 helices interact - remains in place in the S129A mutant. However, the difference in the ellipticity
424 profile of S129A suggests that this mutation introduces some local conformational change
425 (Figure 2E). Consistent with this hypothesis, deconvolution of CD spectra and analysis of the
426 secondary structure content showed that the VP35 S129A oligomerization domain has a lower
427 α -helical percentage with respect to WT at all temperature values tested, and that this content
428 diminishes as temperature increases (Figure 2F). This result indicates that a more flexible and
429 relaxed conformation is applied at the level of the VP35 coiled-coil superhelix upon the
430 substitution of Ser-to-Ala at residue 129.

431 We further investigated the effect of S129A on VP35 oligomerization domain conformation by
432 computational modeling. Starting from the available WT crystal structure, a Ser-to-Ala
433 substitution was made and the oligomerization domain was subjected to energy minimization.
434 The resulting structure was then compared to WT (Figure 3A and B). Conformation was
435 unaffected but did cause a loss of hydrogen bonds between Ser129 and the neighboring Ala125
436 (Figure 3C). The implicated biological effect may contribute to protein-protein interaction or
437 modulatory phosphorylation (Figure 3D). Taken together, these data indicate that VP35-S129A
438 has reduced capacity to oligomerize relative to VP35-WT and suggests a biological effect.

439 *VP35-S129A retains IFN antagonist function*

440 To assess a role of Ser129 in VP35 function, we examined IFN antagonist function, including
441 VP35 inhibition impact on the RIG-I-mediated signaling cascade and subsequent impairment of
442 the interferon stimulated-gene (ISG) expression. Using a luciferase reporter gene assay, we first
443 evaluated VP35-WT and VP35-S129A inhibition of dsRNA-induced RIG-I activation of the IFN- β
444 promoter. HEK 293T cells were co-transfected with pGL-IFN- β -luc, pRL-TK, and various
445 dilutions of a vector control, VP35-WT, or VP35-S129A. Twenty-four h post-transfection, cells
446 were stimulated with influenza A virus (IAV)-RNA. After 24 h, reporter activity was measured to
447 assess IFN inhibition. IFN- β promoter activation was significantly inhibited by both VP35-WT
448 and VP35-S129A at comparable efficiencies (Figure 4A). Next, we tested the effect of VP35-WT
449 and VP35-S129A on expression of ISGs upon stimulation by viral RNA or poly I:C. Using RT-
450 qPCR, we analyzed gene expression of two well characterized ISGs, ISG15 and 2'-5'-
451 oligoadenylate synthetase (OAS 2'-5'). Expression of these ISGs induced by stimulation with
452 vRNA was significantly reduced by the presence of either VP35-WT or VP35-S129A, relative to
453 the vector control. Specifically, ISG15 expression was inhibited 87% by VP35-WT and 90% by
454 VP35-S129A, and OAS 2'-5' expression was inhibited 87% by VP35-WT and 95% by VP35-
455 S129A (Figure 4B). Further, the effect of poly I:C stimulation on ISG56 protein expression was
456 assessed by immunoblot. HeLa cells were transfected with vector control, VP35-WT, or VP35-
457 S129A. Twenty-four h post-transfection cells were left unstimulated or stimulated with poly I:C.

458 Both VP35-WT and VP35-S129A were able to inhibit ISG56 protein expression, with levels
459 comparable to that of the non-stimulated vector control (Figure 4C). Collectively, the data show
460 that VP35-S129A retains IFN antagonist activity, indicating that VP35 Ser129 does not impact
461 IFN antagonist function.

462 *VP35-S129A abrogates EBOV minigenome activity and interaction with L₁₋₅₀₅*

463 Since VP35 is an essential polymerase cofactor, we next evaluated the effect of VP35-S129A
464 on EBOV minigenome activity. Plasmids encoding EBOV minigenome assay components NP,
465 L, VP35, VP30 and the 3E5E-luciferase minigenome, along with *Renilla* luciferase to control for
466 transfection efficiency variability, were co-transfected in HeLa cells. Forty-eight h post-
467 transfection, a dual-luciferase assay was used to measure reporter activity. Notably, the
468 presence of VP35-S129A nearly abolished minigenome activity relative to VP35-WT, with
469 activity level close to that of the no L control (Figure 5A). Additionally, representative lysates
470 from the minigenome assay were subjected to immunoblotting to confirm appropriate
471 expression of the WT and mutant (Figure 5B). While both VP35-WT and VP35-S129A
472 predominantly expressed one major species of the same migration (~38 kD), other bands were
473 observed. Specifically, VP35-WT contained modest upper bands at ~45 kD and ~85 kD),
474 whereas VP35-S129A contained a modest lower band at ~35 kD. This may be representative of
475 differential PTMs between VP35-WT and VP35-S129A, such as differing phosphorylation
476 states. Overall, these data suggest that Ser129 impacts VP35 transcription and replication
477 function.

