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## Characterization of the Interaction Domains between the Phosphoprotein and the Nucleoprotein of Human Metapneumovirus

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1 **Characterization of the interaction domains between the phosphoprotein and the nucleoprotein**  
2 **of human Metapneumovirus**

3  
4 **Running title: Metapneumovirus N-P interaction**

5  
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16  
17 **Keywords** : HMPV, phosphoprotein, nucleoprotein, protein-protein interaction, structural modeling

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19

**20 ABSTRACT**

21 Human metapneumovirus (HMPV) causes severe respiratory diseases in young children. The HMPV  
22 RNA genome is encapsidated by the viral nucleoprotein (N), forming an RNA-N complex ( $N^{Nuc}$ ), which  
23 serves as template for genome replication and mRNA transcription by the RNA-dependent RNA  
24 polymerase (RdRp). The RdRp is formed by the association of the large polymerase subunit (L), which  
25 has RNA polymerase, capping and methyltransferase activities, and the tetrameric phosphoprotein  
26 (P). P plays a central role in the RdRp complex by binding to  $N^{Nuc}$  and L, allowing the attachment of  
27 the L polymerase to the  $N^{Nuc}$  template. During infection these proteins concentrate in cytoplasmic  
28 inclusion bodies (IBs) where viral RNA synthesis occurs. By analogy to the closely related  
29 pneumovirus respiratory syncytial virus (RSV), it is likely that the formation of IBs depends on the  
30 interaction between HMPV P and  $N^{Nuc}$ , which has not been demonstrated yet. Here, we finely  
31 characterized the binding P-  $N^{Nuc}$  interaction domains by using recombinant proteins, combined with a  
32 functional assay for the polymerase complex activity, and the study of the recruitment of these  
33 proteins to IBs by immunofluorescence. We show that the last 6 C-terminal residues of HMPV P are  
34 necessary and sufficient for binding to  $N^{Nuc}$ , that P binds to the N-terminal domain of N ( $N_{NTD}$ ), and  
35 identified conserved N residues critical for the interaction. Our results allowed to propose a structural  
36 model for the HMPV P- $N^{Nuc}$  interaction.

37

**38 IMPORTANCE**

39 Human metapneumovirus (HMPV) is a leading cause of severe respiratory infections in children but  
40 also affects human populations of all ages worldwide. Nowadays, no vaccine or efficient antiviral  
41 treatments are available for this pneumovirus. A better understanding of the molecular mechanisms  
42 involved in viral replication could help the design or discovery of specific antiviral compounds. In this  
43 work we have investigated the interaction between two major viral proteins involved in HMPV RNA  
44 synthesis, the N and P proteins. We finely characterized their domains of interaction, and identified a  
45 pocket on the surface of the N protein, a potential target of choice for the design of compounds  
46 interfering with N-P complexes and inhibiting viral replication.

47

48 **INTRODUCTION**

49 Pneumonia is the leading cause of death among children younger than 5 years worldwide, and severe  
50 pneumonia is more frequently caused by viruses than bacteria (1). After the closely related respiratory  
51 syncytial virus (RSV), human metapneumovirus (HMPV) is recognized to be one of the most important  
52 cause of viral bronchiolitis and pneumonia in young children, causing 7 to 19% of all cases of acute  
53 respiratory tract infections (1, 2). HMPV infects mainly newborn, children, elderly and  
54 immunocompromised individuals worldwide. This virus was first reported in 2001 from Dutch children  
55 with acute lower respiratory tract illness, and serological studies have revealed that virtually every  
56 child has been exposed to HMPV by the age of 5 years (3). The clinical features of HMPV infection  
57 display as mid-to-upper respiratory tract infection, and can be severe enough to cause life-threatening  
58 bronchiolitis and pneumonia. As yet, there is no effective treatment or licensed vaccine for HMPV.

59 Together with respiratory syncytial virus (RSV), HMPV belongs to the *Pneumoviridae* family in the  
60 order *Mononegavirales* (4). HMPV is an enveloped virus that forms pleomorphic or filamentous  
61 virions. The virus genome is composed of a negative-sense single-stranded RNA of approximately  
62 13.3 kb in size, which encodes eight genes in the following order: 3'-N-P-M-F-M2-SH-G-L-5' (5, 6).  
63 The M2 gene of HMPV contains two overlapping open reading frames (ORFs), encoding for M2-1 and  
64 M2-2 proteins which precise functions during HMPV viral cycle remain unclear. The HMPV genome is  
65 encapsidated by multiple copies of the nucleoprotein (N) forming helical nucleocapsids (N<sup>Nuc</sup>). This  
66 N<sup>Nuc</sup> serves as template for genome replication and mRNA transcription by the viral polymerase  
67 complex formed by the large polymerase subunit (L) and its main cofactor the phosphoprotein (P).  
68 After virus binding to the cell surface and virus-cell membrane fusion mediated by the fusion protein F,  
69 nucleocapsids are released into the cytoplasm. Replication and transcription of the viral genome take  
70 place within viro-induced cytoplasmic inclusions named inclusion bodies (IBs) (7). These structures  
71 can be observed upon expression of P and N proteins alone (8), and it was recently shown for RSV  
72 that the interaction between P and N<sup>Nuc</sup> is critical for the formation of IBs (9).

73 Among the components of the polymerase complex, P plays a pivotal role as a cofactor of the L  
74 polymerase, but also as a molecular hub between viral partners. HMPV P, 294 amino acid residues in  
75 length, forms homo-tetramers. The atomic structure of the coiled-coil oligomerization domain (residues  
76 171-194) was resolved by crystallography (10). Small angle X-ray scattering (SAXS) studies indicated  
77 that the flanking N-terminal (residues 1-170) and C-terminal (residues 195-294) regions (named PNT

78 and PCT respectively) are intrinsically disordered, some of them, such as residues 195-237, having  $\alpha$ -  
79 helical propensity (10, 11). More recently, the structure of the LP complex was resolved by cryo-EM  
80 (12). It revealed a tentacular arrangement of P, with each of the four protomers adopting a distinct  
81 conformation, demonstrating a “folding-upon-partner-binding” mechanism. Depending on the  
82 protomer, the L-binding region involved regions encompassing residues 171-236, 172-217, 170-231 or  
83 169-264. On the other hand, by binding to N, P mediates the attachment of the L protein to the N<sup>Nuc</sup>  
84 template for viral RNA synthesis.

85 By analogy to RSV (13), the most C-terminal region of HMPV P is also expected to be the N<sup>Nuc</sup> binding  
86 domain, but this has not been shown yet. It is noteworthy that the C-terminal extremity of P supposed  
87 to bind to N<sup>Nuc</sup> was not visible in the cryo-EM structure of the LP complex, indicating that this region is  
88 disordered in the absence of N<sup>Nuc</sup>. Furthermore, the encapsidation of neosynthesized genome or  
89 antigenome necessitates a pool of monomeric, RNA-free N, termed N<sup>0</sup>, which is kept in an  
90 unassembled state through an interaction with P, which plays the role of molecular chaperone, until  
91 delivery to the sites of viral RNA synthesis. The crystal structures of recombinant HMPV N protein  
92 (394 residues) expressed in *E. coli* and purified either as an RNA-free monomeric N in complex with  
93 the N-terminal residues of P, or as rings of 10 N protomers complexed to RNA were resolved (14). In  
94 both states, the structures show that N presents two globular domains (N<sub>NTD</sub> and N<sub>CTD</sub>) separated by a  
95 flexible linker that forms the RNA groove, and N- and C-arms. In the oligomeric state, the N- and C-  
96 arms play a key role in the interaction between N protomers and oligomerization, the N-arm binding to  
97 the flank of the N<sub>*i*-1</sub> protomer and the C-arm binding atop the N<sub>*i*+1</sub> protomer (*i* corresponding to the  
98 middle subunit of three adjacent N protomers). In the N<sup>0</sup> state, the important conformational changes  
99 consist in packing of the C-arm of N in the RNA groove, impairing RNA binding.

100 In this work, based on the structural data of HMPV P and N proteins and their strong homologies with  
101 RSV N and P proteins, we finely characterized the binding domains involved in HMPV P-N interaction.  
102 By combining biochemical and functional cellular assays, coupled with a rational mutational approach,  
103 we identified residues of P and N critical for this interaction. Our data show that the last C-terminal  
104 residues of P bind to the N<sub>NTD</sub>. These results allowed to establish a structural model of the interaction  
105 that could be used for the rational design of antivirals targeting the N<sup>Nuc</sup>-P interaction of HMPV.

106

107

108 **RESULTS**

109

110 **The HMPV PCT domain allows to purify N-RNA rings expressed in bacteria**

111 HMPV and RSV P proteins present a conserved structural organization, with a central tetramerization  
112 domain flanked by N- and C-terminal highly disordered regions (Fig. 1A). Co-expression of N with the  
113 C-terminal residues 161-241 of RSV P fused to GST in bacteria allowed the purification of ring shape  
114 N-RNA oligomers, showing that the C-terminal disordered region of RSV P (PCT) is involved in N<sup>Nuc</sup>  
115 binding (13, 15). Similarly, when expressed alone in *E. coli*, HMPV N also forms decameric rings  
116 containing RNA (14). In a first attempt to characterize the N binding site of HMPV P, we thus co-  
117 expressed recombinant HMPV GST-PCT (residues 200-294) and N proteins (from CAN 97-83 strain)  
118 in *E. coli*. The purified complexes were analyzed by SDS-PAGE stained with Coomassie blue. As  
119 shown on figure.1B, N was co-purified with GST-PCT to > 95% homogeneity. The PCT was then  
120 separated from GST by thrombin cleavage (Fig. 1B) and the solubilized complex was analyzed by size  
121 exclusion chromatography, following optical density (OD) at 220, 260 and 280 nm. The elution profile  
122 showed a major peak (P1) with a OD<sub>260nm</sub>/OD<sub>280nm</sub> ratio > 1 and an apparent mass of ~ 500 kDa, in  
123 agreement with the expected size of N-RNA decamers (Fig. 1C). A second peak of lower intensity  
124 (P2), with an apparent mass of ~ 10 kDa was detected only at 220 nm. This peak should correspond  
125 to PCT, which does not present aromatic residues and a higher apparent molecular weight than  
126 predicted, likely due to the elongated shape of this fragment. The fractions of P1 peak were pooled,  
127 and sample analysis by SDS-PAGE stained with Coomassie blue showed the presence of a unique  
128 band corresponding to N (Fig. 1C). The separation of PCT and N upon gel filtration reveals the  
129 relatively low affinity of monomeric PCT for N. We then further analyzed the sample collected from the  
130 P1 peak by combining dynamic light scattering (DLS) and electron microscopy (EM) approaches. The  
131 DLS measurement profile confirmed the homogeneity of the sample, with a single peak at 18 nm (Fig  
132 1D), while the observation of purified N by EM confirmed the presence N-rings (Fig 1E). These N-RNA  
133 rings are thus similar to those purified by Renner *et al.*, who determined the 3D structure of N-RNA  
134 rings with a diameter close to 16 nm (14).

