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# 1 Structural landscape of the Respiratory Syncytial Virus nucleocapsids

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## 9 Abstract

10 Human Respiratory Syncytial Virus (RSV) is a prevalent cause of severe respiratory infections in  
11 children and the elderly. The viral genome, enwrapped by the nucleoprotein N into a helical  
12 nucleocapsid (NC), is a template for the viral RNA synthesis and a scaffold for the virion assembly.  
13 Although the structures of NC filaments representative of the other major families of the  
14 *Mononegavirales* order have been solved, a detailed understanding of the RSV NCs is missing.  
15 This cryo-electron microscopy (cryo-EM) analysis highlights the polymorphism of the RSV  
16 nucleocapsid-like assemblies. We reveal in particular the non-canonical arrangement of the RSV  
17 NC helix, composed of 16 N per asymmetric unit, and the resulting systematic variations in the  
18 RNA accessibility. We demonstrate that this unique helical symmetry originates from recurring  
19 longitudinal interactions by the C-terminal arm of the RSV N, whose truncation abrogates the inter-  
20 turn contacts. We report the cryo-EM structures of the full-length helical NC filaments, double-  
21 headed NCs, ring-capped NCs and double-decameric N-RNA rings, as well as those of the  
22 alternative assemblies formed by a C-terminally truncated N mutant. In addition, we demonstrate  
23 the functional importance of the interface involved in the formation of the double-headed and the  
24 ring-capped interactions. We put all these findings in the context of the RSV RNA synthesis  
25 machinery and delineate the structural basis for its further investigation.

## 26 Introduction

27 Human respiratory syncytial virus (RSV) is the most frequent cause of bronchiolitis and  
28 pneumonia in infants and a major cause of childhood death in low-income settings <sup>1,2</sup>. Reinfection  
29 can occur throughout life and is often serious in elderly and immunocompromised. Yet, RSV  
30 remains one of the only major etiological agents of the lower respiratory tract infections-related  
31 mortality for which no licensed vaccine is yet available, with treatment limited to supportive care.  
32 Development of effective therapeutics requires a better understanding of the RSV synthesis  
33 machinery. RSV belongs to the *Mononegavirales* order with the non-segmented negative strand  
34 RNA genome fully coated by the viral nucleoprotein N. The resulting helical nucleocapsid (NC)  
35 shields the viral genetic material from recognition by the innate immune system while serving as  
36 template for replication and transcription by the viral RNA polymerase complex, thereby  
37 constituting a potential drug target.

38 Alongside RSV and human Metapneumovirus (hMPV), belonging to the *Pneumoviridae*  
39 family, *Mononegavirales* contains other important human pathogens such as the *Rhabdoviridae*

40 rabies, the *Filoviridae* Ebola (EV) and Marburg (MaV), and the *Paramyxoviridae* measles (MeV),  
41 mumps (MuV) and Nipah (NiV) viruses. *Pneumoviridae* are equally distant to *Paramyxo-* and  
42 *Filoviridae*<sup>3</sup>. In particular, as far as the NCs are concerned (i) each paramyxo- and filoviral N binds  
43 precisely 6 nucleotides, whereas pneumoviral N binds 7<sup>4</sup>; (ii) the genome size of paramyxo- but  
44 not pneumo- and filoviruses is a strict multiple of 6 nucleotides; (iii) paramyxo- and filo- but not  
45 pneumoviral genomes require bipartite promoters separated by an exact multiple of 6 nucleotides  
46<sup>5</sup>; (iv) paramyxo- and filoviral N possess a very long C-terminal extension involved in replication  
47 and transcription, whereas the pneumoviral N features only a short C-terminal arm (*i.e.* the length  
48 of MeV N is 525, EV NP 739 and RSV N 391 amino acids respectively)<sup>6</sup>. Removal of the C-  
49 terminal extension rigidifies and condenses the helical paramyxo- and filoviral NCs by  
50 strengthening the contacts between successive turns, thus facilitating their structural analysis by  
51 cryo-electron microscopy (cryo-EM) and tomography (cryo-ET).