478 To further investigate the role of Ser129 on VP35 replication function, we next examined
479 whether VP35-S129A retains the ability to interact with NP and L by co-IP experiments. HeLa
480 cells were transfected with FLAG-VP35-WT or HIS-VP35-S129A in the absence or presence of
481 either HA-NP or HA-L₁₋₅₀₅. The previously described HA-L₁₋₅₀₅ truncation mutant includes the
482 VP35 interaction site and was used due to protein detection issues of full length L (Shabman et
483 al., 2013, Trunschke et al., 2013). Vector control was included in transfections with VP35-WT or
484 VP35-S129A alone to keep total DNA amounts constant. After 24 h, proteins were harvested
485 and applied to IP using HA-tagged beads. After IP, WCLs and IP samples were subjected to
486 SDS-PAGE and products were analyzed by immunoblot. Both VP35-WT and VP35-S129A were
487 pulled down only in the presence of HA-NP, demonstrating specificity of interaction and
488 indicating that VP35-S129A retains the ability to interact with NP (Figure 5C). Notably, VP35-
489 WT was pulled down with HA-L₁₋₅₀₅ whereas VP35-S129A was not, indicating that the
490 substitution of Ser129 to Ala results in the loss VP35-L interaction (Figure 5D). Collectively,
491 these data show that Ser-to-Ala substitution at residue 129 of VP35 abolishes minigenome
492 activity through loss of interaction between VP35-S129A and L but does not affect IFN
493 antagonist function, effectively uncoupling IFN antagonist and replication functions. This is the
494 first report to suggest phosphorylation contributes a regulatory role in VP35 function and
495 presents a potential therapeutic target.

496 **Discussion**

497 The most highly phosphorylated protein of NNSVs is generally the P protein, with
498 phosphorylation universally regarded as important to P protein function. While phosphorylation

499 has been shown to modulate the function of several NNSVs including VSV, RSV, CHPV, BDV,
500 RABV, RPV, MV, MuV, and NDV, evidence to support this is lacking for filoviruses
501 (Chattopadhyay and Banerjee, 1987, Barik and Banerjee, 1991, Barik and Banerjee, 1992a,
502 Barik and Banerjee, 1992b, Gao and Lenard, 1995, Gao et al., 1996, Pattnaik et al., 1997, Das
503 and Pattnaik, 2004, Barik et al., 1995, Asenjo and Villanueva, 2000, Villanueva et al., 2000,
504 Asenjo et al., 2006, Asenjo et al., 2008, Asenjo and Villanueva, 2016, Chattopadhyay et al.,
505 1997, Raha et al., 1999, Raha et al., 2000, Schmid et al., 2007, Timani et al., 2008, Moseley et
506 al., 2007, Saikia et al., 2008, Sugai et al., 2012, Pickar et al., 2014, Qiu et al., 2016). The
507 combination of cell-biological, biochemical, and computational studies described here suggests
508 that phosphorylation plays a modulatory role in EBOV VP35 function, thus supporting
509 phosphorylation of the EBOV P protein as functionally significant. Specifically, we identify a
510 highly conserved Ser129 as a key regulatory residue, showing by Ala substitution that Ser129 is
511 important for VP35 replication function, but not IFN antagonist function. NanoDSF results
512 indicate that the S129A mutant possesses a higher degree of flexibility at the level of the VP35
513 oligomerization domain, with secondary structure content analysis indicating that a more flexible
514 and relaxed conformation is applied at the level of the VP35 coiled-coil superhelix. We further
515 show that while interactions with VP35-WT and NP remain intact, VP35-S129A exhibits
516 impaired ability to interact with the viral polymerase.

