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1 STRUCTURAL DESCRIPTION OF THE NIPAH VIRUS PHOSPHOPROTEIN AND ITS
2 INTERACTION WITH STAT1

3

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31

32 **STATEMENT OF SIGNIFICANCE**

33 The intrinsically disordered phosphoprotein of Nipah virus orchestrates viral genome
34 transcription, replication and encapsidation and shares a long N-terminal disordered region
35 with the non-structural V and W proteins involved in blocking the interferon responses. By
36 combining multiple biophysical methods, including X-ray crystallography, NMR
37 spectroscopy and small-angle X-ray scattering, we characterized the structure and flexibility
38 of this protein, identified several putative short linear motifs of interaction with viral and
39 cellular partners and investigated the interaction with one of them. The protein appears as a
40 nanometer-size octopus comprising a central tetrameric core and displaying binding sites on
41 its flexible arms to recruit multiple viral and cellular partners, thus providing new insights
42 into how this protein fulfills its multiple functions.

43

44 **SUMMARY**

45 The structural characterization of modular proteins containing long intrinsically disordered
46 regions (IDRs) intercalated with folded domains is complicated by their conformational
47 diversity and flexibility and requires the integration of multiple experimental approaches.
48 Nipah virus (NiV) phosphoprotein, an essential component of the viral RNA
49 transcription/replication machine and a component of the viral arsenal that hijacks cellular
50 components and counteracts host immune responses, is a prototypical model for such modular
51 proteins. Curiously, the phosphoprotein of NiV is significantly longer than the corresponding
52 protein of other paramyxoviruses. Here, we combine multiple biophysical methods, including
53 X-ray crystallography, NMR spectroscopy and small-angle X-ray scattering (SAXS), to
54 characterize the structure of this protein and provide an atomistic representation of the full-
55 length protein in the form of a conformational ensemble. We show that full-length NiV
56 phosphoprotein is tetrameric, and we solve the crystal structure of its tetramerization domain.
57 Using NMR spectroscopy and SAXS, we show that the long N-terminal intrinsically
58 disordered region and the linker connecting the tetramerization domain to the C-terminal X
59 domain exchange between multiple conformations while containing short regions of residual
60 secondary structure. Some of these transient helices are known to interact with partners, while
61 others represent putative binding sites for yet unidentified proteins. Finally, using NMR
62 spectroscopy and isothermal titration calorimetry, we map a region of the phosphoprotein,
63 comprised between residues 110 and 140 and common to the V and W proteins, that binds
64 with weak affinity to STAT1, and confirm the involvement of key amino acids of the viral
65 protein in this interaction. This provides new insights into how the phosphoprotein and the
66 non-structural V and W proteins of NiV perform their multiple functions.

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72 INTRODUCTION

73 Nipah virus (NiV) is one of the most pathogenic and deadly viruses infecting humans (1). It is
74 an emergent zoonotic virus that naturally infects fruit bats in South-East Asia, but that can
75 also infect other hosts including pigs, dogs, cats, horses and humans, where it becomes highly
76 pathogenic. Since its discovery in 1998 in Malaysia, NiV has caused regular outbreaks of
77 severe human encephalitis and respiratory illness with a fatality rate ranging from 40% to
78 90% mainly in Bangladesh and more recently in Philippines and India (1, 2). With its ability
79 to transmit from man to man and the lack of vaccine or efficient treatment, NiV has the
80 potential to cause a pandemic and to be used in biological warfare (3). It is consequently
81 classified as a biosafety level-4 (BSL-4) pathogen.

82

83 NiV is an enveloped virus with a nonsegmented negative-sense RNA genome, which belongs
84 to the order *Mononegavirales* and the family *Paramyxoviridae*, although, several features in
85 its genome organization and in its protein sequences led to its classification into a new genus,
86 Henipavirus, with the highly pathogenic Hendra virus and the non-pathogenic Cedar virus (4).
87 The multiplication of NiV in a suitable host cell, like that of all members of the
88 *Mononegavirales*, requires both (i) transcription of the single-stranded RNA genome into
89 monocistronic, capped and polyadenylated messenger RNAs (positive-sense RNA) that can
90 be used by the cellular ribosomes to generate new viral proteins and (ii) replication of the
91 negative-sense RNA genome into positive-sense antigenome copies, which are then used to
92 generate a progeny of negative-sense genomes. The enzymatic machinery that carries out
93 these activities is conserved among all members of the *Mononegavirales* and is unique in
94 biology and thus represents an attractive target for the development of much-needed antiviral
95 compounds. It consists of a megadalton-sized multi-molecular complex located in the core of
96 the viral particle and timely released in the cytoplasm of the host cell (5, 6). This
97 transcription/replication complex is composed of the genomic RNA and of three viral
98 proteins: the nucleoprotein (N), the phosphoprotein (P) and the large RNA-dependent RNA
99 polymerase (L). With the help of the phosphoprotein acting as a chaperone, the nucleoprotein
100 assembles into linear homopolymers that coats both negative-sense genomic and positive-
101 sense antigenomic RNAs, forming long, helical nucleocapsids (7). These nucleocapsids
102 constitute the biologically active templates used by the polymerase complex, which consists
103 of the L polymerase and the phosphoprotein acting as a non-catalytic cofactor. Structural and
104 dynamical studies of the unassembled nucleoprotein (N⁰) in complex with the phosphoprotein
105 (8–11) and of nucleocapsids (7, 12) have started to unravel the mechanisms of assembly and

106 remodeling of the nucleocapsids during the transcription and replication processes (13). The
107 multidomain L polymerase catalyzes RNA synthesis, but also the capping, methylation and
108 polyadenylation of the viral messenger RNAs (14). The underlying mechanisms of the
109 functioning of this polymerase remain largely to be deciphered (15), but its structure in
110 complex with its cofactor the phosphoprotein is being revealed by recent cryo-electron
111 microscopic reconstructions of the complex from various *Mononegavirales* (16–19),
112 including a first low-resolution reconstruction of the NiV polymerase (14). The third essential
113 component, the phosphoprotein, is thus a multitasking protein that orchestrates the interplay
114 between the different components of the transcription/replication complex. The
115 phosphoprotein takes its name from early observations showing that it is the most heavily
116 phosphorylated viral protein in cells infected by different members of the *Mononegavirales*
117 (20). In NiV phosphoprotein, residues Ser224 and Thr239 are the major phosphorylation sites
118 (21) and a binding site for Polo-like kinase 1 is located near residue 130 (22), but as for other
119 *Mononegavirales*, the role of these phosphorylations in the viral replication cycle remains
120 elusive (23).

121
122 All phosphoproteins of the *Mononegavirales* share the properties of binding the L
123 polymerase, the unassembled nucleoprotein and the nucleocapsid through independent
124 structural modules (13). In this way, the phosphoprotein promotes the initiation of RNA
125 synthesis, confers processivity to the polymerase, maintains the unassembled nucleoprotein in
126 an RNA-free and soluble form and delivers the latter to the site of RNA synthesis in order to
127 encapsulate the nascent RNAs (9, 10, 12, 24–28). Despite common functions, similarities in
128 the phosphoprotein sequence are hardly found beyond the level of the family and in some
129 cases of the genus (29, 30). However, all phosphoproteins of the *Mononegavirales* are
130 multimeric and share a common architecture with a long N-terminal intrinsically disordered
131 region (P_{NTR}) and a C-terminal region (P_{CTR}) that includes the multimerization domain (P_{MD}),
132 a linker region (P_{CT-LINK}) and most often a folded domain at the C-terminal end (P_{XD}) (31, 32)
133 (**Figure 1B**). They also share a rather common localization of functional modules (14, 32–
134 34), and thus, NiV phosphoprotein exhibits a structural architecture and an organization of the
135 functional modules involved in binding the nucleoprotein and the polymerase that are typical
136 of the family *Paramyxoviridae*, although the protein of henipaviruses is appreciably longer
137 than that of the other genera of *Paramyxoviridae* (25, 33, 35) (**Figure 1B**): (i) The N-terminal
138 disordered region of more than 400 aa contains a short region (aa 1-38) at the N-terminal end
139 that acts as a molecular recognition element or MoRE (*i.e.* a region that is partially disordered

140 in isolation but that adopts a stable conformation upon binding to a partner (36)) for recruiting
141 and chaperoning the RNA-free unassembled nucleoprotein (N^0) required for the assembly of
142 new nucleocapsids during genome replication (9). (ii) The tetramerization domain of NiV (aa
143 475-578) (37) is involved in the interaction with and in the function of the L polymerase (27,
144 34, 38). (iii) A folded domain at the C-terminal end of the protein, named the X domain (P_{XD})
145 (aa 655-709) that binds to a MoRE in the C-terminal disordered tail of the nucleoprotein
146 (N_{TAIL}) (39). (iv) A third region of weak interaction with the nucleoprotein that has recently
147 been identified in measles virus phosphoprotein, appears to be conserved in NiV
148 phosphoprotein (aa 330-350) (10). Despite all this knowledge, we are still lacking a structural
149 characterization of the full-length NiV phosphoprotein.