135 Altogether, these results show that the HMPV GST-PCT fusion protein is sufficient to interact with  
136 nucleocapsid-like N-RNA rings.

137

138

**139 The last 6 residues of HMPV P constitute the minimum domain for N<sup>Nuc</sup> binding**

140 We then investigated the minimal domain of HMPV P involved in N-RNA rings binding. The 9 C-  
141 terminal residues of RSV P were previously shown to be sufficient to interact with N (13). Sequence  
142 alignment of the C-terminal extremity of pneumoviruses P proteins reveals the presence of two distinct  
143 motifs, separated by a highly acidic stretch in the case of Metapneumovirus P proteins (Fig. 2A). Of  
144 particular interest, the last 9 C-terminal residues of P proteins form a partially conserved motif. To  
145 investigate the role of these C-terminal residues of HMPV P in N binding, a series of GST fused  
146 peptides of 9 to 1 amino acid long derived from the C-terminal of P was generated. These constructs  
147 were co-expressed with N in *E.coli*, and their capacity to purify N was analyzed by SDS-PAGE and  
148 Coomassie blue staining. As shown on figure. 2B, the minimal sequence required for N purification  
149 corresponds to the 6 last C-terminal residues of P. In parallel, alanine scanning was performed with  
150 the GST-P[285-294] construct to characterize the residues of P involved in the interaction with N-RNA  
151 rings. Again, GST-P constructs and N were co-expressed in bacteria, followed by purification by  
152 Glutathione-Sepharose beads affinity, and co-purification of N was analyzed by SDS-PAGE and  
153 Coomassie blue staining. Only the four P substitutions I289A, Y290A, L292A and M294A abrogated  
154 the interaction with N (Fig. 2C). In order to further investigate the potential role of P residues Q291 and  
155 I293 in the interaction with N, a double substitution Q291A/I293A was inserted in GST-PCT. These  
156 two mutations did not affect N binding, confirming that these residues may not be directly involved in  
157 the interaction (Fig. 2D). These results confirm that the last residues of P are directly involved in the  
158 interaction with N, and suggest that HMPV P-N interaction mainly involves hydrophobic interactions.  
159 Of particular interest, the last 7 C-terminal residues of the P proteins of HMPV strains NL 99/1 (B1  
160 subgroup), CAN 98-75 (B2 subgroup) and CAN 97-83 (A2 subgroup) are conserved, thus confirming  
161 their critical role. As both hydrophobic and acidic residues of RSV P were previously shown to be  
162 critical for the interaction with N (Fig. 2E), these results suggest that the binding of HMPV P on N  
163 differs from RSV and involves specific interactions. We thus tested the capacity of each PCT  
164 fragments to pulldown heterologous N proteins. Figure 2F shows that HMPV and RSV GST-PCT  
165 constructs did not allow to purify heterologous N. This last result confirms that HMPV and RSV N and  
166 P proteins cannot cross-interact, and that pneumoviruses P-N interactions are specific to each genus.  
167

168

169 **Search for the P binding site on the surface of HMPV N**

170 Although HMPV and RSV P-N interactions have their own specificity and cannot cross-react, the  
171 strong homologies between N proteins suggest that the PCT binding site on N protein could be  
172 partially conserved. For RSV, the P binding site on N<sup>Nuc</sup> is located at the surface of the helical  
173 nucleocapsid, on the N<sub>NTD</sub> (16). The residues of RSV N<sub>NTD</sub> involved in the interaction with P, which are  
174 partially conserved between HMPV and RSV (Fig. 3A), were shown to form a well-defined pocket  
175 constituted of hydrophobic and positively charged residues (16, 17). Corresponding residues of HMPV  
176 N<sub>NTD</sub> also form a pocket exposed at the surface of N-RNA rings (Fig. 3B). In a first attempt to  
177 characterize the HMPV P binding domain on N, we thus tested the capacity of P to interact with the  
178 N<sub>NTD</sub>. The N<sub>NTD</sub> was expressed in *E. coli* and purified using a 6xHis tag, showing that this domain is  
179 soluble when expressed alone (Fig. 4A). In parallel, the GST-P or GST-P<sub>ΔM294</sub> (deletion of the last C-  
180 terminal residue of P) proteins were expressed in bacteria, either alone and purified using the GST  
181 tag, or co-expressed with N<sub>NTD</sub> followed by purification by 6xHis tag. Analysis of the samples by SDS-  
182 PAGE showed that N<sub>NTD</sub> allowed to co-purify the wild type P, and that deletion of the last residue of P  
183 was sufficient to impair the interaction (Fig. 4A). Of note, the presence of numerous bands was  
184 observed in the samples containing GST-P alone or incubated in the presence of N<sub>NTD</sub>. These bands  
185 migrated with a lower apparent mass weight compared to GST-P protein, suggesting a high propensity  
186 of recombinant GST-P to be cleaved and degraded. The HMPV N<sub>NTD</sub> residues identified by analogy  
187 with RSV, i.e. residues L46, L47, E50, D128, R132, M135, R151, P152, and S153 (Fig. 4B), were then  
188 substituted by alanine. We first assessed the impact of these mutations on the expression and  
189 solubility of N proteins fused to a C-terminal poly-His tag expressed in *E. coli*. Analysis of purified N  
190 proteins by SDS -PAGE showed that none of these mutations induced a defect of production or  
191 solubility of N (Fig. 4C). The capacity of GST-P to co-purify mutated N proteins was then tested.  
192 Whereas substitutions L46A, L47A, and P152A did not disrupt N-P interaction P, E50A, D128A,  
193 R132A, M135A, R151A and S153A substitutions strongly affected or abrogated N-P interaction (Fig.  
194 4D, upper panel). Of note, expression of N mutants in bacteria lysates was assessed by western blot  
195 and confirmed that all the N were expressed (Fig 4D, lower panel). These results thus revealed a  
196 critical role of hydrophobic residues, but also of negatively and positively charged residues of N<sub>NTD</sub> in



197 P binding. Overall, these data confirm that the P binding site on N presents strong homologies  
198 between HMPV and RSV.

199

#### 200 **Validation of P-N<sup>Nuc</sup> binding domains in eukaryotic cells**

201 As P-N<sup>Nuc</sup> interaction is required for viral polymerase activity, we then studied the impact of mutations  
202 on P and N on HMPV replication/transcription using a bicistronic minigenome, pGaussia/Firefly. This  
203 construct contains the Gaussia and Firefly luciferase genes under the control of HMPV gene start and  
204 gene stop sequences, as well as Leader and Trailer sequences of HMPV genome (Fig. S1). Briefly,  
205 BSRT7 cells were transfected with plasmids coding for N (WT or mutants), P (WT or mutants), L, and  
206 M2-1 proteins, the plasmid pGaussia/Firefly minigenome, and pSV- $\beta$ -Gal (used to normalize  
207 transfection efficiencies). In this system, the expression of luciferase depends on the HMPV  
208 polymerase complex activity which can thus be quantified by luminescence measurement. As shown  
209 on figure 5A, mutations I289A, Y290A, L292A and M294A of P abrogated the polymerase activity, and  
210 only a residual activity was detected for the mutant Q291A. On the contrary, the P mutation I293A  
211 induced only a slight decrease of the polymerase activity. On the other hand, nearly all the mutations  
212 of on N induced a decrease of  $\geq 50\%$  of the polymerase activity compared to wild type N, with only  
213 mutations D128A and R132A totally impairing the activity (Fig 5A, right panel). For all these P and N  
214 mutants, the levels of protein expression were similar to those of wild type proteins as assessed by  
215 western blot (Fig.5B).

216 We then assessed if the impact of N and P mutations on polymerase activity could correlate with a  
217 defect in IBs morphogenesis. Knowing that co-expression of N and P is sufficient to induce the  
218 formation of pseudo-IBs (8), we used an approach based on fluorescent proteins expression. First, in  
219 order to investigate the impact of P mutations, cells were co-transfected with plasmids encoding P  
220 (WT or mutants) together with plasmids encoding N and GFP-N (at a ratio 2:1). A similar strategy used  
221 for RSV showed that, although GFP-N fusion protein had a dominant negative effect on RSV RNA  
222 synthesis, co-expression of GFP-N with wild type N partially restored polymerase activity (18). In our  
223 HMPV minigenome assay, co-expression of N with GFP-N at a ratio 2:1 allowed to rescue almost 70%  
224 of the polymerase activity (Fig. 5C). Cells transfected with P and N: GFP-N were fixed 24 h post-  
225 transfection and labelled with anti-P antibody. As shown on figure 6, although P labelling revealed a  
226 diffuse cytoplasmic localization of the protein, wild type P was found to co-localize with GFP-N into

227 cytoplasmic inclusions similar to IBs observed upon HMPV infection (7, 8). No impact on IBs  
228 morphology was detected upon alanine substitution of P residue I293. On the contrary, Q291A  
229 substitution seemed to induce a defect in IBs formation compared to wild type condition, as smaller  
230 IBs were observed, and a total loss of IBs formation was observed when mutating residues I289,  
231 Y290, L292 and M294 of P (Fig. 6B and Table 1).

232 In parallel, the impact of N mutations on IBs morphogenesis was investigated by co-transfecting N  
233 (WT or mutants) with P carrying BFP inserted between residues S61 and S62 (NL99-1 strain), in a  
234 region poorly conserved among HMPV strains and predicted as naturally intrinsically disordered. This  
235 P-BFP construct was functional since 65% of the HMPV polymerase activity was recovered when  
236 tested in our minigenome assay (Fig. 5D). Transfected cells were fixed before immunolabelling with  
237 anti-N antibody. Pseudo IBs were detected upon co-expression of P-BFP and WT N, but also upon  
238 alanine substitution of most of the residues (i.e. L46, L47, E50, M135, P252, and S153) (Fig. 7 and  
239 Table 1). As previously observed for the mutant Q291A of P, the mutation R151A of N seemed to  
240 induce a defect of IBs formation compared to the wild type condition, and no IBs were observed when  
241 expressing N mutants D128A and R132A (Fig. 7 and Table 1). Altogether, these results reveal a good  
242 correlation between the polymerase functioning and the capacity of N and P to interact and form IBs.  
243 Besides, these data confirm that the residues of P and N previously identified as critical for N<sup>Nuc</sup>-P  
244 interaction *in vitro* are also critical for polymerase activity and the formation of IBs and in cells.