517 VP35, along with L, NP, and VP30, is an essential component of the filovirus replication
518 machinery, functioning as a non-enzymatic cofactor of the viral polymerase (Muhlberger et al.,
519 1998, Muhlberger et al., 1999, Boehmann et al., 2005, Prins et al., 2010). It is generally
520 accepted that VP35 acts as a bridge between L and NP, thereby serving to mediate L
521 interaction with NP-encapsidated template RNAs. Both VP35-L and VP35-NP interactions are
522 thus likely to be essential for viral RNA synthesis (Becker et al., 1998, DiCarlo et al., 2007,
523 Trunschke et al., 2013, Kirchdoerfer et al., 2015, Leung et al., 2015). It has been shown that
524 VSV P protein must undergo phosphorylation-dependent homo-oligomerization to become
525 transcriptionally active (Gao and Lenard, 1995). Consistent with these findings are the nanoDSF
526 results in this study which indicate that the VP35-S129A mutant possesses a higher degree of
527 flexibility at the level of the VP35 oligomerization domain, and severely decreases EBOV
528 minigenome activity. In addition, the oligomerization domain of MuV and hPIV3 P proteins has
529 been shown to have an enhancing effect on the P-L interaction, which further supports the
530 findings here (Choudhary et al., 2002, Pickar et al., 2015).

531 VP35 has been shown to be phosphorylated by IKK ϵ and TBK-1 in vitro, which suggests the
532 possibility that its function may be modulated by these kinases (Prins et al., 2009). In this case
533 VP35 exerts IFN-antagonist function by preventing TBK-1 and/or IKK ϵ from activating
534 IRF3/IRF7. It has yet to be determined whether VP35 becomes phosphorylated in EBOV-
535 infected cells, though, and whether that modulates its function. The extent to which VP35 is
536 phosphorylated by these kinases in EBOV-infected cells would be influenced by the extent to
537 which VP35 acts as a decoy substrate for IKK ϵ and TBK-1 relative to the extent VP35 merely
538 prevents kinase activation via steric inhibition (Prins et al., 2009). Even so, in our study here the
539 VP35-S129A mutant impaired replication function but did not affect VP35 IFN antagonist
540 function. Given the high virulence of EBOV infection and the multifunctional nature of VP35, it
541 seems unlikely that VP35 phosphorylation by IKK ϵ and TBK-1 would detrimentally affect virus

542 replication. Considering then that the IFN antagonism of the VP35-S129A mutant remained
543 intact instead suggests that another host kinase(s) would be involved in the potential
544 phosphorylation of Ser129.

545 Previous studies have demonstrated phosphorylation of NP and VP30; moreover, a functional
546 significance for VP30 phosphorylation has been described (Elliott et al., 1985, Becker et al.,
547 1994, Modrof et al., 2002, Martinez et al., 2008, Biedenkopf et al., 2016). The data here suggest
548 that phosphorylation of VP35 does play a modulatory role, which aligns with the homologous
549 functions of NNSV P proteins. Even so, the MS analysis herein was limited to predicting
550 phosphorylation of Ser129, rather than definitively identifying the residue as phosphorylated,
551 thus future studies will need to provide such evidence. Given that other P proteins, such as VSV
552 and RSV, have been dramatically affected by single Ser-to-Ala substitutions lends more
553 credence to phosphorylation of VP35 Ser129 exerting a modulatory role, as opposed to other
554 PTMs (Chattopadhyay and Banerjee, 1987, Asenjo and Villanueva, 2000). However, other
555 modifications of Ser are known to occur, including O-linked glycosylation and acetylation (Wang
556 et al., 2015). Future studies must confirm relevance during EBOV infection as well as determine
557 whether Ser129 is post-translationally modified, and, if so, whether the PTM is constitutive or
558 dynamic.

559 EBOV, SUDV, and BDBV infections cause severe disease in humans with high case fatality
560 rates (Feldmann et al., 2013). While there are promising vaccine and therapeutic candidates,
561 there remains an urgent need to develop effective therapeutics against ebolaviruses (Haque et
562 al., 2015, Espeland et al., 2018, Dhama et al., 2018). Development of drugs that interfere with
563 VP35 homo-oligomerization are likely to impair viral gene expression, therefore identification of
564 a potential target is an asset for the design of novel antiviral agents. Because Ser129 is well
565 conserved across ebolaviruses, inhibition of its modulatory role presents a potential pan-filoviral
566 therapeutic strategy.