150

151 In addition, besides its roles in the transcription and replication of the viral genome, the
152 phosphoprotein of many *Mononegavirales* also plays roles in hijacking cellular machineries
153 and in counteracting the host immune responses (40). As in other *Paramyxoviridae*, the NiV P
154 gene codes for multiple proteins through the use of alternative open reading frames, and these
155 additional proteins act as virulence factors, in particular by directly targeting the Signal
156 Transducer and Activator of Transcription (STAT) proteins and blocking the interferon
157 signaling pathway (40). The NiV P gene encodes three proteins, the phosphoprotein and the
158 non-structural V and W proteins. The gene contains an editing site in the part coding for the
159 N-terminal region of the phosphoprotein, where the polymerase stutters during transcription
160 and incorporates one, two or more pseudo-templated Gs resulting in shifts of the reading
161 frame (41). The different messenger RNAs generated through this process lead to the
162 expression of either the phosphoprotein or of one of the non-structural V (+1 ORF) or W (+2
163 ORF) proteins, which share their 405 N-terminal residues with the phosphoprotein but have
164 specific C-terminal moieties of 51 and 44 amino acids, respectively (**Figure 1B**) (41). In
165 addition, a second gene coding for the C protein is overprinted on the initial region of the P
166 gene in the same reading frame as the V protein (+1 ORF) (**Figure 1B**) and is expressed from
167 the three different mRNAs by a leaky ribosome scanning mechanism (42). However, in
168 contrast to some other *Paramyxoviridae*, NiV phosphoprotein, V and W proteins block
169 interferon signaling by preventing the phosphorylation of STAT1 and by sequestering STAT1
170 and STAT2 into non-functional complexes (43). The phosphoprotein and the V protein retain
171 STAT proteins in the cytoplasm, whereas the W protein sequesters them in the nucleus. The
172 N-terminal part comprised between residues 100 and 300, shared by the three proteins, is
173 critical for interferon evasion. The binding site for STAT1 was first localized between

174 residues 100 and 160 (44, 45) and then further mapped down to residues 114 to 140 (6).
175 Mutation of different residues in this region abrogate interferon inhibition (6, 46). The
176 interaction with STAT2 requires the entire 100-300 region, in particular a motif between
177 residues 230 and 237 and the prior binding of STAT1 (44). Furthermore, the region between
178 residues 174 and 192 contains a CRM1-dependent nuclear export signal (NES), which allows
179 the V protein to shuttle between the nucleus and cytoplasm and remove STAT1 out of the
180 nucleus (43, 47–49), whereas the W protein maintains phosphorylated STAT1 in the nucleus
181 through the presence of a nuclear localization signal (NLS) in its unique C-terminal moiety
182 (6, 45). Finally, the region around residue 130, overlapping with the STAT1 binding site
183 interacts with polo-like kinase 1, and this interaction results in the phosphorylation of the V
184 protein (22).

185

186 Here, we investigate the structure of full-length NiV phosphoprotein (P_{FL}). We determine its
187 oligomeric state by biophysical methods and demonstrate that the multimerization domain is
188 the only region of the phosphoprotein involved in its tetramerization. We solve the crystal
189 structure of the tetramerization domain and use NMR spectroscopy to investigate the
190 structural properties of the long N-terminal region, of the C-terminal linker predicted to be
191 intrinsically disordered and of the C-terminal X domain. We demonstrate the independence of
192 the different N-terminal chains in the tetrameric protein and identify several regions with
193 transiently populated secondary structure elements, which could be molecular recognition
194 elements (MoRE) for different viral or cellular partners. We generate an ensemble of
195 conformers of full-length phosphoprotein that incorporates the crystal structure of the
196 tetramerization domain and a homology model of the X domain and that simultaneously
197 accounts for NMR and small-angle X-ray scattering data from three different constructs of the
198 protein. Finally, we investigate the interaction between STAT1 and a fragment of 100 amino
199 acids derived from the N-terminal region of the phosphoprotein. NMR spectroscopy is used to
200 validate the binding site in the phosphoprotein, while isothermal titration calorimetry and cell-
201 based assays are combined with site-directed mutagenesis to map important residues for the
202 interaction.

203

204 **MATERIALS AND METHODS**

205 **Disorder meta-prediction**

206 The location of intrinsically disordered regions (IDRs) in NiV phosphoprotein was predicted
207 by submitting its amino acid sequence to 16 different algorithms accessible through web

208 servers and by calculating a consensus prediction as described previously (32, 50). The
209 calculated disorder score (D-score) varies between 0 and 1, and generally, regions with a D-
210 score lower than 0.5 are intrinsically disordered, whereas regions with a score higher than 0.5
211 are either folded domains (50–52) or potential molecular recognition elements for partner
212 proteins (53).

213

214 **Protein expression and purification**

215 On the basis of our predictions, different fragments of the phosphoprotein (Uniprot: Q9IK91)
216 were cloned between the NcoI and BamHI restriction sites of a pET28 vector, such that each
217 construct included a C-terminal His₆-tag with a Leu-Glu linker. For the constructs lacking the
218 N-terminal end of the protein, with the exception of the P₆₅₅₋₇₀₉ construct which starts at
219 Met655, a Met residue or a Met-Ala dipeptide (construct P₁₇₃₋₂₄₀) was introduced at the N-
220 terminal end of the fragment. Three single mutants of P₉₂₋₁₉₀: Y116A, G120E, G125E and a
221 triple mutant: H117A/Q118A/H1119A, were generated by reverse PCR using the
222 QuickChange mutagenesis protocol (Agilent). All constructs were verified by DNA
223 sequencing.

224

225 The proteins were expressed in *E. coli* BL21-RIL. Cells were grown in Luria Bertoni medium
226 at 37°C until the optical density (O.D.) at 600 nm reached a value of 0.6, and protein
227 expression was then induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG)
228 and cells were allowed to grow for 3 hours. For producing the selenomethionine-substituted
229 form of the P₄₇₁₋₅₈₀ construct, the bacterial cells were grown in minimal medium and SeMet
230 was added before induction of the heterologous protein expression. In each case, the cells
231 were harvested by centrifugation, and the pellet was resuspended in 20 mM Tris–HCl buffer
232 at pH 7.5 containing 150 mM NaCl (buffer A) and EDTA-free protease inhibitor cocktail
233 (Roche). The cells were broken by sonication, and the soluble fraction was loaded onto a Ni²⁺
234 column equilibrated in buffer A. After washing the column with buffer A containing 20 mM
235 imidazole, the protein was eluted with buffer A containing 300 mM imidazole. The protein
236 was further purified by size exclusion chromatography on a Superdex S75 (GE Healthcare)
237 equilibrated in buffer A or in 20 mM Bis-Tris buffer at pH 6.0 containing 150 mM NaCl, 50
238 mM arginine, 50 mM glutamate and 0.5 mM TCEP (buffer B) for NMR experiments or in 20
239 mM sodium phosphate buffer at pH 7.5 containing 150 mM NaCl (buffer C) for ITC
240 experiments. Each protein preparation was checked by SDS-PAGE and SEC-MALLS (see
241 below).

242

243 A fragment of the gene coding for residues 132 to 683 of human STAT1 was cloned in
244 pET28, was expressed in *E. coli* and was purified as previously described (54). Briefly, after
245 ammonium sulfate precipitation, the protein was solubilized in a 20 mM Tris-HCl buffer at
246 pH 8.8 containing 150 mM NaCl, and the solution was loaded onto a SP sepharose column
247 (GE healthcare) equilibrated in the same buffer. The protein was not retained on the column
248 and eluted in the flow-through. After concentration, the protein was further purified by
249 loading the solution containing STAT1₁₃₂₋₆₈₃ onto a Superdex S200 (GE Healthcare) size
250 exclusion column equilibrated in buffer C.

251

252 **SEC-MALLS experiments**

253 Size exclusion chromatography (SEC) combined with on-line detection by multi-angle laser
254 light scattering (MALLS) and refractometry (RI) is a method for measuring the absolute
255 molecular mass (MM) of a particle in solution that is independent of its dimensions and shape
256 (55). SEC was performed with TSK G4000 (Tosohbioscience) or S200 Superdex column (GE
257 Healthcare) equilibrated with 20 mM Tris-HCl buffer containing 150 mM NaCl, and the
258 columns were calibrated with globular standard proteins of known hydrodynamic radius (R_h)
259 (56). Chromatographic separations were performed at 20°C with a flow rate of 0.5 or 0.8
260 mL.min⁻¹, with on-line MALLS detection with a DAWN-HELEOS II detector (Wyatt
261 Technology Corp.) using a laser emitting at 690 nm, and protein concentration was measured
262 on-line by the use of differential refractive index measurements using an Optilab T-rEX
263 detector (Wyatt Technology Corp.) and a refractive index increment, dn/dc , of 0.185 mL.g⁻¹.
264 The weight-averaged molecular mass was calculated using the ASTRA software (Wyatt
265 Technology Corp.).

266

267 **Small angle X-ray scattering experiments**

268 Small angle X-ray scattering (SAXS) data were collected at the BioSAXS beamline (ID14-3)
269 of the ESRF (57). The scattering from the buffer alone was measured before and after each
270 sample measurement and used for background subtraction. All data analyses were performed
271 using the program PRIMUS from the ATSAS 3.0.0 package (58).

272

273 **NMR spectroscopy**

274 The spectral assignment of the different constructs of NiV P protein (P₁₋₁₀₀, P₉₂₋₁₉₀, P₁₇₃₋₂₄₀,
275 P₂₂₃₋₃₁₉, P₃₀₀₋₄₀₁, P₃₈₇₋₄₇₉, P₅₈₈₋₆₅₀ and P_{XD} (residues 655-709)) at 25°C in buffer B using BEST-

276 type triple resonance experiments (59). The NMR experiments were acquired at a ^1H
277 frequency of 600 or 800 MHz (Agilent spectrometers). Six experiments were acquired for
278 each construct: HNCO, intra-residue HN(CA)CO, HN(CO)CA and intra-residue HNCA,
279 HN(COCA)CB and intra-residue HN(CA)CB. All spectra were processed in NMRPipe (60),
280 analyzed in Sparky (61) and automatic assignment of spin systems was done using MARS
281 (62) followed by manual verification. The assigned chemical shifts have been deposited in the
282 BMRB database under accession numbers 50098, 50099, 50100, 50101, 50102, 50103,
283 50105. The completeness of the assignments was above 97% for all constructs with the
284 unassigned residues mostly belonging to the N-termini that undergo fast solvent exchange.
285 Only the construct P₃₈₇₋₄₇₉ had a lower assignment completeness (92%) with residues S399-
286 I402 being unassigned. Secondary structure propensities (SSPs) were calculated from the
287 experimental C $_{\alpha}$ and C $_{\beta}$ chemical shifts according to Marsh et al. (63).