245

#### 246 **Molecular modeling of PCT-N<sub>NTD</sub> interaction**

247 To gain insight into the possible binding mode of P with N<sub>NTD</sub>, we built models of the last 6 residues of  
248 P (peptide I289-YQLI-M294 named P6) bound to N, by analogy with the PCT-N<sub>NTD</sub> complex of RSV.  
249 For RSV, only the last 2 residues of P (D240-F241) were resolved in the PCT-N<sub>NTD</sub> X-ray structures  
250 (16), and are bound to a hydrophobic pocket of N similar to the one we characterized at the surface of  
251 HMPV N. Using comparative modeling and refinement with HADDOCK (19), we obtained three  
252 clusters of HMPV P6/N<sub>NTD</sub> complex structures representing possible binding modes of P6 (Table S1).  
253 The first two clusters in terms of HADDOCK-score were the most populated and displayed similar  
254 Root-mean-square deviation (RMSD) among their members (considering only the last three residues  
255 of P, i.e. residues L292-I293-M294, called P3). In those two clusters, the C-terminal carboxyl group of  
256 M294 is involved in an ionic interaction with the guanidinium of either R131 or R152 side-chains of N.

257 Additionally, only L292 and M294 of P3 consistently display hydrophobic contacts with N. This result is  
258 consistent with our mutation data showing that I293 of P is not required for binding to N, contrary to  
259 L292 and M294. The 10 best-scoring structures from cluster 1 and 2 are shown in [figure 9](#),  
260 superimposed with the RSV P2/N<sub>NTD</sub> structure (residues D240-F241 of P). Owing to the best  
261 HADDOCK-score and non-bonded energies of cluster 2, we considered this cluster as most  
262 representative for the potential binding mode of HMPV P3 to N<sub>NTD</sub>. Interestingly, more than half of the  
263 structures from this cluster display a hydrogen-bond between the backbone oxygen of I293 of P3 with  
264 the side-chain of R151 of N, while the side-chain of R132 of N forms a salt-bridge with the C-terminal  
265 carboxyl group of M294 of P. Thus, the interactions determined by molecular simulation correlate with  
266 the experimental data showing the importance of R132 and R151 of N and of M294 of P for PCT-N  
267 interaction.

268 The interactability propensity of each modeled N<sub>NTD</sub> conformation was further profiled with our in-  
269 house tool InDeep (20) in order to select a subset of N<sub>NTD</sub> models being more likely to interact with the  
270 P3 peptide. On this subset of N<sub>NTD</sub> conformations, the hydrophobic channel of InDeep was also used  
271 to locate regions where hydrophobic moieties of P3 are expected to interact at the N<sub>NTD</sub> surface. Three  
272 hydrophobic patches are detected on the N<sub>NTD</sub> surface, that present a good match with hydrophobic  
273 moieties of P3 structures belonging to cluster 2 of HADDOCK: i) one occupied by L292 of P3; ii)  
274 another occupied by M294 of P3; and iii) the last one located in an unoccupied region of the N pocket  
275 ([Fig.8A](#)). Based on experimental and modeling information, the structure shown in [figure 8](#) represents  
276 a consistent model of P3/N<sub>NTD</sub> complex. The N<sub>NTD</sub> conformation of this structure is predicted to be  
277 favorable to protein-protein interaction by InDeep, and the P3 conformation, belonging to cluster 2 of  
278 HADDOCK, binds to N<sub>NTD</sub> with a combined effect of electrostatic/polar interactions and hydrophobic  
279 contacts in agreement with the mutational experiments. In addition, a procedure of virtual alanine  
280 scanning using the same InDeep approach confirmed some of the mutagenesis results, such as the  
281 importance of R132, M135, and R151 of N for the interaction with P. It also suggests that residue L139  
282 of N may be key for the N<sub>NTD</sub> patch for partners' binding.

283 These analyses reveal that despite the different physico-chemical nature of residues involved in the  
284 interaction, PCT binding at the N surface is structurally similar between HMPV and RSV.

285

286 **DISCUSSION**

287 Beyond its interest in better understanding the molecular mechanisms of virus replication, the study of  
288 protein-protein interactions required for Pneumoviruses' polymerase complex activity also presents  
289 interest for the development of specific antiviral compounds. The activity of this complex depends on  
290 multiple highly specific protein-protein interactions that have no cellular counterparts. Among those,  
291 N<sup>Nuc</sup>-P interaction which is critical for viral polymerase functioning constitutes a singular potential  
292 target for antivirals. We have previously described RSV N<sup>Nuc</sup>-P interaction at the molecular level,  
293 showing that the C-terminus of P binds to a well-defined pocket at the surface of N (9, 13, 17). We  
294 also made the proof of concept that small chemical compounds can target this pocket of N and inhibit  
295 viral replication in cells (16). More recently, Sá et al. showed that flavonoids derived molecules could  
296 also specifically bind to N pocket and block its interaction with P (21). Given the strong homologies  
297 between RSV and HMPV N and P proteins, it could be expected that a similar strategy could be used  
298 to block HMPV replication. It is noteworthy that ideally, the discovery of compounds that could inhibit  
299 replication of these two closely related viruses at the same time would be much more advantageous.

300 In the present study, we investigated the domains involved in HMPV P-N<sup>Nuc</sup> interaction using pulldown  
301 assays between recombinant proteins (either truncated or mutated proteins) expressed in *E. coli*. As  
302 for RSV, the HMPV N protein was purified as nanorings formed by N and bacterial RNA, which can be  
303 considered as N<sup>Nuc</sup>-like complexes (Fig 1). Our results revealed that the mechanisms of binding  
304 between P and N-RNA rings are similar between RSV and HMPV, the C-terminus of P interacting with  
305 a pocket located on the surface of the N N-terminal globular domain N<sub>NTD</sub>. However, it appeared that P  
306 and N amino acid residues involved in these interactions have different contributions/roles between  
307 RSV and HMPV, and that the P-N<sup>Nuc</sup> interaction is specific of each virus. Our results correlate with  
308 these data showing that pneumoviruses' polymerase complexes activity necessitates genus-specific  
309 combination of L, P, N, and M2-1 (22, 23). More specifically, we showed that the minimal domain of  
310 HMPV P required for N binding is constituted by the last 6 C-terminal residues of P which are mostly  
311 hydrophobic residues (IYQLIM), and determined that only Q291 and I293 are not critical for the  
312 interaction *in vitro* (Fig. 2). Analysis of the impact of mutations on polymerase activity and IBs'  
313 formation confirmed these results, but also revealed that residue Q291 of P is critical for the  
314 polymerase activity. This P fragment is thus shorter than for RSV for which N binding minimal domain  
315 involves the 9 C-terminal residues of P and is composed of both hydrophobic and acidic residues (13,  
316 17). Interestingly, sequence analysis of the C-terminal residues of Orthopneumoviruses and

317 Metapneumoviruses P proteins highlights the conservation of residues critical for N-P interaction (Sup  
318 10A), confirming a strong conservation of the interaction mode for each virus. We also identified 6  
319 residues of N involved in P binding, *i.e.* residues E50, D128, R132, M135A, R151A and S153A, which  
320 form a well-defined pocket at the N<sub>NTD</sub> surface (Fig 4). Using combined approaches, we revealed a  
321 correlation between the impact of the mutations on N-P interaction, and the level of polymerase  
322 activity (Table 1). However, only mutations of residues D128 and R132 abrogated IBs' formation and  
323 polymerase functioning. Although residues of N proteins involved in P binding are poorly conserved  
324 between RSV and HMPV, sequence analysis of N proteins of Orthopneumoviruses and  
325 Metapneumoviruses shows that 3 essential residues for N-P interaction present the same physico-  
326 chemical properties (132R/R, 135Y/M and 151R/R, Fig 10B). Of most interest, residues R132 and  
327 R150 of RSV N were also shown to play a key role in P-N<sup>Nuc</sup> interaction, the aliphatic part of the R132  
328 side chain being involved in the stacking of the aromatic ring of the residue F241 of P. In order to  
329 better understand the binding mode of HMPV P to N, molecular modeling was performed and the best  
330 structural models support that R132 and R151 of N and of M294 of P are critical for PCT-N interaction  
331 (Fig. 7). Nevertheless, molecular modeling was mainly targeted towards the binding of the last 3 C-  
332 terminal residues of P, and did not allow to reliably predict the interaction of all the 6 critical residues of  
333 P on N. As also observed in the crystal structures of RSV PCT/N<sub>NTD</sub> complexes (16), the last C-  
334 terminal residue of P seems to drive the binding to a well-defined N<sub>NTD</sub> pocket and this primary binding  
335 could allow transient contacts with upstream P residues outside of the N pocket. Remarkably, the  
336 acidic stretch in RSV PCT upstream of the main N binding domain was shown to contribute also to N  
337 binding (13, 24). Compared to RSV P, HMPV PCT is characterized by the presence of an 18 residues  
338 long sequence upstream the last 10 C-terminal residues, mainly composed of acidic residues (Fig.  
339 2A), which could, as for RSV, contribute to HMPV P-N interaction. This sequence, located between L  
340 and N binding sites of P could also be involved in the modulation of the interaction of P with L and N.  
341 In conclusion, our data confirm the strong structural homologies between HMPV and RSV P-N  
342 complexes but also highlight some singularities. Altogether, our study suggests that the HMPV P  
343 binding pocket at the N<sub>NTD</sub> surface could represent a new target for the rational design of antivirals.

344

345

346 **MATERIALS AND METHODS**

347 **Plasmid constructions**

348 The N and P sequences used for bacterial expression were derived from HMPV CAN97-83 strain. The  
349 sequence of N or N<sub>NTD</sub> (residues 30-255 of N) were cloned into pET-28a(+) vector (Novagen) at  
350 NcoI/XhoI or BamHI/XhoI restriction sites respectively, to allow bacterial expression of full-length N or  
351 N<sub>NTD</sub> domain with a C-terminal or N-terminal 6xHis tag. The sequence of P or PCT (residues 200-294  
352 of P), were cloned into pGEX-4T3 vector, at BamHI and SmaI sites to express in bacteria GST-P and  
353 GST-PCT fusion proteins. For the construction GST-P[285-294], complementary antiparallel  
354 oligonucleotides were hybridized and inserted at the BamHI/SmaI sites in the pGEX-4T-3 vector.