567 In recent years, novel VP35 activities beyond IFN antagonist, polymerase cofactor, and
568 nucleocapsid component have been discovered, including repression of stress granules and
569 NTPase and helicase-like activities (Le Sage et al., 2017, Shu et al., 2019). Though these
570 studies have provided more insight into VP35 biology, the regulation of these activities is
571 underexplored, particularly regarding the homology of VP35 as a P protein. Here, our data
572 indicate that the Ser129 residue is important for VP35 polymerase cofactor function but not for
573 IFN antagonist function, which effectively uncouples the major function of VP35. Biochemical
574 characterization indicated that VP35-S129A has reduced capacity to oligomerize, and colPs
575 showed that the interaction between VP35 and L1-505 was abolished upon Ala substitution at
576 residue 129. Future studies will address host kinases and incorporate viral infections to shed
577 light on how VP35 function is modulated.

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872 **Figure 1.** EBOV VP35 homo-oligomerization domain contains a putative regulatory serine
873 phosphorylation site. (A) A schematic representation of VP35 domain organization. (B) Multiple
874 sequence alignment of the homo-oligomerization domain of all ebolavirus species. Dashed gray
875 box indicates the coiled coil region. Ser129 is highlighted in yellow. (C) MS/MS spectrum of the
876 VP35 peptide 111-133 from affinity purified FLAG-VP35 for PTM prediction. FLAG-VP35 was
877 overexpressed in HeLa cells, immunoprecipitated, resolved on 10% SDS-PAGE, stained with
878 colloidal Coomassie Blue, and in-gel digested with trypsin. Eluted peptides were analyzed by a
879 Thermo Orbitrap Fusion Lumos Tribrid mass spectrometer. The spectrum gives positive
880 identification of ITSLENGLKPVYDMAKTISSLNR with a phosphorylation site predicted for
881 Ser129.

882 **Figure 2.** VP35-S129A has moderately diminished oligomerization capacity. (A) CoIP
883 experiment demonstrating that VP35-S129A retains the ability to interact with VP35-WT. IP was
884 performed with anti-FLAG beads after expression of indicated plasmid in HeLa cells. Vector was
885 used to keep amount of DNA transfected constant. (B) DSP cross-linking experiment
886 demonstrating that VP35-S129A retains the capacity to oligomerize at levels near that of WT.
887 HeLa cells were transfected with VP35-WT or VP35-S129A. Twenty-four h after transfection,
888 cells were left untreated or treated with 1 mM of DSP. Asterisks denote oligomeric forms. (C)
889 Quaternary structure analysis of EBOV VP35-WT oligomerization domain compared to S129A
890 mutant by SEC-MALS. Absorbance peaks (280 nm) of protein retention volumes and absolute
891 molecular masses under the main peak of each sample are indicated by continuous and dashed
892 lines, respectively. (D) Thermal stability analysis of EBOV VP35-WT and S129A oligomerization
893 domain by nanoDSF. Variation in the protein intrinsic 330/350 nm fluorescence ratio upon
894 thermal denaturation and the corresponding first derivative curves are indicated by continuous
895 and dashed lines, respectively. T_m values are defined by the first derivative curve peak of each
896 sample. (E) Far-UV CD spectra of EBOV VP35-WT and S129A oligomerization domain at
897 different temperature values. The 222/208 nm MRE ratio value indicating the coiled-coil folding
898 of each sample is annotated. (F) Secondary structure content analysis of EBOV VP35-WT and
899 S129A oligomerization domain by CD spectra deconvolution with the CONTINLL method.
900 Structure composition of α -helices are indicated as percentages of the entire amino acid
901 sequence.

902 **Figure 3.** Predicted phosphorylation at Ser129 of VP35 oligomerization domain implicates
903 biological effect. (A) Comparison by computational modeling of VP35-WT (maroon) and VP35-
904 S129A (grey) oligomerization domain. (B) Close-up. (C) Hydrogen bond network. (D)
905 Phosphorylation at Ser129 of VP35 oligomerization domain.