288

289 **X-ray crystallography**

290 Initial crystallization conditions for the tetramerization domain (aa 471-580) were identified
291 at the High Throughput Crystallization platform of the PSB, (<https://htxlab.embl.fr>). The
292 crystals used for data collection were obtained in 7% PEG 3350, 1 M LiCl, 0.1 M sodium
293 citrate, 0.1 M arginine, 4% hexanediol, 0.001% NaN₃ at pH 5.0, and were frozen with 15%
294 glycerol as cryo-protectant. X-ray diffraction data were collected at the ID29 beamline of the
295 ESRF at a wavelength of 0.9793 Å and at Proximal beamline of SOLEIL at a wavelength of
296 0.97895 Å and at a temperature of 100 K and were processed with the XDS package (64).
297 Initial phases were obtained using the anomalous scattering from selenium atoms by the SAD
298 method with the program HKL2MAP (65). A model was initially constructed with the
299 Autobuild program (66) from the phenix suite (67) and subsequently refined with the
300 phenix.refine program (68) and Coot (69). The geometry of the final model was checked with
301 MolProbity (70). In the model, 98.04% of residues had backbone dihedral angles in the
302 favored region of the Ramachandran plot, 1.83% fall in the allowed regions and 0.13% were
303 outliers. Figures were generated with PyMol (71) and Chimera (72).

304

305 **Modeling of NiV phosphoprotein as an ensemble of conformers**

306 An ensemble of 10,000 full-length NiV phosphoprotein conformers was generated with the
307 program Flexible-Meccano (73). These included the high-resolution three-dimensional
308 structures of the tetramerization domain (P_{MD}) and an homology model of the C-terminal X
309 domain based on the known HeV P_{XD} structure (74) by using Modeller (75). To accurately

310 model the disordered N-terminal region, the ASTEROIDS selection algorithm (76) was first
311 applied to each of the sub-constructs of the phosphoprotein in order to obtain an ensemble of
312 conformers of these regions in agreement with experimental $^1\text{H}^{\text{N}}$, ^{15}N , $^{13}\text{C}\alpha$, $^{13}\text{C}\beta$, and $^{13}\text{C}'$
313 chemical shifts. Briefly, in successive rounds of selection, 5×200 pairs of (ϕ, ψ) dihedral
314 angles were selected for each residue from a pool of 10,000 conformers assembled by
315 Flexible-Meccano such that the difference between ensemble-averaged ^1H , ^{15}N and ^{13}C
316 chemical shifts predicted by the program SPARTA (77) and corresponding experimental
317 values was minimized. The selected dihedral angles were then used in generation of a new set
318 of 10,000 conformers for the next round of selection. The (ϕ, ψ) databases for each sub-
319 construct of the phosphoprotein thus obtained after four rounds of selection were combined
320 and used by Flexible-Meccano to build conformations of full-length protein. Residues in the
321 linker region between the tetramerization domain and the X domain were stochastically
322 assigned amino-acid specific backbone dihedral angles in agreement with random coil
323 statistics. In the disordered regions, side chains were constructed using the program SCCOMP
324 (78). Scattering curves were calculated with CRY SOL (ATSAS 3.0.0) (79) and ensemble
325 selections were performed using ASTEROIDS (80) by combining both the experimental
326 NMR and SAXS data. In order to account for conformational heterogeneity within different
327 regions of the full-length protein, the ASTEROIDS target function combined SAXS data from
328 three constructs: P_{FL} , P_{1-580} and $\text{P}_{471-709}$, representing the full-length tetramer, and the
329 tetramerization domain with the N-terminal and C-terminal parts of the protein, respectively.

330

331 **Isothermal titration calorimetry**

332 Isothermal titration calorimetry (ITC) experiments for characterizing the interaction between
333 the five fragments P_{92-190} (WT, Y116A, G120E, G125E, H117A/Q118A/H1119A) and
334 $\text{STAT1}_{132-683}$ were performed with a MicroCal iTC200 system (North Hampton, MA). The
335 STAT1 solution at a concentration of 100 μM in buffer C was loaded in the calorimetric cell
336 and titrated at 25°C, typically, by performing 16 injections of 2.5 μl of the wild-type (WT)
337 P_{92-190} solution or of one of the variants (at concentrations ranging between 1.5 and 2.5 mM)
338 in the same buffer. Protein samples and buffers were degassed before data collection. The
339 heat generated for each injection was obtained by integrating the calorimetric signal. The heat
340 resulting from the binding reaction between the two proteins was obtained as the difference
341 between the heat of the reaction and the corresponding heat of dilution. The data were
342 processed using Origin 7.0 (Malvern Panalytical, Malvern, UK) and fitted to a single-site
343 binding model.

344

345

346 **STAT1 localization in NiV infected cells**

347 Generation of recombinant NiV was described elsewhere (6). NiV-G121E and NiV-G135E
348 contain a single amino acid change in the P protein at position 121 or 135. To monitor the
349 effect of NiV infection on endogenous STAT1, Vero E6 cells were grown on polymer
350 coverslips of the μ -Slide VI^{0.4} and were mock infected or infected with recombinant NiVs
351 (WT, G121E or G135E) at a multiplicity of infection (m.o.i.) of 0.5. At 1 h post-infection, the
352 virus inoculum was replaced with DMEM containing 2.5% fetal bovine serum and the cells
353 were incubated at 37°C for 28 h. The cells were washed with serum-free medium and then
354 treated with 1,000 U/mL of human interferon- β (PBL) in serum-free DMEM for 40 min at
355 37°C. For untreated controls, infected cells were incubated with IFN-free medium. Cells were
356 washed twice with cold phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde-
357 PBS solution for 20 min at room temperature, and subsequently treated with 0.1 M glycine for
358 10 min. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and washed
359 with a blocking solution (2% BSA in PBS). Immunofluorescence staining was performed
360 with mouse anti-phospho-STAT1 (pY701) (BD Biosciences) (1:250 dilution) antibody or
361 rabbit anti-NiV matrix protein (1:500) antibody.

362

363 **RESULTS**

364 **NiV phosphoprotein has a modular architecture**

365 The presence of intrinsically disordered regions (IDR) in a protein can be predicted from its
366 amino acid sequence by different computational approaches (81, 82). Previously, we devised
367 a meta-prediction method that calculates a consensus disordered score (D-score) between 16
368 independent disorder predictors by a simple voting procedure (32) and found that by applying
369 a threshold at 0.5 to distinguish between ordered and disordered regions we were able to
370 precisely predicts boundaries between folded domains and IDRs (32, 50, 52, 83) as well as the
371 existence of MoREs (36).

372

373 The D-score profile calculated from NiV phosphoprotein sequence reveals three disordered
374 regions of D-score < 0.5 (named IDR1-3) and several regions of D-score > 0.5 predictive of
375 structural order (**Figures 1A and 1B**) in agreement with the Hydrophobic Cluster Analysis
376 plot (**Supplementary Figure S1**) (84). Two of these structured regions of more than 100 aa
377 between residues 475 and 598 (averaged D-score of 0.82) and between residues 658 and 709

378 (averaged D-score of 0.69) coincide with the known localization of the folded tetramerization
379 (P_{MD}) and X domains (P_{XD}), respectively (37, 39). A shorter region of D-score > 0.5 at the N-
380 terminal end (aa 1-29) corresponds to the α -MoRE known to bind the unassembled
381 nucleoprotein N^0 (9). However, the D-score also identifies other putatively ordered regions. A
382 region of D-score > 0.5 , also spanning more than 100 aa between residues 102 and 219,
383 encompasses the putative binding sites for STAT1, STAT2 and Polo-like kinase 1 as well as
384 the CRM1-dependent NES (**Figure 1B**). A shorter region of D-score > 0.5 is localized within
385 the C-terminal linker (aa 607-658). Finally, borderline regions appear between residues 310
386 and 450 in the form of clearly visible peaks in the D-score even if the score remains under the
387 threshold of 0.5. One of these peak encompasses the conserved ultra-weak nucleoprotein
388 binding motif recently discovered in measles virus phosphoprotein (10), while two other ones
389 overlap with the regions encoding the specific moiety of the V and W proteins (**Figure 1B**).

390

391 **Protein production and quality control**

392 We expressed the full-length NiV phosphoprotein and its different fragments as recombinant
393 proteins in *Escherichia coli* (**Figure 1C**). The full-length protein and the different protein
394 fragments were soluble and eluted as single peaks from the SEC column. The molecular mass
395 determined by on-line multi-angle laser light scattering (MALLS) detection was constant
396 throughout the chromatographic peak (polydispersity index, M_w/M_n lower than 1.01)
397 indicating that each sample was monodisperse (**Table 1**).

398

399 **NiV full-length phosphoprotein is a tetramer of large size**

400 The molecular mass of full-length NiV phosphoprotein (P_{FL}) measured by on-line MALLS is
401 306 ± 30 kDa, in agreement with the molecular mass calculated for the tetramer ($4 \times 79.367 =$
402 317.468 kDa) (**Figure 2 and Table 1**). Its hydrodynamic radius (R_h) measured by SEC is 9.3
403 ± 0.2 nm, a value that is intermediate between those expected for a compact globular protein
404 ($R_{h,theo} = 6.0$ nm) or an unfolded chain ($R_{h,theo} = 16.8$ nm) of the same molecular mass (**Table**
405 **1**). Analyses of the different protein fragments by SEC-MALLS indicate that the central
406 region is solely responsible for the tetramerization of the protein in accordance with cross-
407 linking experiments (85) and the recent crystal structure of the NiV phosphoprotein
408 multimerization domain (37). The fragments P_{1-580} and $P_{471-709}$, which both encompass the
409 tetramerization domain, and the fragment $P_{471-580}$, which closely corresponds to this domain,
410 have molecular masses determined by SEC-MALLS of 250 ± 25 kDa, 113 ± 10 kDa and $58 \pm$
411 6 kDa (**Figure 2**), respectively, in agreement with the theoretical molecular masses expected

412 for their tetrameric forms (**Table 1**). Similarly to the full-length protein, the hydrodynamic
413 radius of these fragments is intermediate between those expected for compact globular
414 proteins and for unfolded chains of the same molecular mass (**Table 1**). By contrast, all other
415 fragments are monomeric and those from the N-terminal region have a hydrodynamic radius
416 close to that of an unfolded chain (**Table 1** and see below).