355 A HMPV minigenome plasmid containing Gaussia/Firefly luciferases was designed and synthesized  
356 by Genscript, cloned in pUC57 vector, and containing the trailer, leader, gene start (GS) and gene end  
357 (GE) sequences derived from HMPV CAN97-83 strain (see Fig S1). The first ORF of this  
358 pGaussia/Firefly minigenome codes for the Firefly luciferase and the second one codes for the  
359 Gaussia luciferase. The plasmids pP, pN, pL, and pM2-1 corresponding to the sequences of NL99-1  
360 HMPV strain cloned into pCite vector. For expression of GFP-N fusion protein, the mGFP gene was  
361 amplified by PCR and cloned in frame at the 3' end of N at the EcoRI site of pN, using In-fusion HD  
362 cloning kit (Takara Bio). For expression of P-BFP fusion protein, a BamHI cleavage site was inserted  
363 between position S61 and S62 of P by site-directed mutagenesis. The BFP gene was then amplified  
364 by PCR and cloned at the BamHI site of modified pP, using In-fusion HD cloning kit (Takara Bio).  
365 Point mutations were introduced in the P and N sequences by site-directed mutagenesis using the Q5  
366 site-directed mutagenesis Kit (New England Biolabs). Sequence analysis was carried out to check the  
367 integrity of all constructs. All the oligonucleotides sequences are available on request.

368

369 **Antibodies**

370 Antisera used in this study included polyclonal rabbit antisera raised against recombinant HMPV N  
371 and P expressed in bacteria. A mouse monoclonal anti- $\beta$ -tubulin (Sigma) and secondary antibodies  
372 directed against mouse and rabbit Ig G coupled to HRP (P.A.R.I.S) were also used for  
373 immunoblotting. Secondary antibodies directed against rabbit Ig G coupled to Alexafluor-594 or to  
374 Alexafluor-488 (Invitrogen) were used for immunofluorescence experiments.

375

376 **Cell culture and transfections**

377 BHK-21 cells (clone BSRT7/5) constitutively expressing the T7 RNA polymerase (25) were grown in  
378 Dulbecco Modified Essential Medium (Lonza) supplemented with 10% fetal calf serum (FCS), 2 mM  
379 glutamine, and antibiotics. The cells were grown at 37°C in 5% CO<sub>2</sub>, and transfected using  
380 Lipofectamine 2000 (Invitrogen) as described by the manufacturer.

381

### 382 ***Minigenome replication assay***

383 BSRT7/5 cells at 90% confluence in 48-well dishes were transfected using Lipofectamine 2000  
384 (Invitrogen) with a plasmid mixture containing 0.125 µg of pGaussia/Firefly minigenome, 0.125 µg of  
385 pN, 0.125 µg of pP (WT and mutants), 0.06 µg of pL, 0.03 µg of pM2-1, as well as 0.03 µg of pSV-β-  
386 Gal (Promega) to normalize transfection efficiencies. Cells were harvested at 24 h post-transfection  
387 and lysed in Firefly lysis buffer (30 mM Tris [pH 7.9], 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (DTT), 1%  
388 [vol/vol] Triton X-100, and 15% [vol/vol] glycerol). The Firefly luciferase activity was determined for  
389 each cell lysate with an Infinite 200 Pro (Tecan, Männedorf, Switzerland) and normalized based on β-  
390 galactosidase (β-Gal) expression. Transfections were done in triplicate, and each independent  
391 transfection experiment was performed three times. The significance of the differences between wild  
392 type condition and mutants was determined using Student's t-test (GraphPad Prism software).  
393 Significance is represented: \*\*\*p < 0.001; \*\*\*\*p < 0.0001; ns, not significant. For proteins expression  
394 analysis, cells were lysed in Laemmli buffer and analyzed by Western blotting (WB) using anti-N, anti-  
395 P, and anti-tubulin antibodies according to standard protocols.

396

### 397 ***Fluorescence microscopy***

398 Immunofluorescence microscopy was performed with cells grown on coverslips and co-transfected  
399 with either pP (WT or mutant) and pN and pGFP-N (ratio pN:pGFP-N of 2:1), or pP-BFP and pN (WT  
400 or mutants). At 24 h post transfection, cells were fixed with 4% paraformaldehyde (PFA) for 25 min,  
401 made permeable, and blocked for 30 min with PBS containing 0.1% Triton X-100 and 3% bovine  
402 serum albumin (BSA). Cells were then successively incubated for 1 h at room temperature with  
403 primary (rabbit anti-N or rabbit anti-P) and secondary antibodies mixtures diluted in PBS containing  
404 0.3% BSA. For nucleus labeling, cells were exposed to Hoechst 33342 stain (Invitrogen) during  
405 incubation with secondary antibodies. Coverslips were mounted with ProLong Gold antifade reagent  
406 (Invitrogen) and observed with a Nikon TE200 microscope equipped with a CoolSNAP ES2



407 (Photometrics, Tucson, USA) camera. Images were processed with ZEN software (Zeiss) and ImageJ  
408 software.

409

#### 410 ***Expression and purification of recombinant proteins***

411 *E. coli* BL21 bacteria (DE3) (Novagen) transformed with pGEX-P plasmids (WT or mutant) or pET-  
412 N<sub>NTD</sub> were grown at 37°C for 8 hours in 100 ml of Luria Bertani (LB) medium containing 100µg/ml  
413 ampicillin or 50µg/ml kanamycin, respectively. Bacteria transformed with pET-N-derived plasmids  
414 together with pGEX-P derived plasmids were grown in LB medium containing ampicillin (100 µg/ml)  
415 and kanamycin (50 µg/ml). The same volume of LB was then added and protein expression was  
416 induced by adding 80µg/ml isopropyl-β-D-thio-galactoside (IPTG) to the medium. The bacteria were  
417 incubated for 15 hours at 28°C and then harvested by centrifugation. For purification using the GST-  
418 tag, bacteria were re-suspended in lysis buffer (50 mM Tris-HCl pH 7.8, 60 mM NaCl, 1 mM EDTA, 2  
419 mM DTT, 0.2% Triton X-100, 1 mg/ml lysozyme) supplemented with complete protease inhibitor  
420 cocktail (Roche), incubated for 1 hour on ice, sonicated, and centrifuged at 4°C for 30 min at 10,000g.  
421 Glutathione-Sepharose 4B beads (GE Healthcare) were added to clarified supernatants and incubated  
422 at 4°C for 3 hours. Beads were then washed two times in lysis buffer and three times in PBS 1X, then  
423 stored at 4°C in an equal volume of PBS. To isolate GST-free P fragments or N rings, beads  
424 containing bound proteins were incubated with thrombin (Novagen) overnight at 20°C. Purified  
425 recombinant N proteins were loaded onto a Superdex 200 16/30 column (GE Healthcare) and eluted  
426 in 20 mM Tris/HCl pH 8.5, 150 mM NaCl.

427 For purification of 6xHis-N and N<sub>NTD</sub>-6xHis fusion proteins purification, bacterial pellets were re-  
428 suspended in lysis buffer (20 mM Tris-HCl pH8, 500 mM NaCl, 0.1% TritonX-100, 10 mM imidazole, 1  
429 mg/ml lysozyme) supplemented with complete protease inhibitor cocktail (Roche). After sonication and  
430 centrifugation, lysates were incubated 30 min with chelating Sepharose Fast Flow beads charged with  
431 Ni<sup>2+</sup> (GE Healthcare). Beads were then successively washed in the washing buffer (20 mM Tris-HCl,  
432 pH 8, 500 mM NaCl) containing increasing concentration of imidazole (25, 50, and 100 mM). For SDS-  
433 PAGE analysis of proteins on beads, samples were prepared in Laemmli buffer, denatured 5 min at 95  
434 °C, separated on 10% polyacrylamide gel and detected by Coomassie brilliant blue.

435

#### 436 ***Dynamic light scattering (DLS)***



437 Size measurement of purified N oligomers was performed at 20 °C using a helium-neon laser  
438 wavelength of 633 nm and detection angle of 173° with a Zetasizer Nano (Malvern). Ten  
439 measurements were made, with an acquisition time of 10 s for each measurement. Hydrodynamic  
440 diameters ( $D_h$ ) were calculated using the DLS software provided by the instrument manufacturer. The  
441 results were presented as size distribution (nm).

442

#### 443 ***Negative stain electron microscopy observations of recombinant nucleoproteins***

444 Three microliters of sample were applied to the clean side of carbon on a carbon–mica interface and  
445 stained with 2% sodium silicotungstate. Micrographs were recorded on a FEI Tecnai T12 microscope  
446 operated at 120 kV with a Gatan Orius 1000 camera. Images were recorded at a nominal  
447 magnification of 23 000 × resulting in a pixel size of 2.8 Å.

448

#### 449 ***Molecular modeling***

450 In the X-ray structures of HMPV N-RNA rings (PDB 5fvc) or N<sup>0</sup>-P (PDB 5fvd), the N<sub>NTD</sub> residues L110  
451 to M113, corresponding to a long beta-hairpin close to the PCT binding site, are not modeled. We  
452 used Modeller (26) to first construct a complete model HMPV N<sub>NTD</sub> (residues 32 to 251) using as  
453 templates the X-ray structures of HMPV N-RNA rings (residues 32 to 109 and 114 to 251, PDB) and  
454 RSV N<sub>NTD</sub> (PDB 4uc6) for the missing beta-hairpin (residues 110 to 113). Next, using Modeller again,  
455 we generated 100 models of HMPV N<sub>NTD</sub> complexed with the last six residues of P (peptide I289-  
456 YQLI-M294 called P6) using the RSV N<sub>NTD</sub> structure in complex with the last two residues of RSV P  
457 (PDB 4uc9), *i.e.* D240-F241 (called P2), as template for HMPV P6 by aligning the last 2 residues of  
458 HMPV-P and RSV-P. To compensate for possible alternate conformations of exposed residues in apo  
459 and holo-form of N<sub>NTD</sub>, no template was used for R132 and R151 side-chains. Next, P6-N<sub>NTD</sub> models  
460 where M294 side-chain of P is pointing towards the N<sub>NTD</sub> hydrophobic pocket (by analogy with the  
461 binding of F241 of P on N RSV) were selected, to obtain an ensemble 41 P6-N<sub>NTD</sub> models. Finally, the  
462 models were submitted to a water refinement procedure using the refinement interface of the  
463 HADDOCK2.2 server (27). Each input P6-N<sub>NTD</sub> complex model was refined 20 times to obtain 820  
464 refined models. The best 400 models with the lowest HADDOCK-score were clustered based on  
465 similarity of the L292-I293-M294 residues of P, called P3 (I289-Y290-Q291 were not considered for  
466 clustering since they appear very floppy in the refined models and had no structural templates during

467 homology modeling). Models were first superposed on their  $N_{\text{NTD}}$  backbone atoms and the RMSD was  
468 computed on the backbone atoms of L292-I293-M294 of P. Clustering was performed using the  
469 HADDOCK tool *cluster\_struc* (28) with a 2Å cut-off and a minimal cluster size of 10.