906 **Figure 4.** VP35-S129A retains IFN antagonist function. (A) IFN- β promoter activity in the
907 presence of vector control (VC), VP35-WT (WT) or VP35-Ser129Ala (S129A). HEK293T cells
908 were co-transfected with 60 ng of pGL-IFN- β -luc, 10 ng pRL-TK, and decreasing amounts (100,
909 10, 1.0, 0.1, 0.01 ng) of VC, WT, or S129A plasmid. Twenty-four h after transfection, cells were
910 additionally transfected with IAV-RNA. After 24 h cells were lysed and luciferase activity was
911 measured. (B) RT-qPCR assay of interferon-stimulated gene (ISG) expression upon IAV-RNA

912 stimulation in the presence of VC, WT, or S129A plasmids. HEK293T were transfected with
913 2500 ng of VC, WT, or S129A plasmid. Twenty-four h after transfection, cells were stimulated
914 with 2500 ng of IAV-RNA. After 24 h, total RNA was extracted, reverse transcribed, and
915 subjected to quantitative real-time PCR (RT-qPCR) for the analysis of ISG15 and 2'-5'-
916 oligoadenylate synthetase (OAS 2'-5') levels. mRNA expression levels were normalized to the
917 level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data represent mean \pm SD
918 (n=3) of two independent experiments. (C) Effect of VP35-WT and VP35-S129A on ISG56
919 expression upon poly I:C stimulation. HeLa cells were left untransfected or transfected with
920 VP35-WT or VP35-S129A. Twenty-four h after transfection, cells were left untreated or treated
921 with 10 μ g/mL of poly I:C. After 24 h, cells were harvested and subjected to immunoblotting.
922 Data represent mean \pm SD. *p \leq 0.05, **p \leq 0.01, *** p \leq 0.001, **** \leq 0.0001.

923 **Figure 5.** VP35-S129A abrogates EBOV minigenome activity and interaction with L₁₋₅₀₅. A)
924 Effect of VP35-WT and VP35-S129A on minigenome activity. HeLa cells were transfected with
925 minigenome components (125 ng pCAGGS-HA-NP, 125 ng pCAGGS-FLAG-VP35, 50 ng
926 pCAGGS-V5-VP30, 50 ng pRL-TK, 500 ng pCAGGS-L, and 750 ng of pCAGGS-3E5E-
927 luciferase). Forty-eight h post-transfection, reporter activity was measured. Data represent
928 mean \pm SEM from one representative experiment (n=3) of at least three independent
929 experiments (B) Immunoblot confirmation of VP35-WT and VP35-S129A protein levels in the
930 minigenome (C) CoIP experiment demonstrating that VP35-S129A retains the ability to interact
931 with NP. (D) Representative IF images of FLAG-VP35-WT or HIS-VP35-S129A in the presence
932 of HA-NP. (E) CoIP experiment demonstrating a lost interaction between VP35-S129A and L₁₋₅₀₅.
933 IPs in (C and E) were performed with anti-HA beads. FLAG-VP35-WT or HIS-VP35-S129A
934 (red), HA-NP or HA-L₁₋₅₀₅, Hoechst 33342 nuclear stain (blue) were visualized by confocal
935 microscopy. Scale bars = 20 μ M. *p \leq 0.05.