417

418 SAXS curves were recorded at three different protein concentrations for the full-length
419 phosphoprotein (**Supplementary Figure S2**) and for both tetrameric constructs: P₁₋₅₈₀,
420 (**Supplementary Figure S3**) and P₄₇₁₋₇₀₉ (**Supplementary Figure S4**). For each protein, the
421 scattering profile shows an absence of concentration dependence and the Guinier plots at low
422 Q values are linear, indicating the absence of aggregation or of significant interchain
423 interactions (**Supplementary Figures S2, S3 and S4**). We note, however, an unexplained
424 overestimation of more than 30% in the molecular mass of full-length phosphoprotein and of
425 P₄₇₁₋₇₀₉ calculated by using the calibration with bovine serum albumin (BSA) (Table 2). The
426 dimensionless Kratky plot and the pair-distance distribution function revealed ambivalent
427 features for the three proteins, supporting the presence of both structured and disordered parts
428 (**Supplementary Figures S2, S3 and S4 and Table 2**) (86). A peak slightly above 1.1 is
429 visible in the Kratky plot at $Q \cdot R_g \approx 2$ likely reflecting the presence of the folded
430 tetramerization and X domains. For full-length phosphoprotein and the fragment P₁₋₅₈₀, the
431 value evolves towards a plateau at higher $Q \cdot R_g$ indicating the presence of a large fraction of
432 disordered regions in agreement with the asymmetric bell-shape curve in the P(r) plots. For
433 the fragment P₄₇₁₋₇₀₉, a maximum is also present at $Q \cdot R_g \approx 5$ in the Kratky plot, and two
434 maxima at $r \approx 4$ nm and $r \approx 8.5$ nm, likely reflecting on the long-range contacts between the
435 tetramerization domain and the four X domains. The radius of gyration determined from the
436 Guinier plot and the longest pair distance D_{\max} were almost identical for the full-length
437 protein and the N-terminal fragment P₁₋₅₈₀ indicating the overall size of these molecules is
438 mainly determined by the N-terminal disordered region (**Table 1**). The corresponding values
439 for the C-terminal fragment P₄₇₁₋₇₀₉ was clearly smaller in agreement with the largest fraction
440 of residues involved in structured domains (~65% of folded residues) (**Table 1**).

441

442 **Crystal structure of the tetramerization domain**

443 The fragment P₄₇₁₋₅₈₀ crystallized in different conditions and different space groups. We
444 solved the structure by the SAD method using selenomethionine labeled protein crystallized

445 in space group P12₁1 (see **Table 3** for details of data collection and statistics) The asymmetric
446 unit contains two copies of a tetramer (**Supplementary Figures S5A and S5B**), in which
447 each monomer is composed of two short helices (helices α_1 and α_2) and a long C-terminal
448 helix (helix α_3 - aa 508-570) (**Figures 3A and 3B**). The long helix of each monomer is
449 composed of eight complete heptad repeats and a stammer at position 540-542 resulting in a
450 kink near residue Pro544 (**Figure 3C**). Within one tetramer, the four long helices fold into a
451 parallel coiled-coil forming a central shaft, while the short helices form a crown around the N-
452 terminal end of this shaft (**Figures 3A and 3B**). The global net charge of the tetrameric
453 domain is negative at neutral pH, and the calculated electrostatic surface potential shows an
454 asymmetrical distribution, with a negative head and a patch of negative charges at the C-
455 terminal end of the coiled coil shaft, whereas the central part of the coiled-coil shaft is rather
456 neutral (**Supplementary Figure S5C**).

457
458 The N-terminal part of the tetrameric domain exhibits four-fold symmetry, but in the coiled-
459 coil shaft, the four-fold symmetry is progressively replaced by a two-fold symmetry when
460 going from the N-terminal end to the C-terminal end (**Figures 3D and 3E**). The monomers
461 within a tetramer superimpose by pairs with C_α r.m.s.d. values around 0.6 (two-fold
462 symmetrical mate) and 1.0 Å (other two chains). Each protomer N_i contacts the three other
463 protomers. The long helix α_3 of N_i is packed between helix α_3 of the N_{i-1} and N_{i+1} protomers,
464 while helix α_2 contacts the N_{i+1} protomer and helix α_1 contacts the N_{i+2} protomer (**Figure 3B**).
465 In the corresponding tetrameric domain of Sendai virus phosphoprotein, each protomer only
466 interacts with two other adjacent protomers (38).

467
468 The tetrameric structure of NiV tetramerization domain is stabilized by multiple interchain
469 interactions, each protomer forming on average 19 H-bonds and 11 salt bridges with the
470 others protomers and burying a surface area of 2,200 Å² between each pair of adjacent
471 protomers (for a total of 8,800 Å² of accessible surface area buried in the tetrameric
472 structure). The interior of the coiled-coil shaft is composed of 19 sidechain layers, mainly
473 hydrophobic but with four layers of polar residues (C512, S515, G519 and A522) at the N-
474 terminal end and one near the C-terminal end (T560) (**Figure 3C**).

475
476 The structure is very similar to the crystal structure of NiV tetramerization domain published
477 by Sapphire and coworkers (37) with, however, two noticeable differences: (1) Whereas the
478 constructs are similar in length, the coiled-coil region in our structure is six residues shorter

479 than in the previous structure (62 aa instead of 68 aa), with a length intermediate between that
480 of Sendai virus (64 aa) (38) and that of measles virus (60 aa) (87), suggesting crystal contacts
481 play a role in the stabilization of this part of the structure. (2) The C-terminal part of the
482 coiled coil exhibits a slightly different superhelical frequency (-3.39° versus -3.63°) resulting
483 in a different superhelical pitch (158 Å *versus* 148 Å) (**Supplementary Figure S5D**). The
484 mapping of crystallographic B-factors onto the structure confirms that the C-terminal end of
485 the coiled-coil shaft is more flexible than the remaining part of the molecule (**Supplementary**
486 **Figure S5E**) as shown previously (34, 37).

487

488 **The N-terminal region and the C-terminal linker are globally disordered**

489 Several fragments of about 100 amino acids covering the entire N-terminal region but also the
490 linker in the C-terminal part (P_{CT-LINK}) that is predicted to be at least partially disordered
491 (IDR3 in **Figure 1A**) and the X domain were produced as ^{13}C , ^{15}N -labeled proteins and
492 purified (**Figure 1C**). Each fragment from the N-terminal region has a hydrodynamic radius
493 (R_h) significantly larger than that expected for a globular protein of the same molecular mass
494 and slightly smaller than that expected for a fully unfolded protein in a good solvent (**Table**
495 **1**). SAXS data recorded for these fragments revealed no concentration dependence. For the
496 different N-terminal fragments and for the fragment corresponding to the C-terminal linker
497 (P₅₈₈₋₆₅₀), the experimental radius of gyration (R_g) values determined using the Guinier
498 approximation were close to the R_g values calculated for a random coil according to Flory's
499 power-law dependence on chain length using parameters for chemically unfolded proteins
500 (88) or for intrinsically disordered proteins (89) (**Table 1**), in line with the proposal that these
501 regions are disordered. By contrast, the radius of gyration for the X domain is significantly
502 smaller than that of a disordered chain of the same length, as expected for a folded X domain
503 similar to that of Hendra virus (74).

504

505 To characterize the structure of these fragments in solution, we carried out assignment of the
506 backbone NMR resonances and calculated their secondary structure propensities (SSP) from
507 the experimental $\text{C}\alpha$ and $\text{C}\beta$ chemical shifts. The ^1H - ^{15}N two-dimensional heteronuclear
508 single quantum coherence (HSQC) spectra of the different N-terminal fragments exhibit poor
509 chemical shift dispersion of the amide ^1H resonances as is typical for a disordered protein (90)
510 (**Supplementary Figure S6**). The combined SSP score for the entire N-terminal region
511 (P_{NTR}) reveals the presence of several transient α -helices (**Figure 4A**). In particular, we
512 observe two transiently populated helical elements at the very N-terminus that make up the

513 binding region for N⁰ (9). In the N⁰-P complex, these helical elements (aa 1-29) bind to the
514 C-terminal domain of the nucleoprotein mainly through hydrophobic interactions, forming a
515 stable helix, kinked at residue Q21 (9). In addition, a third helical element with a population
516 of around 40% is observed at residues 340-355, which coincides with the putative additional
517 weak binding region for the nucleoprotein recently discovered in measles virus (10). The ¹H-
518 ¹⁵N HSQC spectrum of the fragment P₅₈₈₋₆₅₀ corresponding to the C-terminal linker (P_{CT-LINK})
519 is also typical of a disordered protein (**Supplementary Figure S6**) and the combined SSP
520 score also revealed the presence of low populated α -helices (aa 600-625) (**Figure 4B**). By
521 contrast, the ¹H-¹⁵N HSQC spectrum of the isolated X domain, (P_{XD}, aa 655-709) confirms
522 that this domain is folded in solution, and the chemical shift assignments clearly show that a
523 three-helix bundle structure is predominant in solution (**Figure 4C**) as also observed in the
524 corresponding domains of measles, mumps, Sendai, and Hendra viruses (74, 91–93) .

525
526 The HSQC spectrum of the tetrameric full-length phosphoprotein (a tetrameric protein
527 complex of 2,868 aa with our construct) contains resonances with limited chemical shift
528 dispersion that superposed well with the resonances from the residues of the N-terminal
529 disordered part of the protein (**Figure 4D** and **Supplementary Figure S6**). This clearly
530 indicates that the N-terminal part is flexible with similar conformational behavior in the
531 tetramer as in the isolated peptides and negligible interchain contacts. No resonances for the
532 tetrameric domain, C-terminal linker or the X domain could be identified in the spectrum of
533 full-length phosphoprotein, possibly reflecting slower rotational tumbling caused by a
534 hydrodynamic drag due to the connection to the tetrameric domain and the presence of the X
535 domain at the C-terminus, although we cannot rule out the possibility of interactions between
536 the four C-terminal regions that may also give rise to broadening.

537
538 Overall, these results show that (i) the N-terminal region of NiV phosphoprotein is
539 intrinsically disordered and exchanges between multiple conformers on a fast timescale, (ii)
540 this region contains transient α -helical elements, (iii) there are no significant interactions
541 between the N-terminal region of the four protomers within the tetrameric phosphoprotein and
542 (iv) the C-terminal linker also seem to be disordered and to contain transient α -helical
543 elements.