470 In addition, the 820 P6- $N_{\text{NTD}}$  conformations generated by HADDOCK were profiled using InDeep  
471 software (20) in order to select the most suitable  $N_{\text{NTD}}$  conformations to bind P3. InDeep is a deep-  
472 learning model based on a FCN (Fully Convolutional Network) trained from known structures of  
473 protein complexes capable of predicting protein-protein interaction interfaces  
474 (<https://ippidb.pasteur.fr/targetcentric/>) (20). Each  $N_{\text{NTD}}$  pocket conformation is placed within a 3D grid  
475 composed of 1 Å<sup>3</sup> voxels. The model computes a probability value for each voxel representing its  
476 interaction propensity. The predictions were performed on the putative P6 binding site on  $N_{\text{NTD}}$   
477 surface. An “interactability-score” of the pocket is computed by taking the mean of the 150 best voxels  
478 probabilities. This value represents the binding propensity of a given  $N_{\text{NTD}}$  pocket conformation. Then,  
479 on the 200-top conformations with the highest “interactability-score” the hydrophobic channel of  
480 InDeep was applied in order to locate the most probable hydrophobic patches on the  $N_{\text{NTD}}$  pocket  
481 where P3 hydrophobic side chains could interact. In addition, once a set most promising  
482 conformations was identified, a virtual alanine scanning was performed using the same tool but by  
483 mutating one by one the residues surrounding the patch of interaction of P3. The results are provided  
484 as a set of correlations, one for each residue, highlighting the impact of the simulated mutation on the  
485 interactability of the  $N_{\text{NTD}}$ .

486

487

488

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497

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499 H.D., M.G. and J.-F.E. designed experiments. H.D., M.B. performed molecular, and cellular assays.

500 B.B., L.C.R. and O.S. performed molecular simulations. I.G performed electron microscopy

501 observations. C.-A.R. performed gel filtration and *in vitro* study. M.G. wrote the paper with

502 contributions from all authors, and J.-F.E. and M.G. edited the manuscript. All authors commented on

503 the manuscript.

504

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- 606
- 607
- 608

609 **Figure legends**

610

611 **Figure 1:** Purification of HMPV N-RNA rings using GST-PCT. A/ Schematic representation of HMPV  
612 and RSV P proteins. The oligomerization domain is represented by a rectangle and disordered N- and  
613 C-terminal domains (PNT and PCT respectively) by lines. PCT is indicated. Residues flanking the  
614 structural domains are indicated by numbers. B/ GST-PCT and N proteins were co-expressed in *E.*  
615 *coli*, followed by purification using the GST tag. The product of purification was analyzed by SDS-  
616 PAGE and Coomassie blue staining (left lane). The sample was then incubated in the presence of  
617 thrombin in order to cleave GST (remaining on beads) and isolate PCT-N in the supernatant (right  
618 lane), C/ Gel filtration profile of purified PCT-N complex. The curves corresponding to OD spectra at  
619 220 nm, 260 nm, and 280 nm are presented. P1 and P2 indicate the two peaks detected. The  
620 fractions corresponding to P1 were pooled and the sample was analyzed by SDS-PAGE colored with  
621 Coomassie blue, D/ Dynamic light scattering (DLS) analysis of the purified N protein, showing a  
622 homogenous peak at 18 nm, corresponding to N oligomers. E/ Image of purified HMPV N-RNA rings  
623 as observed by negative stain electron microscopy. Bar, 100 nm.

624

625 **Figure 2:** Identification of the residues of P involved in N binding. A/ Sequence alignment of  
626 *Pneumoviridae* P C-terminus (PCT), from the Orthopneumovirus and Metapneumovirus genus: hRSV-  
627 A/-B (human RSV strains A and B, Uniprot entries PHOSP\_HRSVA and PHOSP\_HRSVB,  
628 respectively), oRSV (ovine RSV, Uniprot entry PHOSP\_ORSVW), bRSV (bovine RSV, Uniprot entry  
629 PHOSP\_BRSPA), mPV (murine pneumonia virus, Uniprot entry PHOSP\_MPV15), aMPV-A/-C (avian  
630 metapneumovirus type A and C, Uniprot entries Q65032 and PHOSP\_AMPV1, respectively) and  
631 hMPV (human metapneumovirus strain CAN97-83, Uniprot entry PHOSP\_HMPVC). Sequence  
632 numbering and secondary structures are indicated on top for hRSV and at the bottom for hMPV. B/  
633 GST-P fragments and C/ GST-P[285-294] (WT or mutants) (sequences indicated on the left) were co-  
634 expressed with N in bacteria, followed by purification using the GST tag. The products of purification  
635 were analyzed by SDS-PAGE and Coomassie blue staining (right). D/ GST-PCT or GST-PCT<sub>ALAM</sub>  
636 corresponding to the double substitution of residues Q291 and I293 residues of P by alanine were co-  
637 expressed with N. The products of purification by GST were analyzed by SDS-PAGE and Coomassie  
638 blue staining. E/ Sequence alignment of the 9 last C-terminal residues of HMPV and RSV P proteins.

639 The residues critical for the interaction with the N protein are indicated in red bold. F/ HMPV and RSV  
640 GST-PCT (residues P[200-294] for HMPV and P[161-241] for RSV) constructs were co-expressed  
641 with either HMPV or RSV N proteins. Copurification of N proteins by GST-PCT fragment was analyzed  
642 by SDS-PAGE and Coomassie blue staining

643

644 **Figure 3:** Search for the PCT binding site on HMPV N. A/ Amino acid sequence alignments between  
645 the N-terminal domains of N (residues 1-252/253) proteins of HMPV (strain CAN 97-83) and human  
646 RSV (HRSV, strain Long VR-26). Invariant residues are highlighted in white font on a red background.  
647 The secondary structure elements observed in the crystal structure of HMPV N protein (11) are  
648 indicated above the sequence. Green asterisks below the sequence indicate the residues constituting  
649 the P binding pocket of RSV N. Uniprot accession codes: NCAP\_HRSVA (Human RSV);  
650 A1DZS3\_9MONO (Human MPV). Sequences were aligned with Clustal W and treated with ESPrpt 3.  
651 B/ Side view (left) and bottom view (right) of the HMPV N-RNA rings (PDB accession number 5fvc)  
652 showing one N monomer coloured according to domains: N-arm in blue, C-arm in green, and N- and  
653 C-terminal domains ( $N_{\text{NTD}}$  and  $N_{\text{CTD}}$ , respectively) are in yellow and red. The residues identified by  
654 analogy with RSV as involved in P binding on  $N_{\text{NTD}}$  are indicated by black spheres.

655

656 **Figure 4:** Identification of the residues of  $N_{\text{NTD}}$  involved in P binding. A/ GST-P or GST- $P_{\Delta M294}$  and  
657  $N_{\text{NTD}}$ -6xHis proteins were co-expressed in *E. coli*, followed by purification using the 6xHis tag. GST-P  
658 or GST- $P_{\Delta M294}$  alone were purified using the GST-tag. The products of purification were analyzed by  
659 SDS-PAGE and Coomassie blue staining, B/ Surface representation of HMPV  $N_{\text{NTD}}$  (from Gly30 to Gly  
660 253, indicated in green) showing the potential P binding pocket, with acidic amino acids colored in red,  
661 basic residues in blue, and hydrophobic residues in orange, C/ The impact of N mutations on the  
662 expression and solubility of the protein was assessed by expressing 6xHis-N protein (WT or mutants)  
663 in *E. coli*, followed by purification and SDS-PAGE analysis. D/ GST-PCT and mutant N proteins were  
664 co-expressed in *E. coli*, followed by purification using the GST tag. The products of purification were  
665 analyzed by SDS-PAGE and Coomassie blue staining (upper panel), and the expression of N proteins  
666 in bacteria was validated by western blot (lower panel).

667

668 **Figure 5:** The residues involved in P-N-RNA rings interaction are critical for the polymerase activity. A/  
669 HMPV polymerase activity in the presence of P and N mutants. BSRT7/5 cells were transfected with



670 plasmids encoding the P (WT or mutants), N (WT, or mutants), L, and M2-1 proteins, the  
671 pGaussia/Firefly minigenome, together with pCMV- $\beta$ Gal for transfection standardization. Viral RNA  
672 synthesis was quantified by measuring the Gaussia luciferase activity after cell lysis 24 h after  
673 transfection. Each luciferase minigenome activity value was normalized based on  $\beta$ -galactosidase  
674 expression and is the average of three independent experiments performed in triplicate. Error bars  
675 represent standard deviations calculated based on two independent experiments made in  
676 quadruplicate. B/ Western blot showing the expression of P and N proteins (WT and mutants) in  
677 BSRT7/5 cells. C and D/ HMPV polymerase activity in the presence of pN:pGFP-N at a ratio 2:1, or P-  
678 BFP, respectively. Error bars represent standard deviations calculated based on two independent  
679 experiments made in triplicate. The statistical significance of differences was calculated using  
680 Student's *t* test. \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.001$ ; ns, not significant.

681

682 **Figure 6:** Impact of P mutations on the formation of HMPV cytoplasmic IBs. BSRT7/5 cells transfected  
683 with plasmids encoding for P (WT or mutants) and N + GFP-N (ratio 2:1) proteins were fixed 24 h  
684 post-transfection, labeled with anti-P antibody, and the distribution of viral proteins was observed by  
685 fluorescence microscopy. Nuclei were stained with Hoechst 33342. Scale bars, 10  $\mu$ m.