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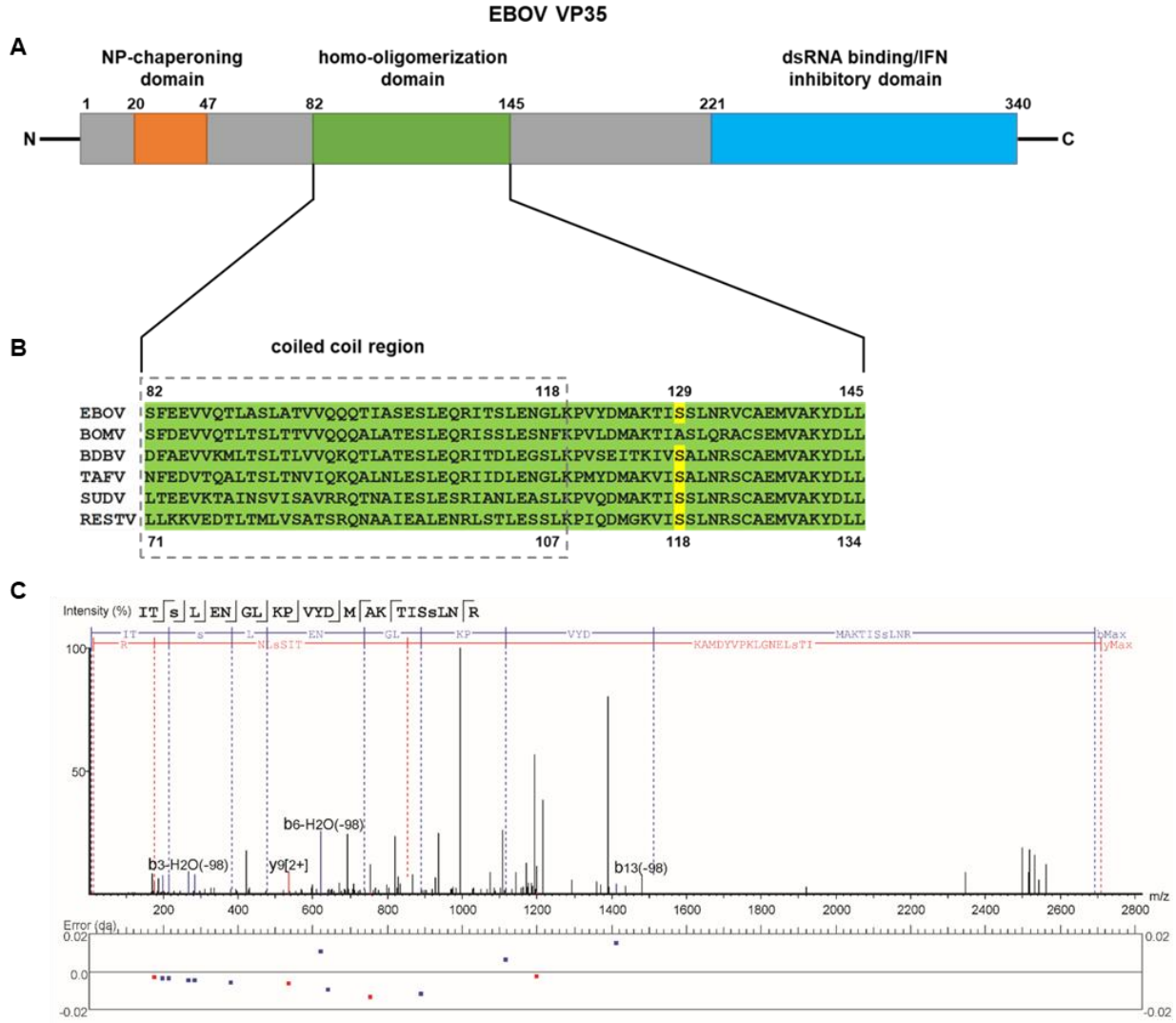
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947 **Figure 1**



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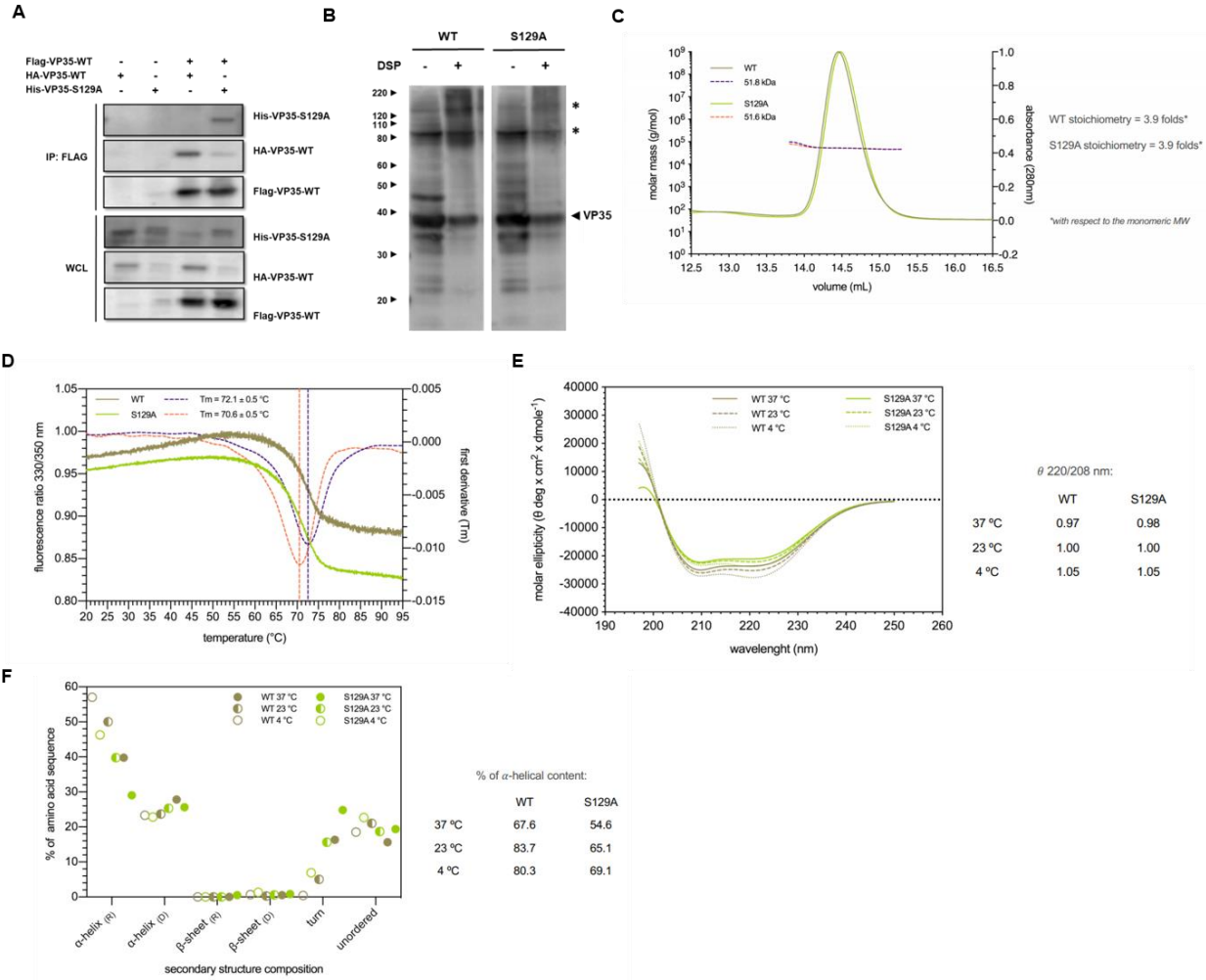
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957 **Figure 2**