544

545 **Ensemble description of full-length NiV phosphoprotein tetramer**

546 To study the conformational diversity of NiV phosphoprotein, we generated ensembles of all-

547 atom conformations of the full-length protein and of both tetrameric fragments: P₁₋₅₈₀ and
548 P₄₇₁₋₇₀₉. We used our crystal structure of the tetrameric domain (**Figure 3**), a homology model
549 of the X domain based on the known structure of the X domain of Hendra virus (74),
550 heteronuclear backbone chemical shifts (C α , C β , C' and ¹⁵N) for the N-terminal fragments
551 (**Figure 4**) and SAXS data for the three proteins (**Supplementary Figures S2, S3 and S4**) as
552 experimental constraints to delineate the contours of conformational space sampled by the
553 full-length protein. We used a combine fit of multiple curves to select conformational sub-
554 ensembles of 10 conformers for which the three average theoretical scattering curves
555 reproduced the three experimental curves. The average SAXS curve calculated for this
556 ensemble is comparable to the experimental SAXS curve measured for the full-length
557 phosphoprotein, while the average curves calculated with the properly truncated forms of the
558 same 10 conformers are comparable to the experimental SAXS curves obtained for P₁₋₅₈₀ and
559 P₄₇₁₋₇₀₉ (**Figure 5A**). A global χ^2 value of 1.53 and the plot of reduced residuals indicate the
560 quality of the fit (**Figure 5A**). It should be noted that the independent fits performed for each
561 of the three proteins yielded ensembles of conformers with averaged R_g values in agreement
562 with the experimental values (full-length P: 13 \pm 3 nm; P₄₇₁₋₇₀₉: 6.3 \pm 0.5 nm; P₁₋₅₈₀: 12 \pm 3
563 nm), but did not improve the overall quality of the fits (**Supplementary figure S7**). Some
564 discrepancies between the experimental and fitted curves are visible. The downward deviation
565 observed at low Q values for full-length phosphoprotein and to a lesser extent for the P₁₋₅₈₀
566 fragment can be due to intermolecular interactions, whereas the discrepancy observed
567 between the experimental and calculated curves for the fragment P₄₇₁₋₇₀₉ at Q values > 1.0 nm⁻¹
568 ¹ (**Figure 5A**) might result from non-optimal [buffer subtraction as already observed for](#)
569 [proteins such as this fragment, which comprises both folded and unfolded domains that](#)
570 [may have distinct electron densities \(94, 95\)](#). Alternatively, it can also result from a bias
571 in the construction of the initial ensemble of the full-length protein. As the initial ensemble of
572 conformers was built for full-length protein and was then truncated to generate the initial
573 ensembles for the fragments P₁₋₅₈₀ and P₄₇₁₋₇₀₉, it is possible that the large N-terminal
574 disordered region has constrained the X domains to position at one pole of the tetramerization
575 domain, whereas such constrain is absent in the fragment P₄₇₁₋₇₀₉. Finally, additional structural
576 organization or inter-chain interactions might be present in the C-terminal part of the
577 phosphoprotein, which has not been taken into account in our models. It is thus remarkable
578 that the same set of conformers is able to represent the curves obtained for the three
579 constructions. The R_g values of the initial ensemble for the full-length protein exhibited broad
580 Gaussian distribution centered around 12 nm, and the R_g values of the selected ensembles of

581 10 conformers exhibit a similar broad distribution (**Figure 5B**), suggesting the absence of
582 constraints on the overall size of the protein. **Figure 5C** shows a typical model of the full-
583 length phosphoprotein and **Figure 5D** shows the overlays of the 10 conformers of each
584 construct selected in the same fitting process for reproducing the data shown panel A.

585 **The N-terminal region of NiV phosphoprotein binds human STAT1 with low affinity**

586 The region of NiV phosphoprotein that binds STAT1 was previously mapped between
587 residues 100 and 160 (43, 47), which contains several highly conserved residues among
588 henipaviruses (**Figure 6A**). Point mutations within residues 81 to 120 of the phosphoprotein
589 affected the expression of a minireplicon suggesting that this region is involved in the
590 functioning of the polymerase machine, while mutations within residues 111 and 140
591 prevented the inhibition of interferon signaling (6).

592

593 To investigate the interaction between NiV phosphoprotein and STAT1, we used the fragment
594 P₉₂₋₁₉₀ that contains the entire putative binding site for STAT1, and we expressed and purified
595 a fragment of human STAT1 that comprises residues 132 to 683 encompassing the coiled-coil
596 domain, the DNA binding domain, the linker domain and the SH2 domain (96) (**Figure 6B**).
597 The missing 131 N-terminal residues and 38 C-terminal residues from the STAT1 α isoform
598 are not required for interaction with the phosphoprotein, V or W proteins of NiV (44). The
599 protein STAT1₁₃₂₋₆₈₃ eluted from the SEC column as a single peak and its molecular mass
600 determined by SEC-MALLS of 64 ± 6 kDa is in good agreement with the theoretical mass of
601 64.1 kDa (**Figure 6C**). The fragment P₉₂₋₁₉₀ alone also eluted as a single peak and its
602 molecular mass of 11 ± 1 kDa is in agreement with the theoretical mass of the monomer. In a
603 first approach, we mixed STAT1₁₃₂₋₆₈₃ ($\sim 54 \mu\text{M}$) with P₉₂₋₁₉₀ ($\sim 540 \mu\text{M}$) and loaded the
604 mixture on a SEC column after 2 hour incubation at 20°C. Both proteins eluted independently
605 at elution volumes and with molecular masses corresponding to isolated STAT1₁₃₂₋₆₈₃ and P₉₂₋₁₉₀
606 (**Figure 6C**). In a second approach, we used NMR spectroscopy and recorded ¹H-¹⁵N
607 HSQC spectra of ¹⁵N labeled P₉₂₋₁₉₀ at a final concentration of $\sim 22 \mu\text{M}$ in the absence or
608 presence of unlabeled STAT1₁₃₂₋₆₈₃ ($\sim 44 \mu\text{M}$ final concentration) (**Figure 6D**). In a complex
609 of the size expected for the heterodimer STAT1₁₃₂₋₆₈₃-P₉₂₋₁₉₀ (~ 75 kDa), NMR signals are
610 strongly broadened in protonated samples, thus precluding their detection. By comparing the
611 HSQC spectra, we find a partial reduction of the intensity in the presence of STAT1₁₃₂₋₆₈₃ for
612 resonances corresponding to residues 100 to 150 (**Figure 6E**). The most affected resonance
613 intensities are within the region of residues 110 to 140, which contains several residues of

614 crucial importance for blocking STAT1 signaling in cell-based assays (6). We interpret this
615 result as evidence that, under these experimental conditions, only a fraction of P₉₂₋₁₉₀
616 molecules bind STAT1₁₃₂₋₆₈₃, and we estimate the dissociation constant for STAT1₁₃₂₋₆₈₃ in
617 the range of 10 to 100 μM. We note that conformational exchange contributions from the
618 exchange between free and STAT1-bound P₉₂₋₁₉₀ could also be the source of some of the
619 observed line broadening in the interaction experiments.

620

621 We used isothermal titration calorimetry (ITC) and site-directed mutagenesis to further
622 characterize the interaction between the two protein fragments. We used STAT1₁₃₂₋₆₈₃ as
623 receptor at a concentration of 100 μM and performed titration with P₉₂₋₁₉₀ as ligand at an
624 initial concentration between 1.5 and 2.5 mM. Despite that complete saturation was not
625 achieved, the binding isotherm can be fitted to a single binding site model, yielding a
626 dissociation constant (K_d) of 100 ± 15 μM ($\Delta G_{Bind}^0 = + RT \ln K_d = -5.4 \text{ kcal.mol}^{-1}$) (from five
627 independent measurements) (**Figure 7A**), in good agreement with the NMR interaction
628 experiment (**Figure 6E**). The favorable enthalpy of binding (ΔH_{Bind}^0) of -9.5 kcal.mol⁻¹ is
629 partially balanced by an unfavorable entropic contribution of 4.1 kcal.mol⁻¹ ($\Delta S_{Bind}^0 = -14$
630 cal.mol⁻¹.K⁻¹) (**Table 4**).

631

632 Point mutations Y116A and G125E as well as a triple alanine mutation
633 H117A/D118A/H119A (¹¹⁷AAA¹¹⁹) in full-length NiV phosphoprotein were previously
634 shown to block interferon signaling and to disrupt the interaction with STAT1 (6, 46). By
635 contrast, the single amino-acid mutation G120E had no effect (6). We expressed and purified
636 NiV phosphoprotein fragments encompassing residues 92 to 190 (P₉₂₋₁₉₀) and harboring these
637 different mutations (Y116A, G120E, G125E and H117A/Q118A/H119A). Each protein
638 eluted as a single peak from the size exclusion chromatography column with a molecular
639 mass calculated from MALLS in agreement with the expected molecular mass for a
640 monomeric protein. We performed calorimetric titrations of STAT1₁₃₂₋₆₈₃ with these different
641 variants, but we were unable to detect any interaction with the variants Y116A and
642 H117A/Q118A/H119A. With the G120E variant, the affinity was comparable to that of the
643 WT protein (**Figure 7B-F and Table 4**). With the G125E variant, the affinity is slightly lower
644 than that of WT protein, with a K_d value of 210 ± 90 μM, although the difference in ΔG_{Bind}^0 of
645 0.4 kcal/mol is not significant (**Table 4**). These results confirm that the interaction between
646 the N-terminal region of NiV phosphoprotein and STAT1 is direct but weak.

647

648 In order to further highlight the importance of the STAT1-binding domain (amino acids 110
649 to 140) that is present in the phosphoprotein but also in the V and W proteins, we also used
650 recombinant WT or mutant viruses, which were generated by reverse genetics (6) and possess
651 either intact or a defective P gene. Two mutant viruses containing substitutions at positions
652 121 and 135 of the phosphoprotein, V and W proteins (NiV-G121E and NiV-G135E) were
653 compared with NiV WT for their ability to block translocation into the nucleus of endogenous
654 tyrosine-phosphorylated STAT1 activated by interferon (**Figure 7G**). NiV matrix protein was
655 used as a marker of infection and a specific anti tyrosine-phosphorylated STAT1 antibody
656 was used to monitor the localization of NiV P gene specific proteins (**Figure 7G**). Little or no
657 tyrosine-phosphorylated STAT1 was detectable in the nucleus of cells infected with the WT
658 NiV, whereas adjacent, uninfected cells exhibited clear nuclear phospho-STAT1 staining. By
659 contrast, a loss of STAT1 binding ability in NiV G121E resulted in strong and apparent
660 tyrosine-phosphorylated STAT1 signal present in the nuclei (**Figure 7G**). Interestingly, NiV-
661 G135E mirrored NiV WT and showed a similar pattern of disruption of normal STAT1
662 trafficking. Earlier it had been shown that the V protein harboring the same substitution failed
663 to detectably bind STAT1 in immunoprecipitation studies but inhibited ISG54-driven reporter
664 induction, albeit less efficiently (6). Our data suggest that this mutant retains sufficient
665 STAT1-binding activity but that the interaction is too weak to be seen in simple co-expression
666 co-immunoprecipitation assays. The mechanism for such behavior will need to be further
667 investigated, in particular with respect to the implication of STAT2.