52 Despite a recent massive increase in the number of medium and high resolution cryo-EM  
53 structures of the helical paramyxo- and filoviral NCs<sup>7-16</sup>, a detailed cryo-EM characterisation of the  
54 pneumoviral NCs is still lacking. Here we present an exhaustive cryo-EM analysis of the structural  
55 landscape of RSV NCs in solution. We reveal in particular the non-canonical helical symmetry of  
56 the RSV NC, with 16 nucleoproteins per asymmetric unit, and demonstrate that this unique  
57 organisation results from inter-turn interactions by the C-terminal arm of N and leads to periodic  
58 variations in the RNA accessibility along the NC filament.

## 59 Results

### 60 RSV nucleocapsids are flexible and polymorphic

61 The current structural information about RSV NCs comes mostly from the 3.3 Å resolution  
62 X-ray crystal structure of decameric N-RNA rings<sup>4</sup> (N<sub>10</sub> ring, PDB: 2WJ8), a negative stain electron  
63 tomography analysis of purified helical NCs<sup>17</sup> and two cryo-ET studies of the RSV virion<sup>18,19</sup>. Our  
64 cryo-EM images of recombinant RSV N purified from insect cells displayed a polymorphic  
65 ensemble in which ring-like particles and filaments could be distinguished and classified (Figure  
66 1a, b). A map of a bottom-to-bottom assembly of two decameric N-RNA rings, termed N<sub>10</sub> double  
67 ring, was derived from the ring-like classes. In parallel, the filaments were split into sets of classes  
68 showing either continuous or discontinuous course. The former were used for 3D reconstruction of  
69 a helical NC and its ~1.5-turn subsection, whereas the latter yielded reconstructions of a double-  
70 headed NC and a ring-capped NC. Thus, five different 3D maps - a double ring, a helical NC and  
71 its short subsection, a double-headed NC and a ring-capped NC - were obtained from the same  
72 data set (Figure 1c-g; Supplementary Figure 1).

### 73 Unique tripartite stabilisation of the RSV N oligomerisation inside a conserved N-hole

74 The 2.86 Å resolution map and the resulting atomic model of the N<sub>10</sub> double ring show that  
75 the N protomer and the entire N<sub>10</sub> ring are identical to the crystal structure, with 0.5-Å RMSD over  
76 378 backbone residues and the density of the last twelve residues (380-391) largely disordered.  
77 Accordingly, the RNA binding groove formed by the interface between the N-terminal and the C-  
78 terminal domains of N (NTD and CTD), and the “4-bases-in, 3-bases-out” RNA conformation  
79 remain unaltered.

80 Similarly to other *Mononegavirales*<sup>20,21</sup>, the N- and C-terminal extensions of RSV N, termed  
81 NTD-arm (residues 1-36) and CTD-arm (residues 360-391) (Figure 1h), interact with the laterally  
82 adjacent N protomers thereby stabilising their oligomeric assembly on the RNA strand by  
83 subdomain swapping (Figure 2a). The visible part of the CTD-arm of  $N_i$  lies on top of the CTD of  
84  $N_{i+1}$  implying that in a helical NC it should be situated in between consecutive turns<sup>4</sup>. In parallel, the  
85 NTD-arm of  $N_{i+1}$  inserts into a compact fold of the CTD of  $N_i$  from the ring interior and extends  
86 towards the CTD-arm of  $N_{i-1}$ . In this regard, a “latch-bolt type” interaction formed by an insertion of  
87 a loop from the NTD of  $N_{i-1}$  into an  $N_i$  cavity, termed N-hole, has been recently described for  
88 paramyxoviral NCs<sup>12,13,21</sup>, and is also present in filoviral NCs (Supplementary Figure 2). The  
89 structures of RSV and hMPV  $N_{10}$  rings<sup>22</sup> (PDB: 5FVC) indicate that pneumoviral NCs do actually  
90 possess a cognate N-hole formed by an Supplementary NTD-arm-proximal loop (residues 19-32 in  
91 RSV N), together with two short loops from the NTD (86-92) and the CTD (300-307). Likewise, in  
92 RSV and hMPV rings, a short loop from the NTD of  $N_{i-1}$  (residues 230-238 in RSV N) protrudes into  
93 the N-hole of  $N_i$  (Figure 2a, b), which demonstrates that the N-hole based interaction is conserved  
94 between *Paramyxo*-, *Filo*- and *Pneumoviridae* families (Supplementary Figure 2).