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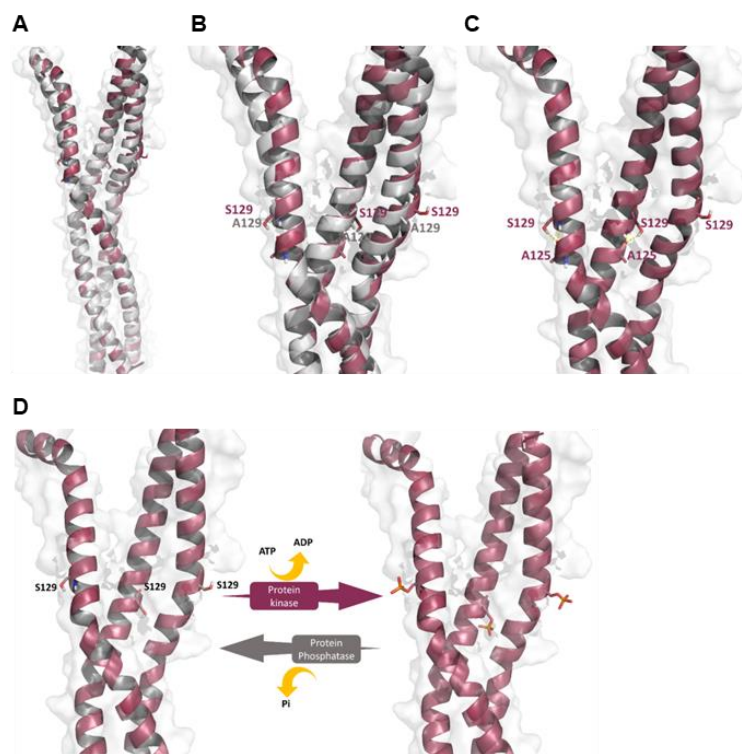
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968 **Figure 3**