668

669 **DISCUSSION**

670 The highly disordered, tetrameric NiV phosphoprotein is an integral component of the
671 transcription/replication machinery of this dangerous virus and of all members of the
672 *Mononegavirales* and a hitherto largely unexplored target for viral inhibition. Due to the size
673 and flexible nature of the phosphoprotein its conformational behavior has remained
674 unexplored.

675

676 Here, we present the first atomistic representation of full length NiV phosphoprotein in
677 solution. We show that, under our experimental conditions, full-length NiV phosphoprotein as
678 well as two other long fragments containing the tetramerization domain also form tetramers in
679 solution (**Figure 2**), and we have obtained a second crystal structure of this tetramerization
680 domain (37, 85). The phosphoprotein of other paramyxoviruses also contains a tetrameric
681 domain (38, 92, 97, 98), although the existence of a trimeric form in solution has been

682 described (98, 99). NiV tetramerization domain has dimensions and crystal structure similar
683 to those of Sendai virus tetramerization domain with a parallel tetrameric coiled-coil and a
684 crown of helices at its N-terminal end (38). By contrast, the tetramerization domains of
685 measles and mumps viruses lack the crown of helices, and while the structure of measles
686 domain is also a parallel tetrameric coiled-coil, that of mumps domain is composed of two
687 pairs of parallel helices packed in opposite orientation (92, 97). Phylogenetic trees based on
688 the phosphoprotein sequences but also on the whole genome sequences place the genus
689 Henipavirus in an intermediate position between the genera Morbillivirus (measles virus) and
690 Respirovirus (Sendai virus), while the genus Rubulavirus (mumps virus) is more distant (100,
691 101). The crown of helices may thus be an ancestral feature lost by some viruses or a modern
692 one acquired by others, that stabilizes the central coiled coil shaft or provides additional
693 function. Up to now, no function has been specifically associated with the N-terminal part of
694 the tetramerization domain, and mutations that perturb the crystallographic water molecules
695 inside the coiled-coil shaft at the crown level had no effect on phosphoprotein function in a
696 minireplicon system (34). Mapping the crystallographic B-factors on the domain structure
697 shows that the N-terminal part of the central coiled-coil, upstream of the kink, is more rigid
698 than the downstream part (**Supplementary Figure S5E**). Although the crystallographic
699 packing can influence B factors, the conservation of this dynamical feature in the
700 tetramerization domain of the related measles virus and Sendai virus argues for functional
701 relevance. The presence of inter-helical networks of h-bonds and salt-bridges in the crown
702 and in the upper part of the coiled-coil preceding the kink region could explain this additional
703 rigidity of the structure. By contrast, a conserved basic patch in the C-terminal part of the
704 shaft is known to interact with the polymerase and mutations in this part affect polymerase
705 activity (34) (**Figures 3C and 3F**). Multiple sequence alignment of Henipavirus
706 phosphoprotein shows that the crown is not the most conserved part of the tetramerization
707 domain, while it reveals a highly conserved region following the basic patch and straddling
708 the C-terminal end of the coiled coil, and the N-terminal part of the P_{CT-LINK} (aa 565-600)
709 (**Figures 3E and 3F**). This region includes the less rigid part of the structure
710 (**Supplementary Figure S5E**), and in related *Mononegavirales*, it interacts directly with L
711 polymerase (16, 17). Because the polymerase changes conformation while it operates and
712 moves along its RNA template, the interaction between the phosphoprotein and the
713 polymerase is submitted to tensional, shear and/or rotational stresses and thus likely requires
714 some flexibility in the protein interfaces to maintain the attachment.

715

716 Previous sequence analyses predicted that the N-terminal part of the phosphoprotein
717 encompassing the region common to the V and W proteins and extending up to the
718 tetramerization domain, is an intrinsically disordered region except for the N-terminal end
719 (31, 102), which consists of a MoRE that binds and chaperones unassembled N⁰ (9). The D-
720 score calculated as a consensus of multiple disorder predictions reveals a more complex
721 picture with intervening putatively ordered regions and undefined regions (**Figure 1A**).
722 Experimentally, we confirm the overall disorder of the N-terminal region of the
723 phosphoprotein. The hydrodynamic radius determined by SEC and the radius of gyration
724 determined by SAXS indicate that each fragment in the N-terminal region (**Table 1**) is rather
725 extended, in accordance with the behavior of intrinsically disordered polypeptide chains in
726 water (89). The poor chemical shift dispersion of the NMR ¹H-¹⁵N HSQC spectra also
727 supports the high flexibility of the different fragments, but the assignment of the spectra
728 revealed the presence of transient secondary structure elements (**Figure 4D and**
729 **Supplementary Figure S6**), and an overlay of the SSP parameter (**Figure 4A**) and D-score
730 (**Figure 1A**) plots shows that in several instances, short regions with an intermediate D-score
731 (moderately above or below 0.5) are transiently folded into helical conformation and could
732 correspond to α -MoRE involved in recruiting different partners, in particular the polo-like
733 kinase 1, the exportin or the viral nucleoprotein (**Supplementary Figure S8**). The
734 comparison of the NMR spectra from the different N-terminal fragments with the full-length
735 phosphoprotein shows that most resonances overlap (**Figure 4D**), thereby showing that the
736 structural properties of fragments are the same in the full-length protein. In other words, the
737 overall disorder of N-terminal region is not a result of our divide-and-conquer approach, but
738 is an intrinsic property of this region of the protein. This view is also confirmed by the
739 ensemble modeling, which shows that an ensemble model of full-length phosphoprotein,
740 where the N-terminal region and the C-terminal linker are highly flexible and adopt random
741 coil conformation, is in agreement with the SAXS and NMR data (**Figure 5**).

742

743 The ensemble model of full-length phosphoprotein provides new insights about the
744 conformation of this protein in solution (**Figure 5**). Overall, the molecule appears elongated
745 in the direction of the tetramerization domain shaft with the four X domains clustering at the
746 C-terminal end (**Figure 5D**). This spatial arrangement certainly provides an ideal positioning
747 of the four X domains for interacting with the polymerase or the nucleocapsid, and for
748 "walking" along the latter, driven by the polymerase. The four N-terminal chains mainly
749 occupy the other end and the sides of the tetramerization domain, likely creating the steric

750 hindrance that pushes the X domains towards one end. When multiple decoys are overlaid
751 with respect to the tetramerization domain, thus mimicking the time average spatial
752 occupancy of the four flexible N-terminal chains and four C-terminal linkers (**Figure 5D**), the
753 central tetramerization domain including the basic site of putative interaction with the L
754 polymerase is masked. The recent structure of the polymerase complexes of two related
755 viruses revealed that one of the chain of the phosphoprotein is mainly involved in the
756 interaction with the L polymerase, but the three other chains are also binding (16, 17). These
757 multiple interactions could be required to move the N-terminal chains of the phosphoprotein
758 away from the multimerization domain and to allow access of the polymerase to the central
759 tetrameric shaft.

760

761 The tetrameric NiV phosphoprotein is thus a large molecule, with a hydrodynamic radius of
762 9.3 ± 0.3 nm and a radius of gyration of 10.9 ± 0.2 nm. The R_g/R_h ratio is a sensitive indicator
763 of chain compactness; experimental values for this ratio lie between limits of about 0.8 for
764 globular proteins (103) and of about 1.5 or higher for random coils (104). The value of 1.18
765 found here for NiV full-length phosphoprotein is in agreement with an elongated molecule
766 and/or the presence of long intrinsically disordered regions and with the values of 1.0 and
767 1.14 that we previously reported for the full-length phosphoproteins of vesicular stomatitis
768 virus and rabies virus (105), respectively. Despite that these phosphoproteins of the family
769 *Rhabdoviridae* are dimeric and less than half its size, they share a similar architecture with
770 NiV phosphoprotein (32). Assuming a partial specific volume of $0.73 \text{ cm}^3 \cdot \text{gr}^{-1}$, the intrinsic
771 molecular volume of a protein the size of tetrameric NiV phosphoprotein should be about 390
772 nm^3 , whereas the volume of the equivalent sphere calculated by using the experimental R_h
773 value is $3,400 \text{ nm}^3$, almost ten-fold larger. Multiple copies of this protein are packaged in the
774 virion and might occupy an important part of the particle volume if not otherwise compacted.
775 With respect to the nucleocapsid, the length of the disordered C-terminal linker (70 aa) allows
776 an extension of the order of 50 nm ($2 \times 70 \times 0.35$ nm) between two X domains, which would
777 allow to connect nucleoproteins at least 10 subunits apart along the nucleocapsid, and
778 probably even more distant if the possible extension of the C-terminal disordered region of
779 the nucleoprotein (N_{TAIL}) is taken into account. In the virion, the nucleocapsids have a
780 diameter of 18-19 nm and an average pitch of 5 nm (7), and thus one phosphoprotein could
781 connect nucleoproteins across the nucleocapsid. The larger size of tetrameric NiV
782 phosphoprotein may thus be of importance in the packaging of the viral particle and in the

783 functioning of the polymerase machine, catching at long distance and directing partners to
784 their site of use.