686

687 **Figure 7:** Impact of N mutations on the formation of HMPV cytoplasmic IBs. BSRT7/5 cells  
688 transfected with plasmids encoding for P-BFP and N (WT or mutants) proteins were fixed 24 h post-  
689 transfection, labeled with anti-N antibody, and the distribution of viral proteins was observed by  
690 fluorescence microscopy. Scale bars, 10  $\mu$ m.

691

692 **Figure 8:** Model of HMPV P3/N<sub>NTD</sub> complex from HADDOCK refinement. A/ The N<sub>NTD</sub> protein is  
693 colored by electrostatic surface and the P3 peptide (residues L292-I293-M294 of P) is shown as  
694 sticks. The residues of N<sub>NTD</sub> critical for the interaction with P are indicated, with residues for which  
695 mutation to alanine abrogate P binding are labeled in bold. Yellow Surface shows InDeep hydrophobic  
696 channel prediction where hydrophobic contacts are expected to occur at the N<sub>NTD</sub> surface. B/  
697 Interaction diagram between P3 peptide (Leu292-Ile293-Met294) and N<sub>NTD</sub>. Legend indicated below  
698 the diagram.

699



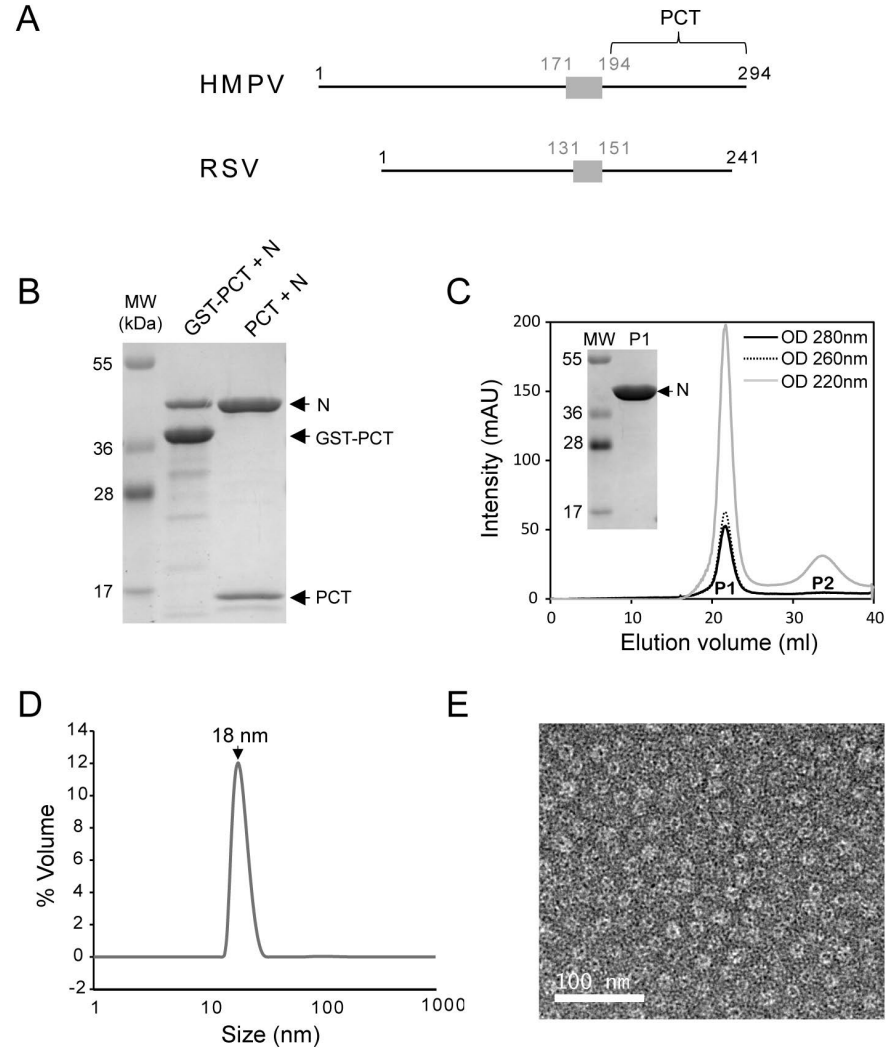
700 **Figure 9.** 10 best-scoring structures of clusters 1 (A) and 2 (B) from HADDOCK refinement of HMPV  
701 P3 (residues L292-I293-M294 of P)/N<sub>NTD</sub> complex superimposed with RSV P2/N<sub>NTD</sub>. N<sub>NTD</sub> is colored in  
702 beige (HMPV) or yellow (RSV), and HMPV P3 peptide in cyan (HMPV) or RSV P2 peptide  
703 (corresponding to residues D240-F241 of P) in pink. Side-chains of residues in contact with the P  
704 peptide in HADDOCK models are also shown, along with their RSV equivalent.

705

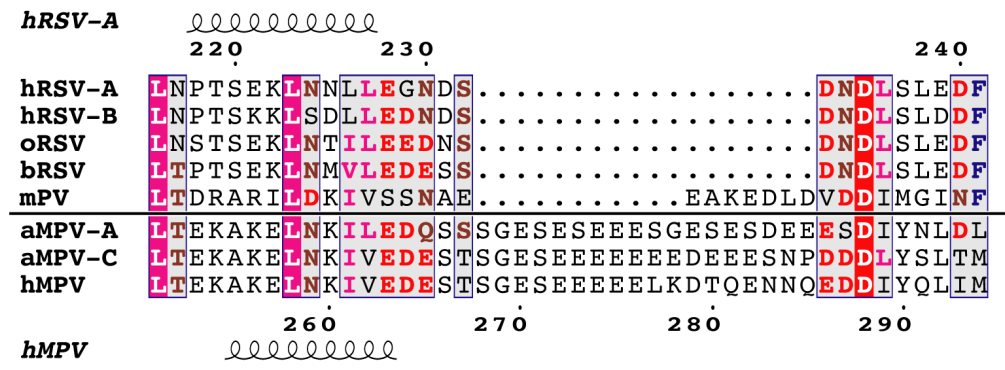
706 **Figure 10.** Conservation of pneumoviruses N and P residues. A/ Sequence logo of the last 9 C-  
707 terminal residues of P of Metapneumovirus (left) and Orthopneumovirus (right) sequences. P  
708 sequences were collected from the Interpro entry IPR003487 (464 unique sequences) and logo were  
709 created from non-redundant peptides sequences of the last 9 amino acids. B/ Sequence logo of the  
710 residues forming the P-binding site on N of the Metapneumovirus (left) and Orthopneumovirus (right)  
711 genus. N sequences were collected from the Interpro entry IPR004930 (359 unique sequences) and  
712 aligned. Classification as Ortho- and Metapneumovirus was based on Uniprot taxonomy (taxon ids  
713 1868215 for OrthoPV and 162387 for MetaPV). Residue numbers are shown at the bottom, and grey  
714 markers indicate residues identified as essential for P-N interaction. Logo were generated with  
715 WebLogo (29).

716

717

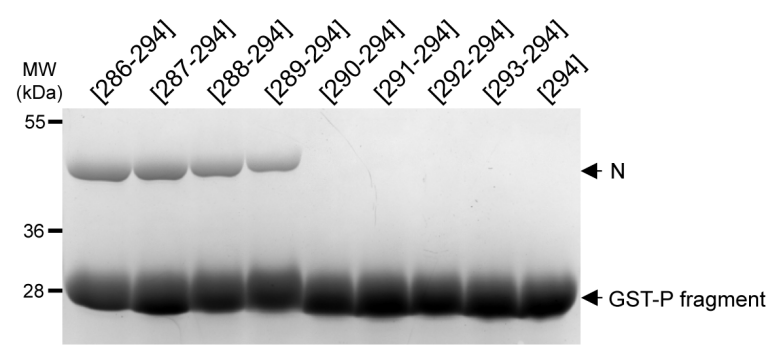


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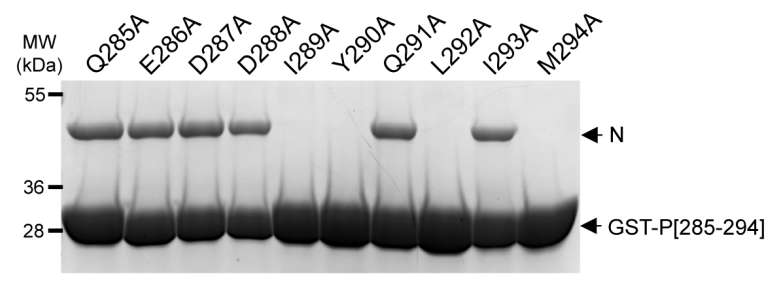
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P segment	Sequence	Interaction with N
P [286-294]	EDDIYQLIM	+
P [287-294]	DDIYQLIM	+
P [288-294]	DIYQLIM	+
P [289-294]	IYQLIM	+
P [290-294]	YQLIM	-
P [291-294]	QLIM	-
P [292-294]	LIM	-
P [293-294]	IM	-
P [294]	M	-

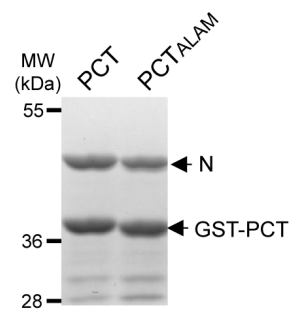


C

P segment	Sequence	Interaction with N
<b>P[285-294]</b>	<b>QEDDIYQLIM</b>	
Q285A	A - - - - -	+
E286A	- A - - - - -	+
D287A	- - A - - - -	+
D288A	- - - A - - -	+
I289A	- - - - A - - -	-
Y290A	- - - - - A - -	-
Q291A	- - - - - A - - -	+
L292A	- - - - - A - -	-
I293A	- - - - - A -	+
M294A	- - - - - A	-