95 Deeper into the N-hole matter, the first atomic model of an RSV NC helix (PDB: 4BKK),  
96 derived from a crystal structure fit into a tomography-based featureless 68-Å pitch spiral,  
97 suggested a fascinating direct interaction between three consecutive protomers<sup>17</sup>. Specifically,  
98 R234 of  $N_{i-1}$  was predicted to bind both D221 of  $N_i$  and Y23 from  $N_{i+1}$ . Such a tripartite interaction  
99 between  $N_{i-1}$ ,  $N_i$  and  $N_{i+1}$  does not exist in paramyxo- and filoviral NCs, and to our knowledge has  
100 not been explicitly investigated for the hMPV  $N_{10}$  ring. Examination of our RSV double ring  
101 structure verifies the presence of the tripartite Y23-D221-R234 interaction and shows that it occurs  
102 inside the N-hole of  $N_i$  which carries D221; the loop 230-238 of  $N_{i-1}$  provides R234 while Y23 is  
103 contributed by the loop 18-32 of  $N_{i+1}$ , whereas Y23 on the equivalent loop of  $N_i$  points into the N-  
104 hole of  $N_{i-1}$  so that to bind R234 of  $N_{i-2}$  (Figure 2a). Surprisingly, despite a great resemblance  
105 between the RSV and the hMPV N-RNA rings, the latter contains no tripartite contact (Figure 2b).  
106 Indeed, although all loops are in place and Y23 is conserved, in hMPV N both D221 and R234 are  
107 replaced by serines making the interaction impossible (Figure 2c). Thus, an additional tripartite  
108 stabilisation of the “latch-bolt type” interaction seems to be a signature of the RSV NCs.

### 109 **Molecular determinants of the longitudinal NTD-NTD interaction**

110 In the double ring, the NTD-NTD stacking of two  $N_{10}$  rings, whose centers of gravity are 67  
111 Å apart, is assured by D1-symmetry-related  $\beta$ -sheets providing two opposing interacting H100  
112 residues and two R101-E122 hydrogen bonds (Figure 3). Interestingly, examination of the crystal  
113 structures of RSV and hMPV rings (Figure 3a-c; Supplementary Figure 3) reveals their bottom-to-  
114 bottom (NTD-NTD) stacking but with a tighter packing, with an inter-ring distance of 61 Å and 60 Å  
115 respectively. This compaction arises from an inter-ring rotation accompanied by a  $\beta$ -sheet insertion  
116 into inter-protomer grooves of the opposite ring (Figure 3a-c), which leads to a difference between  
117 the crystallographic inter-ring interface, based on a K91-D96 interaction, and the solution one.

118 2D classification of segments of filamentous RSV NC produced some 2D class averages  
119 featuring a clear seam, either across the filament stem or close to its end (Figure 1b). Particles with  
120 the stem-crossing seam yielded a 3.9 Å resolution map with a barbed end-to-barbed end junction  
121 of two NC helices (Figure 1e; Supplementary Figure 1), similar to the spiral clams described for  
122 *Paramyxoviridae* Sendai (SeV)<sup>13</sup>, NiV<sup>23</sup> and Newcastle disease (NDV)<sup>10</sup>. The particles with an

123 end-proximal seam gave a 3.8 Å resolution map of a helical NC capped by an N<sub>10</sub> ring (Figure 1d;  
124 Supplementary Figure 1), reminiscent of the semi-spiral clam observed for NiV NCs<sup>23</sup>.  
125 Remarkably, the mode of the longitudinal NTD-NTD interaction in the double rings, the double-  
126 headed and the ring-capped RSV NCs is conserved (Figure 3a, d, e), confirming that the interface  
127 delineated by cryo-EM is more reflective of the native structures than the crystal structure interface  
128 constrained by the crystal packing. In RSV, the NTD-NTD interface is however distinct from the  
129 one in the NiV, SeV and NDV clams, mediated by NTD loops which are absent in pneumoviral N  
130 (Supplementary Figure 4).

131 All *Mononegavirales* NCs are left-handed helices, with the CTDs and the 3'-end of the RNA  
132 oriented towards the pointed end of the filaments and the NTDs and the 5'-end towards the barbed  
133 ends<sup>20</sup>. The paramyxoviral clam-shaped assemblies were proposed to seed the growth of the  
134 double-headed helices from the 5' to the 3' end, protect the 5' end from nucleases<sup>10</sup> and support  
135 encapsidation of several NCs per virion<sup>24</sup>, also documented for RSV<sup>19,25</sup>. Thus, based on our  
136 structures, we designed two double mutants of N – H100E-R101D and H100E-E122R – and  
137 assessed their phenotypes in an RSV minigenome assay. While the first construct behaved  
138 similarly to the wild type N, the H100E-E122R mutation resulted in a circa 90% reduction of the  
139 polymerase activity (Supplementary Figure 5), which suggests a possible functional role of the  
140 NTD-NTD interactions in the RSV RNA synthesis.