785

786 The architecture of the phosphoprotein is conserved among *Mononegavirales*, including the
787 localization of functional and structural modules, but the length of the protein varies
788 considerably between families and even between genera within the same family. As
789 illustrative examples, VSV in the family *Rhabdoviridae* is 267 aa long, whereas measles virus
790 and NiV phosphoproteins in the family *Paramyxoviridae* are 507 and 709 aa long,
791 respectively. The reason why NiV phosphoprotein is so long remains enigmatic, considering
792 that VSV phosphoprotein, for example, perfectly assumes similar functions in the life cycle of
793 the virus. In a previous study, we compared the structure of the C-terminal N-binding domain
794 of the phosphoprotein from different *Mononegavirales* and discovered that the variation in
795 length of this protein domain correlated with the capacity to perform additional functions,
796 suggesting a model where the protein evolves by accretion of functional modules (52).
797 Henipavirus phosphoproteins are significantly longer than the phosphoproteins from other
798 genera of *Paramyxoviridae* (106). Because the L polymerases of the *Mononegavirales* have a
799 high error-rate, the genome of these viruses has the capacity to rapidly evolve and trim
800 superfluous parts of the genome, suggesting that the very existence of additional lengths in
801 NiV phosphoprotein sequence is a sign that they perform additional functions or are required
802 for the fitness of the virus. We can therefore speculate that the larger size of the N-terminal
803 domain correlates with its implication in other functions, and that the transient α -helical
804 elements evidenced by NMR spectroscopy and predicted by sequence analysis represent short
805 linear motifs that recruit different and yet unidentified partners. Some of them clearly
806 encompass regions of the protein known to bind viral or cellular proteins (**Supplementary**
807 **Figure S8**). A comparison with measles virus phosphoprotein shows that most functional and
808 structural regions are conserved but that NiV phosphoprotein contains an additional region in
809 the N-terminal region between residues 150 and 300 that is common to the V and W proteins,
810 and is required for binding STAT2 (**Supplementary Figure S9**). The binding site of STAT1
811 appears to be conserved between NiV and measles virus, being localized between residues
812 110 and 140, but in measles virus the binding of STAT2 is mediated by the specific C-
813 terminal region of the V protein (107). We speculate that NiV phosphoprotein, V and W
814 proteins have thus evolved by acquiring an additional extension within their common N-
815 terminal region that recruits STAT2. The first 405 N-terminal amino acids of N-terminal
816 region are common to NiV phosphoprotein, V and W proteins (**Figure 1B**), and these three

817 proteins block interferon signaling by preventing the phosphorylation of STAT1 and by
818 sequestering both STAT1 and STAT2 proteins in the cytoplasm and the nucleus (6, 43–45).
819 The primary binding site of STAT1 was localized between residues 110 and 140 of the N-
820 terminal region by co-immunoprecipitation (6) and this localization was confirmed here by a
821 direct interaction assay. The short section of only 30 amino acids forming this binding site in
822 the NiV phosphoprotein suggests that it is a typical example of a short linear motif that folds
823 upon binding to recruit a protein partner (108) as used by numerous viruses to hijack cellular
824 machineries (109) by mimicking cellular proteins, such as STAT1 itself, which uses a short
825 linear motif within its transactivation domain (TAD) to interact with the TAZ1 domain of the
826 CBP/p300 transcriptional coactivator protein (110).

827

828 The Y116A mutant and the H117A/D118A/H119A triple mutant lost their ability to bind
829 STAT1. Similarly, the mutant G121E prevents the accumulation of phosphorylated STAT1 in
830 the nucleus of infected cells. Surprisingly, the G125E mutant, which was unable to inhibit
831 IFN signaling and failed to co-precipitate STAT1 and STAT2 (6, 46), shows similar binding
832 affinity for STAT1 as the WT protein by ITC measurement. By contrast, the G135E mutant
833 conserves the ability to block the accumulation of phosphorylated STAT1 in the nucleus,
834 while it also fails to co-precipitate STAT1. Because the affinity between the phosphoprotein
835 and STAT1 is weak, it is possible that the co-precipitation with STAT1 also requires the
836 presence of STAT2 as found for measles virus (107), and that the mutations of Gly125 and
837 Gly135 perturb the co-interaction between STAT1 and STAT2 rather than the direct
838 interaction with STAT1. It is also possible that the difference in affinity, although small, is
839 sufficient to produce an effect *in vivo* and that interactions with WT and the G125E variant
840 are influenced differently by intracellular conditions such as molecular crowding.

841

842 In summary, our results revealed that the NiV phosphoprotein behaves as a nanometer-size
843 octopus-like protein that uses its long flexible arms to expose binding sites for multiple viral
844 and cellular partners and to orchestrate their participation in virus replication, in hijacking
845 cellular machines and in interfering with the interferon response.

846

847 **AUTHOR CONTRIBUTIONS**

848 MRJ, FY, JMB, VV, MB and MJ conceived the experiments; MRJ, FY, GC, EC, CM, VVa,
849 JMB performed the experiments; MRJ, FY, CM, NT, JMB, VV, MB and MJ analyzed and
850 interpreted the data; MRJ, JMB, MB and MJ wrote the paper.

851

852 **ACCESSION CODES**

853 Coordinates and structure factors have been deposited in the Protein Data Bank under
854 accession code [4GJW](#). SAXS data have been deposited in the Small Angle Scattering
855 Biological Data Bank under accession code SASDHT4.

856

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Table 1. Mass and size of the different phosphoprotein constructs

Protein	N _{aa} ¹	pI _{calc} ²	MM _{calc} ³ (kDa)	MM _{exp} ⁴ (kDa)	R _{h,folded} ⁵ (nm)	R _{h,unfolded} ⁵ (nm)	R _{h,exp} ⁶ (nm)	R _{g,theo} ⁷ (Bernado) (nm)	R _{g,theo} ⁸ (Fitskee) (nm)	R _{g,exp} ⁹ (nm)	D _{max} ¹⁰ (nm)
P ₁₋₁₀₀	108	5.1	12.347	13.5 ± 1.5	1.8	3.1	2.6 ± 0.2	2.9	3.4	3.2 ± 0.1	12
P ₉₂₋₁₉₀	108	4.9	11.875	12.0 ± 0.5	1.8	3.0	2.6 ± 0.2	2.9	3.4	3.1 ± 0.2	12
P ₁₇₃₋₂₄₀	78	6.4	8.691	8.0 ± 2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
P ₉₂₋₂₅₀	168	4.6	18.583	21.0 ± 2.0	n.d.	n.d.	n.d.	3.7	4.5	4.0 ± 0.3	15
P ₂₂₃₋₃₁₉	106	4.5	11.536	12.9 ± 1.5	1.8	3.0	3.1 ± 0.2	2.9	3.4	3.1 ± 0.1	11.5
P ₃₀₀₋₄₀₁	111	5.9	12.421	11.5 ± 1.5	n.d.	n.d.	n.d.	2.9	3.5	n.d.	n.d.
P ₃₈₇₋₄₇₉	102	7.1	11.151	12.4 ± 1.5	1.8	3.0	2.5 ± 0.2	3.3	3.3	3.2 ± 0.1	12.5
P ₄₇₁₋₅₈₀	119	5.2	13.757 (55.028)	58 ± 6	3.1	6.7	4.3 ± 0.2	n.r.	n.r.	n.d.	n.d.
P ₆₅₅₋₇₀₉	63	5.3	7.364	8 ± 1	n.d.	n.d.	n.d.	2.2	2.5	1.6 ± 0.1	5.5
P ₅₈₈₋₆₅₀	72	5.3	8.289	8 ± 2	n.d.	n.d.	n.d.	2.4	2.7	2.6 ± 0.1	10
P ₅₈₈₋₇₀₉	131	4.9	15.041	15.3 ± 1	1.6	2.5	1.9 ± 0.2	n.d.	n.d.	n.d.	n.d.
P ₁₋₅₈₀	588	4.7	64.756 (259.024)	250 ± 25	5.5	14.8	9.2 ± 0.3	n.r.	n.r.	10.8 ± 0.3	41
P ₄₇₁₋₇₀₉	248	4.9	28.369 (113.476)	113 ± 10	4.1	9.8	7.8 ± 0.3	n.r.	n.r.	6.3 ± 0.1	22
P _{FL}	717	4.7	79.367 (317.468)	306 ± 30	6.0	16.8	9.3 ± 0.2	n.r.	n.r.	10.9 ± 0.1	39

¹ Number of amino acids in the used constructs including C-terminal His tag and linker (LEHHHHHH) and in some cases a N-terminal M.

² Isoelectric point of the used constructs calculated using the webserver ProtParam. In parentheses, isoelectric point calculated without the C-terminal His tag.

³ In parentheses, calculated mass for a tetramer.

⁴ Measured by SEC-MALLS.

⁵ The theoretical R_h values are calculated from R_h values of folded and chemically unfolded standard proteins taken from Uversky et al. (1997) and using the experimental molecular mass determined by SEC-MALLS.

⁶ Measured from SEC data by calibrating the system with standard globular proteins.

⁷ Calculated using the power law and parameters defined by Bernado et al.

⁸ Calculated using the power law and parameters defined by Fitskee et al.

⁹ Measured from SAXS data at low Q using the Guinier approximation.

¹⁰ Determined from SAXS data using the program PRIMUS.

n.d. = not determined

n.r. = not relevant

Gray shaded lines show proteins that were found to be tetrameric in solution.

Table 2. Data collection and structure statistics for small angle X-ray scattering

	P _{FL}	P ₁₋₅₈₀	P ₄₇₁₋₇₀₉
Data collection parameters			
Instrument	ID14 with PILATUS detector (ESRF, Grenoble, France)		
Beam geometry (mm)	0.7x0.7		
Sample distance (m)	2.43		
Wavelength (Å)	0.931		
Q range (nm ⁻¹)	0.0406-5.0000		
Exposure time	10 x 10 s		
Temperature (K)	277		
Conc. range (mg.mL ⁻¹)	1.0-1.8	0.5-1.0	3.5-5.8
Structural parameters			
R _g (nm) (Guinier)	10.9 ± 0.1	10.8 ± 0.3	6.3 ± 0.1
R _g (nm) (P(r))	11.2	11.3	6.4
D _{max} (nm)	39	41	22
Molecular mass determination (kDa)			
From BSA calibration	432	240	164
Calculated from seq.	317	259	113
Software employed			
Primary data	BsxCuBE		
reduction			
Data processing	PRIMUS (ATSAS 3.0.0)		
Ensemble modeling	ASTERIODS		
3D representations	PYMOL (1.8.6)		

Table 3. Crystallographic data collection and refinement statistics

Parameter	
Resolution (Å)	48.75 - 3.00 (3.06-3.00)
Space group	P1 2 ₁ 1
Unit cell	
a, b, c (Å)	59.5, 85.60, 122.6
α, β, γ (°)	90.0, 94.7, 90.0
Total reflections	195091
Unique reflections ¹	47924
Rfree test set	1257
Multiplicity	4.1 (1.9)
Completeness (%)	99.5 (99.9)
I/σ(I)	5.3 (2.27)
Rwork	0.175 (0.253)
Rfree	0.229 (0.286)
RMSD (bond lengths) (Å)	0.013
RMSD (angles) (°)	1.16
Ramachandran favored (%)	98.04
Ramachandran allowed (%)	1.83
Ramachandran outliers (%)	0.13
Average B factor (Å ²)	55

Values in parentheses are for the highest- resolution shell.