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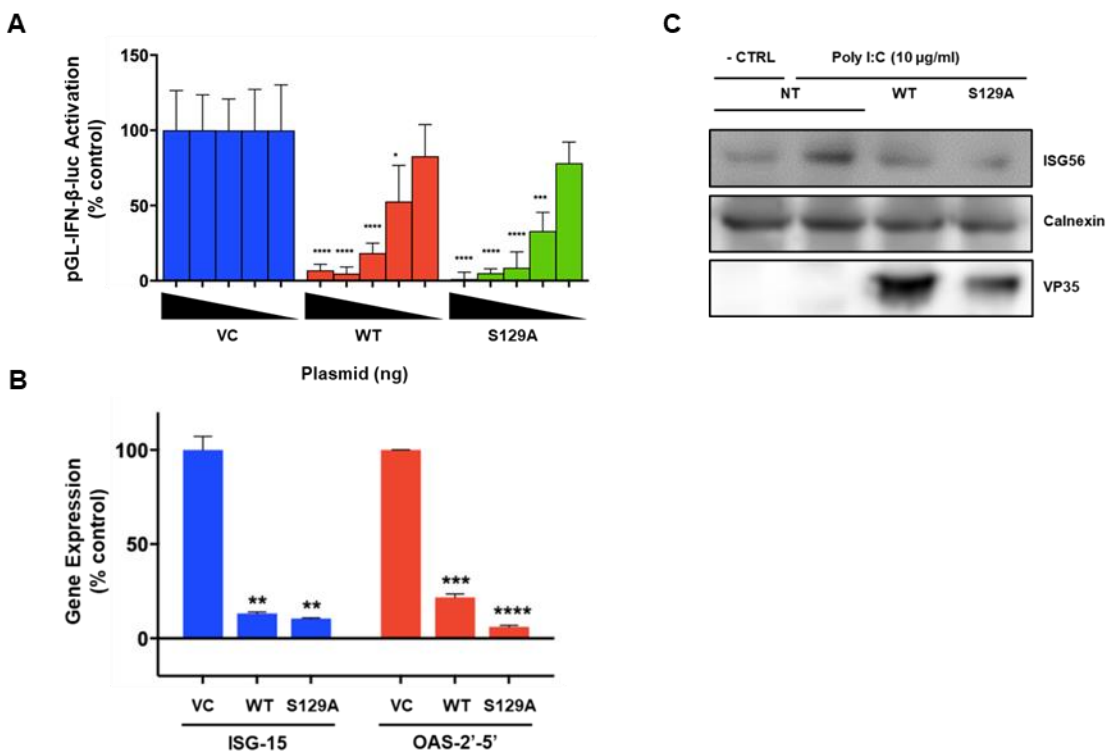
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978 **Figure 4**



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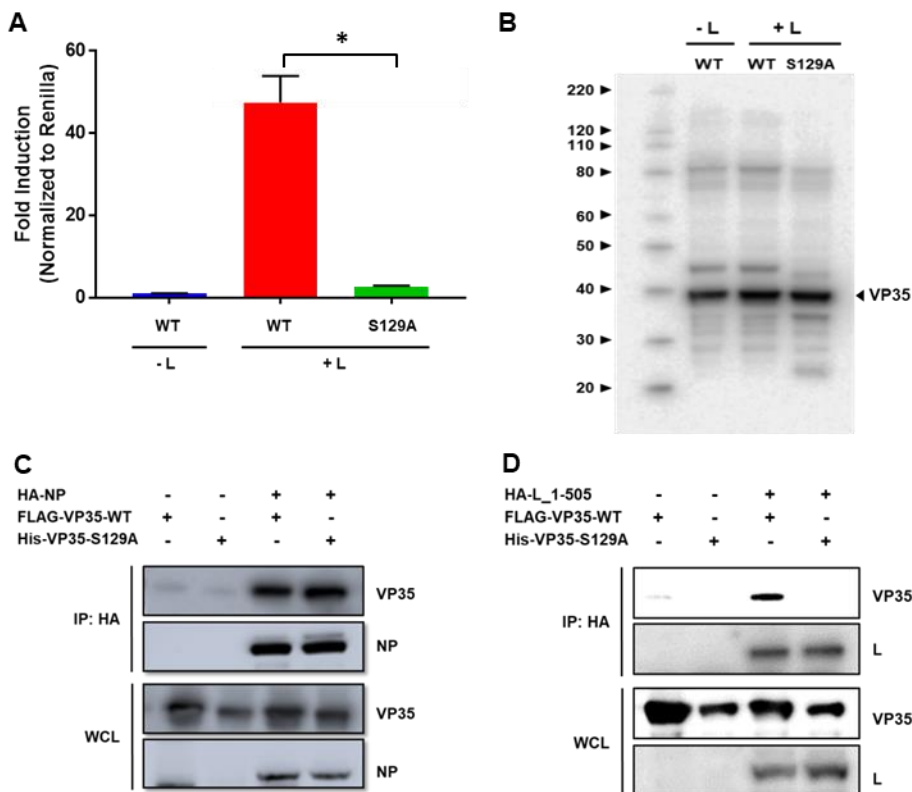
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Figure 5



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