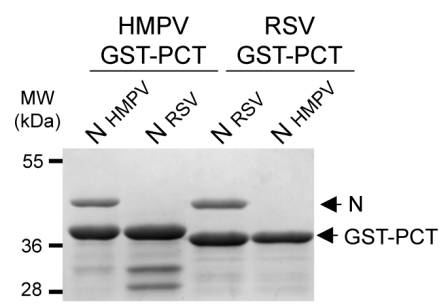
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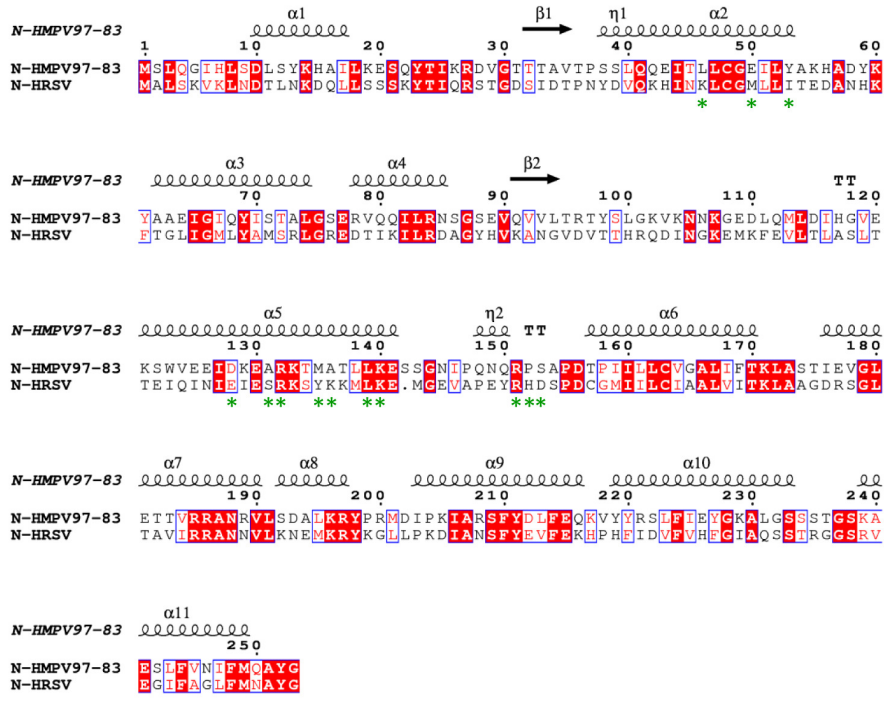
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HMPV EDDIYQLIM  
RSV DNDLSLED F

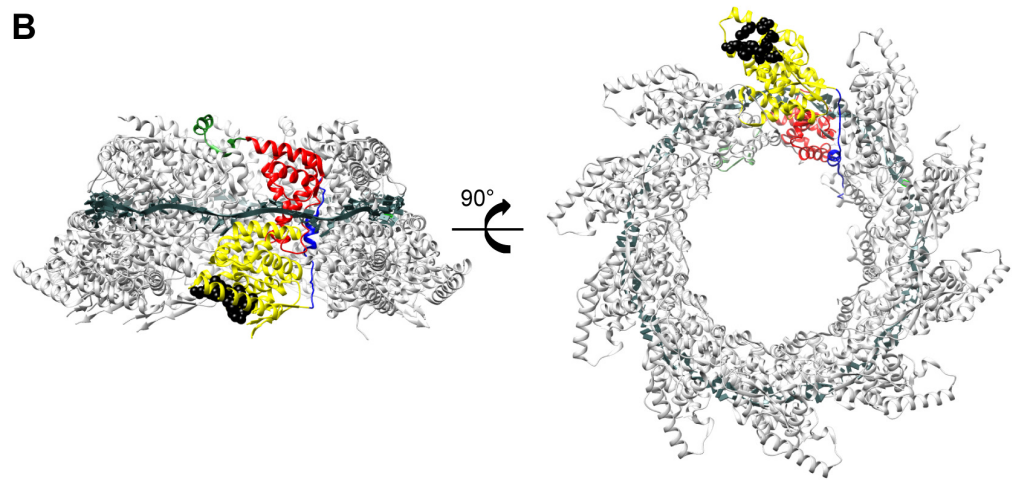
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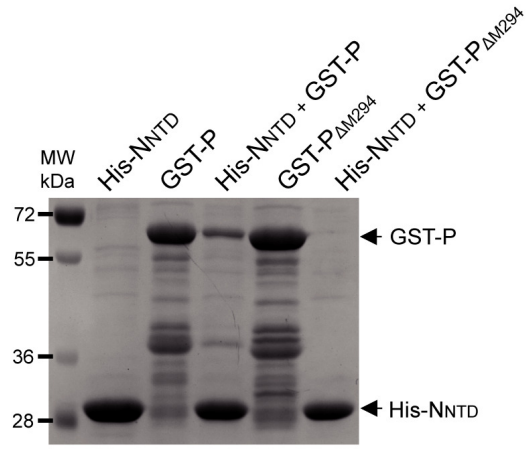
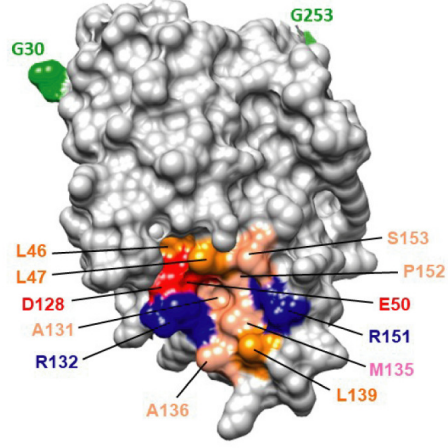
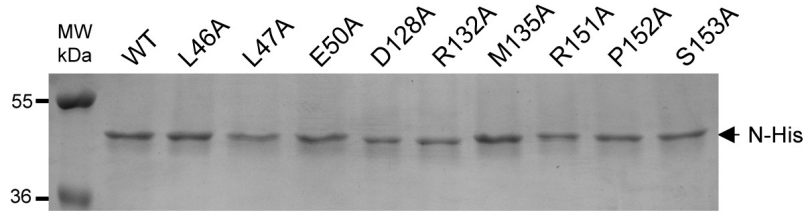
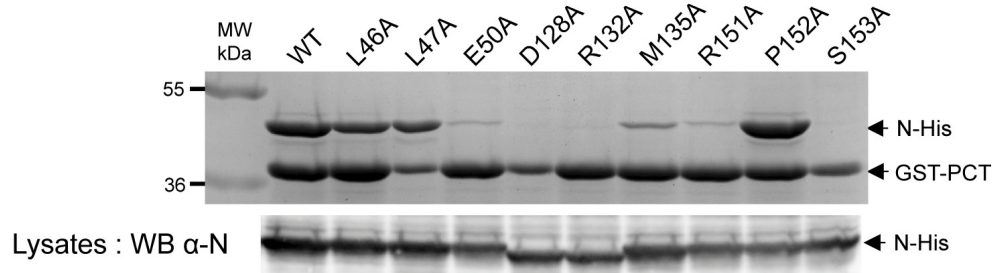