¹ Friedel's pairs unmerged

Table 4. Isothermal titration calorimetry data.

Ligand	n	K_d (μM)	ΔG_{Bind}^0 (kcal.mol^{-1})	ΔH_{Bind}^0 (kcal.mol^{-1})	$-T\Delta S_{\text{Bind}}^0$ (kcal.mol^{-1})	ΔS_{Bind}^0 ($\text{cal.mol}^{-1}.\text{K}^{-1}$)
wt	1.19	100 ± 15	-5.4 ± 0.8	-9.5 ± 1.2	4.2 ± 1.2	-14 ± 4.0
Y116A	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
H117A/D118 A/H119A	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
G120E	1.23	70 ± 9	-5.7 ± 0.7	-8.4 ± 0.6	2.7 ± 0.6	-8.9 ± 2.1
G125E	1.31	210 ± 90	-5.0 ± 2.0	-7.5 ± 2.6	2.5 ± 2.7	-8.4 ± 9.2

T = 298 K

n.d. : not determined

LEGENDS FOR THE FIGURES

FIGURE 1. Modular architecture of Nipah virus phosphoprotein. (A) Consensus disordered prediction. The D-score was calculated as described in (32). The threshold to distinguish between ordered and disordered region is set at 0.5. The shaded areas indicate the known positions of the folded tetramerization domain and X domain. (B) Modular organization of NiV phosphoprotein. The upper part shows the structural organization of the phosphoprotein encoded in the main open reading frame (0 ORF). Boxes indicate the localization of folded domains, undulated lines the localization of predicted MoRE and lines the localization of intrinsically disordered regions (IDR 1-3). The green dots show two identified phosphorylation sites. In the middle part, the lines show the location of the known functional regions. In the lower part, the blue lines show the position of the C gene and of the regions coding for the specific parts of the V and W proteins in the alternative open reading frames (+1 ORF and +2 ORF). (C) Divide-and-conquer strategy. The lines indicate the position of the different fragments of the phosphoprotein used in this study.

FIGURE 2. Molecular masses and size of full length phosphoprotein (P_{FL} in black) and of different fragments (P₁₋₅₈₀ in red; P₄₇₁₋₇₀₉ in green; P₄₇₁₋₅₈₀ in blue) in solution. Size exclusion chromatography was performed at 20 °C on a TSK G4000 column equilibrated with 20 mM Tris/HCl pH 7.5 containing 150 mM NaCl using a flow rate of 0.8 mL.min⁻¹. The elution was monitored on-line by multi-angle laser light scattering and differential refractometry. The lines show the chromatograms monitored by differential refractive index measurements. The crosses indicate the molecular mass calculated from static light scattering across the elution peak and the numbers indicate the weight-averaged molecular mass (kDa) with standard deviations. The hydrodynamic radius (R_h) was determined from the elution volume by calibrating the column with standard globular proteins (**Table 1**).

FIGURE 3. Crystal structure of NiV phosphoprotein tetramerization domain (P_{MD}). (A) Cartoon representation of tetrameric NiV P_{MD} (4GJW.pdb). The different chains are shown in different colors and the overall dimensions are indicated (black arrows). The N and C-terminal end are indicated for each chain and the three α -helices, α_1 to α_3 , are shown for chain D (in red). The position of the stammer in the long coiled-coil is shown and the dotted lines indicate the location of the a-layers displayed in panels D and E. (B) Contacts between

the chains within the crown. Cartoon representation of the NiV tetramerization domain showing that each chain contacts the other three chains. (C) Sequence alignment of the tetramerization domain of members of the genus Henipavirus. (HeV: Hendra virus; CeV: Cedar virus; GBHV: Ghanaian bat henipavirus). The letters under the sequences indicate the position of the different heptad repeats (h_{1-8}) and of the stammer. A and D positions in the heptad repeats, which correspond to residues pointing inside the coiled-coil, are shown in green to indicate hydrophobic residues or in blue to indicate polar residues. (D and E) Symmetry of layers in the coiled coil. Stick representations of two A-layer (Cys512 and Leu557) showing the transition from four-fold to two-fold symmetry along the coiled-coil (see positions in panel A). (F) Sequence conservation map. Space filling model of NiV tetramerization domain where surface residues are colored according to the degree of conservation (using the multiple sequence alignment of panel C). Figures were drawn with PYMOL.

FIGURE 4. NMR characterization of NiV phosphoprotein N-terminal region (P_{NTR}), C-terminal linker and X domain. (A) Secondary structure propensities (SSP) of the N-terminal region of the phosphoprotein obtained on the basis of experimental $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ chemical shifts. The propensities were obtained by combining spectral assignments of individual sub-constructs of this region. (B) Secondary structure propensities of the C-terminal intrinsically disordered linker region of the phosphoprotein (P_{CT-LINK}) obtained from experimental $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ chemical shifts. (C) Secondary structure propensities of the C-terminal X domain of the phosphoprotein (P_{XD}) obtained from experimental $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ chemical shifts. (D) ^1H - ^{15}N HSQC spectrum of full-length NiV phosphoprotein (red) superimposed with HSQC spectra (blue) of individual sub-constructs of the protein (P₁₋₁₀₀, P₉₂₋₁₉₀, P₁₇₃₋₂₄₀, P₂₂₃₋₃₁₉, P₃₀₀₋₄₀₁, P₃₈₇₋₄₇₉).

FIGURE 5. Ensemble structure of NiV phosphoprotein. (A) Multiple-curve fitting of SAXS data. The upper panel shows experimental SAXS curves of the three tetrameric proteins: P_{FL} (in red), P₁₋₅₈₀ (in blue) and P₄₇₁₋₇₀₉ (in orange), with experimental standard deviations shown as error bars. The black lines show the multiple-curve fit obtained for the three SAXS curves for a selected ensemble of 10 conformers. The reduced χ^2 value is 1.53 over all three constructs. The lower panels show the reduced residuals ($\Delta I / \sigma$). (B) R_g distribution for an ensemble of 10 conformers of full-length phosphoprotein. The thin black

bars show the distribution for the initial ensemble of conformers. The large red bars show the R_g distribution for selected ensembles of 10 conformers. (C) Single model of NiV phosphoprotein structure. The tetramerization domain and X domain are shown as surface representations. The different chains are shown in different colors and the N-terminal end and X domain (P_{XD}) are indicated for each chain. (D) Ensemble representations of full-length NiV phosphoprotein (P_{FL}) and of the two tetrameric fragments: P_{1-580} and $P_{471-709}$. The 10 different models are aligned by superimposing their tetramerization domain. The tetramerization domain and X domain are shown as surface representations and the different chains are shown in same colors as in panel C. The models of the $P_{471-709}$ fragment are shown in two orientations.

FIGURE 6. Interaction of the N-terminal region of NiV phosphoprotein (V and W proteins) with STAT1. (A) Sequence conservation between residues 85 and 140 of the phosphoprotein (V and W proteins) across different Henipaviruses (see Figure 3C). The labels above the alignment indicate the mutants used in the ITC experiments (in blue) and in the generation and assays of recombinant viruses (in green). (B) Schematic representation of STAT1 architecture. The color boxes show the different domains present in our construct, which encompasses residues 135 to 683 of STAT1, whereas the gray boxes indicate the missing part. (C) SEC-MALLS analysis. The lines show the chromatograms monitored by refractometry and the red crosses show the molecular mass calculated from on-line static light scattering measurements (MALLS). The upper panel shows the results for $STAT1_{132-683}$, the middle panel for the fragment P_{92-190} , and the lower panel for the mixture of the two protein fragments in a 10:1 ratio after 2 hour incubation at 20°C. (D) Overlay of a region of the 2D 1H - ^{15}N HSQC NMR spectra of P_{92-190} (~540 μ M) in the absence (in red) and presence of $STAT1_{132-683}$ (~54 μ M) (in blue). (E) Intensity profile calculated as the ratio of peak intensities in the 1H - ^{15}N HSQC spectrum of P_{92-190} in isolation (I_0) and in the presence of $STAT1_{132-683}$ (I).

Figure 7. Effect of mutations on the interaction between the phosphoprotein and STAT1. Calorimetric titration of $STAT1_{132-683}$ with P_{92-190} W.T. (A), G120E (B), G125E (C), Y116A (D) and the triple mutant (H117A/Q118A/H119A) (E). The upper panels show the heat effects associated with the injection of P_{92-190} (2.5 μ L per injection of about 2 mM solution) into the calorimetric cell (200 μ L) containing $STAT1_{132-683}$ at a concentration of 100 μ M. All experiments were performed in 20 mM phosphate buffer (pH 7.5) containing 150

mM NaCl at 25 °C. The lower panels show the binding isotherm corresponding to the data in the upper panels and the lines show the best fitted curves for a 1:1 binding model. (F) Average dissociation constant obtained from three experiments for WT P₉₂₋₁₉₀ and the different variants. AAA = H117A/Q118A/H119A. (G) Impact of NiVs infections (NiV-WT, NiV-G121E, NiV-G135E) on the localization of phosphorylated endogenous STAT1. Vero E6 cells were infected with the indicated viruses and treated with interferon- β at 28 h post-infection. The phospho-Y701 form of endogenous STAT1 was visualized in paraformaldehyde fixed cells 40 min after interferon- β addition (phospho-STAT1, green staining). NiV matrix (M) protein expression is a marker for infection (red). Arrowheads point to representative non-infected cells and nuclei stained for phospho-STAT1. Arrows indicate cells infected with NiV-G121E mutant defective in STAT1 binding, and again nuclei stained for phospho-STAT1.