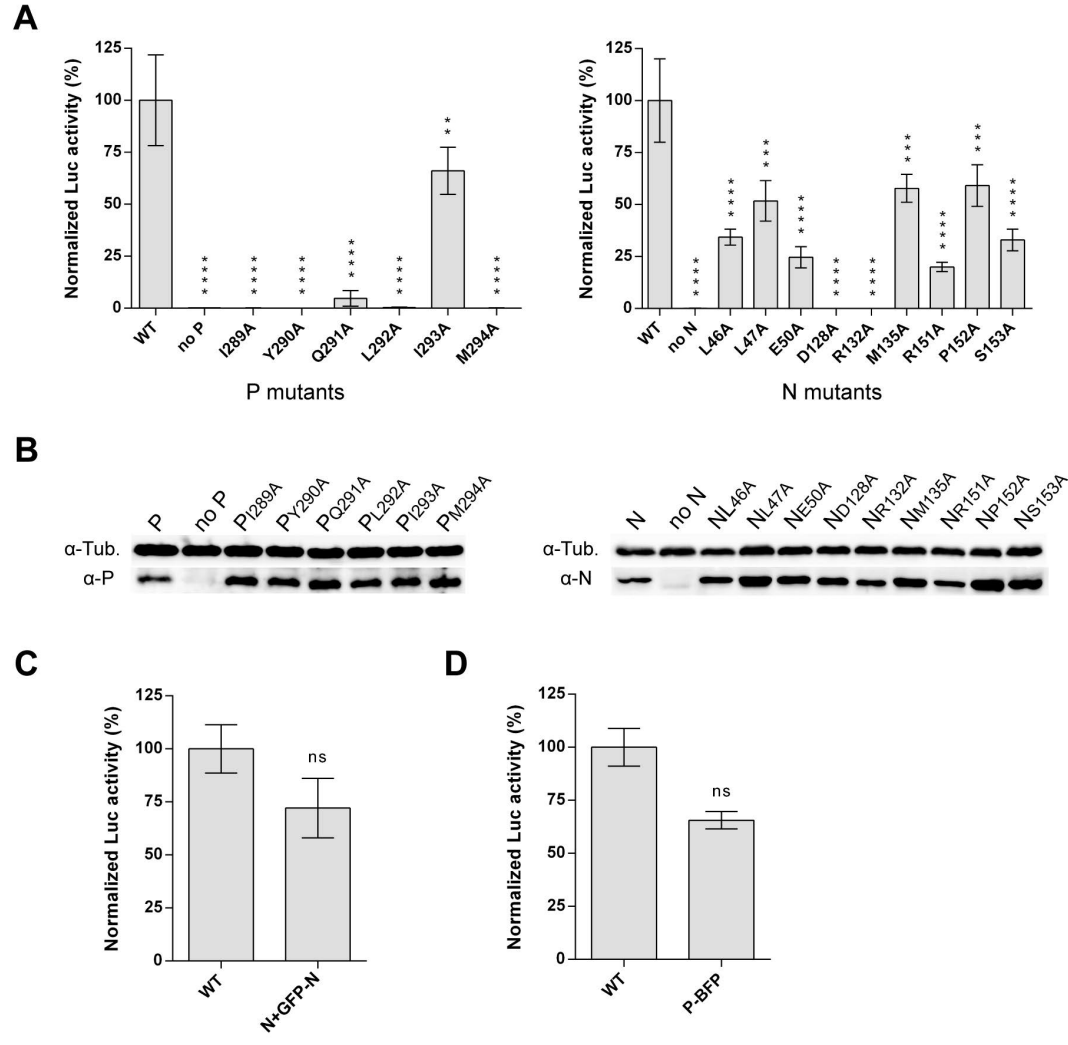
**A**



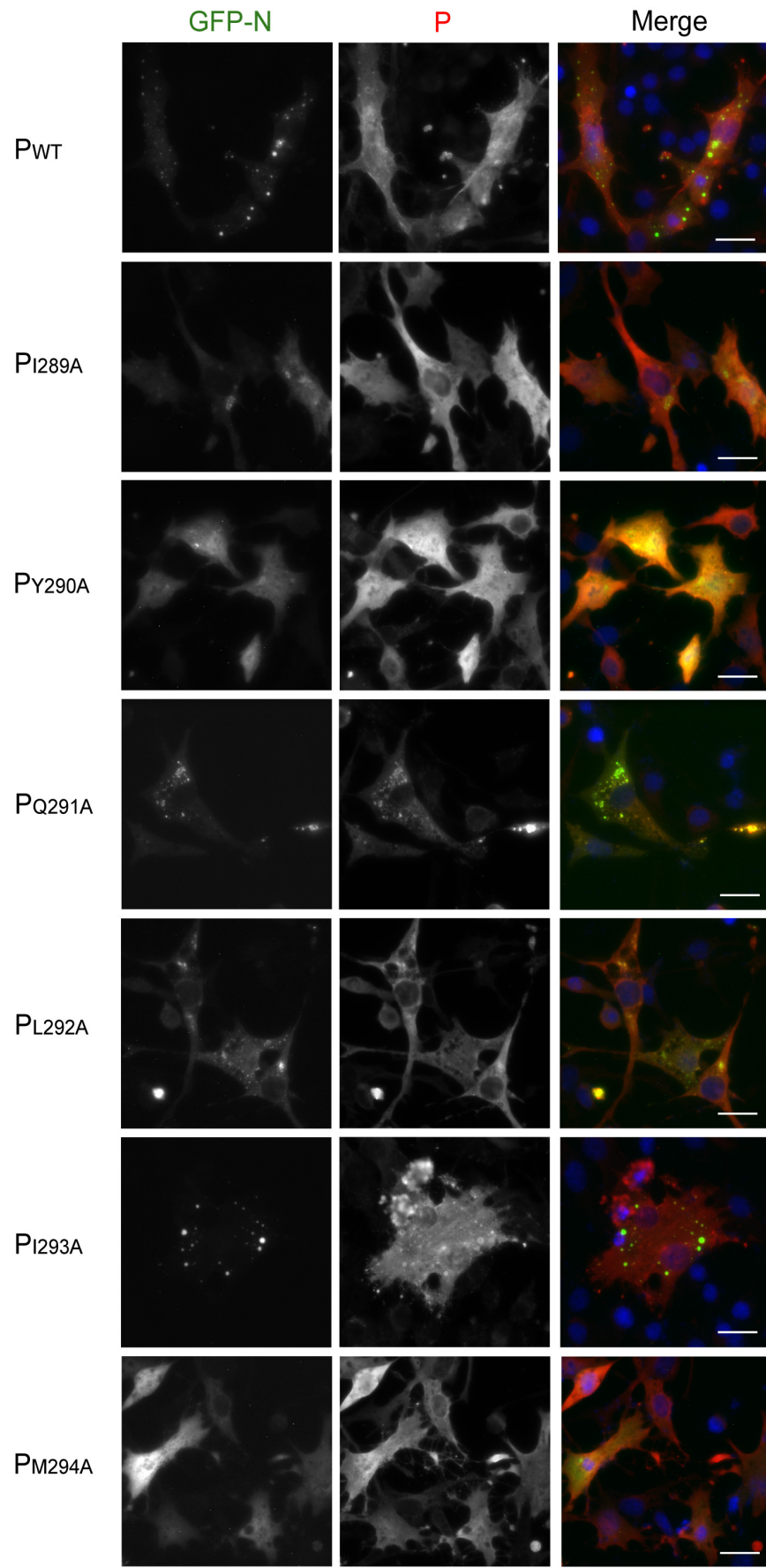
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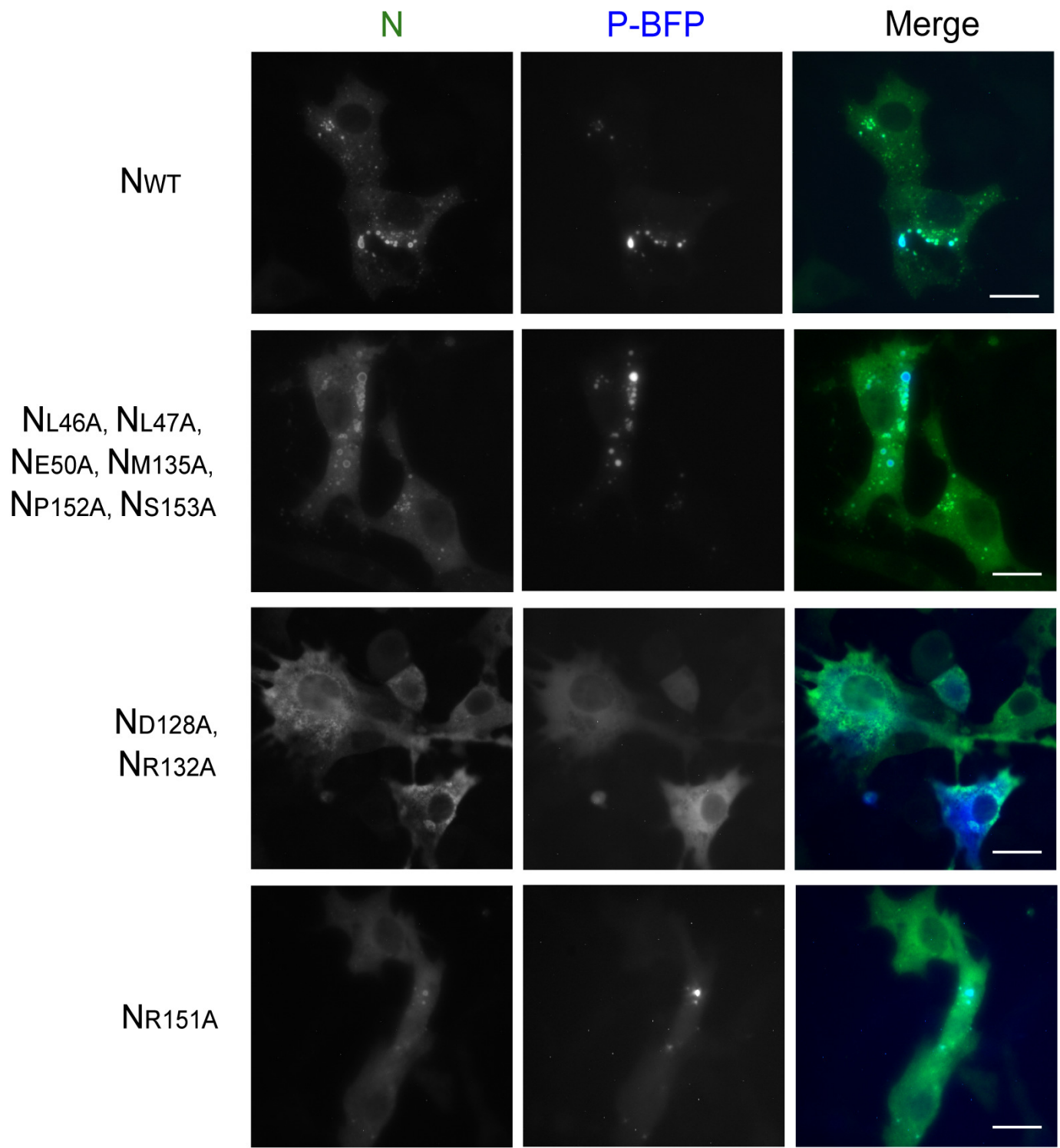


**A****B****C****D**









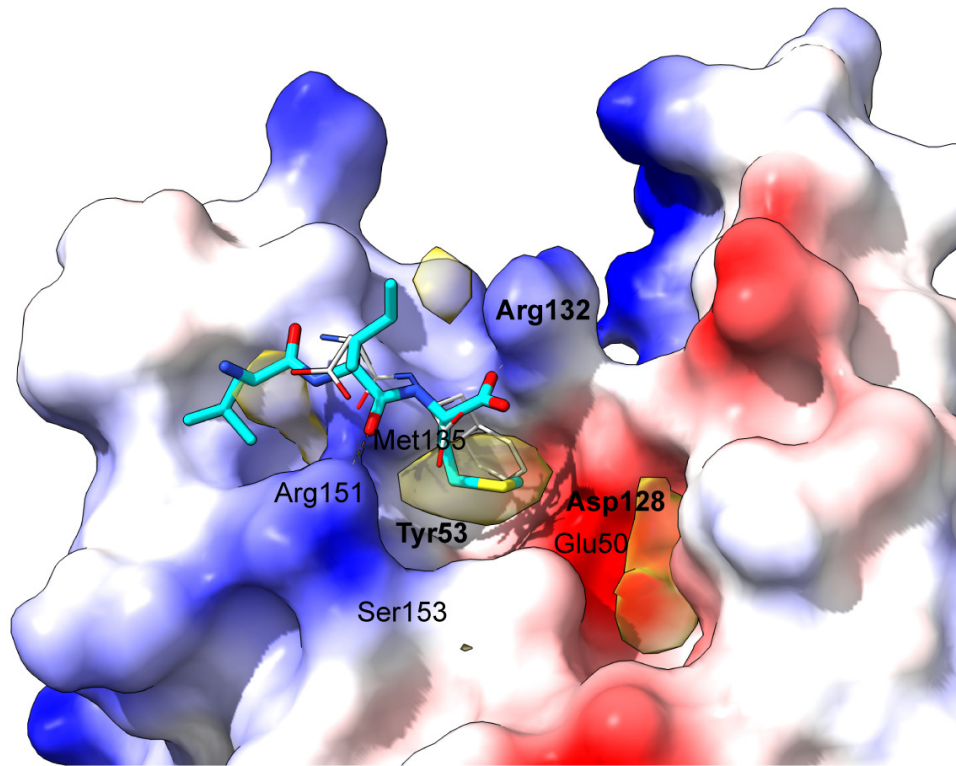


**Table 1.** Characterization of P and N mutants

	Mutation	Pulldown of N by GST-PCT	Polymerase activity *	Observation of IBs
<b>P mutants</b>	I289A	-	-	-
	Y290A	-	-	-
	Q291A	+	5%	+/-
	L292A	-	-	-
	I293A	+	65%	+
	M294A	-	-	-
<b>N mutants</b>	L46A	+	35%	+
	L47A	+	50%	+
	E50A	+/-	25%	+
	D128	-	-	-
	R132A	-	-	-
	M135A	+/-	60%	+
	R151A	+/-	20%	+/-
	P152A	+	60%	+
	S153A	-	30%	+

\* Values correspond to rounded-up averages ; -, no polymerase activity.

A



B