#### 141 **Cryo-EM analysis reveals a non-canonical symmetry of the helical RSV NC**

142 Although at first glance, the 2D class averages of the RSV NCs with a continuous filament  
143 course suggest a paramyxoviral-like arrangement with a herringbone appearance and a ~70 Å  
144 pitch, their careful scrutiny shows that every ~1.5 turns (or ~100 Å) densities at either the left- or  
145 the right-hand side of the pattern are shifted inwards (Figure 1b; Supplementary Figure 6). The  
146 power spectrum (PS) of the 2D classes exhibit an expected layer line with the maximum close to  
147 the meridian at ~1/70 Å, attributable to the estimated pitch. Surprisingly however, the PS also  
148 features an additional layer line, with a strong maximum on the meridian, at ~1/100 Å, pinpointing a  
149 periodicity that should correspond to a ~100 Å rise (Supplementary Figure 6). Since geometrically  
150 the rise cannot be larger than the pitch, this implies that the measured value of the rise does not  
151 reflect the axial shift between two consecutive protomers. In principle, the ~100 Å periodicity could  
152 arise from stacking of short ~1.5-turn helices with a 70 Å pitch; however, no discontinuity and no  
153 isolated ~1.5-turn helices were observed in our cryo-EM images despite exhaustive particle picking  
154 and extensive 2D classification. Alternatively, if the NCs are continuous, they would be organised in  
155 ~1.5-turn asymmetric units composed of multiple N protomers.

156 3D reconstructions with a 100 Å rise as a starting value and a variable twist led to a solution  
157 with correct secondary structures of N, and a subsequent isolation of the straightest NCs yielded a  
158 final 3D map at an average resolution of 6.2 Å and a continuous RNA density (Figure 1f;  
159 Supplementary Figure 1; Figure 4; Supplementary Figure 6). This moderate resolution lies in the  
160 short-range order of the helical RSV NC. Indeed, an additional 3D refinement within a mask  
161 enclosing ~1.5 turns resulted in a 3.5 Å average resolution map of a five protomer-subsection in  
162 the middle of the mask, which however rapidly deteriorates towards the mask periphery due to a  
163 progressive loss of regularity (Figure 1g; Supplementary Figure 1). The structure of the N-RNA  
164 protomer is again largely the same as in the crystal, with RMSD less than 1 Å over 378 backbone  
165 residues for each of the five protomers, and the inter-protomer contacts maintained.

166 The determined helical parameters and an inspection of the map and the model of the NC  
167 helix (Figure 1f; Supplementary Figure 1; Figure 4a, b) allows to interpret the peculiar experimental  
168 class averages and the PS (Supplementary Figure 6). Indeed, the RSV NC reveals itself as a right-  
169 handed “super-helix”, defined by a 105.3 Å rise and a 149.5° twist, generated by helical repetition  
170 of asymmetric units composed of 16 N protomers forming a ~1,5 turns left-handed spiral staircase.  
171 Inside each asymmetric unit, the protomer arrangement is similar to that observed in paramyxoviral  
172 helical NCs. Amazingly however, the position and the orientation of the protomers relative to the  
173 filament axis as well as the axial shift between two consecutive protomers undergo a specific and  
174 coordinated variation (Figure 4c). For example, the tilt of the protomers varies between ~40° for the  
175 most “standing” (N<sub>6</sub> and N<sub>7</sub>) and ~65° for the most “lying” (N<sub>11</sub>, N<sub>12</sub> and N<sub>13</sub>), whereby the most lying  
176 subunits are the closest to the helical axis. The combination between the helical parameters and  
177 the variation profile of the protomer poses in the asymmetric unit engenders an axial alternation of  
178 regions where two neighbouring turns are the closest to each other and regions where they are  
179 spread further apart. This alternation occurs circa every 105 Å/1.5 turns/16 protomers and  
180 manifests itself by clamping the helix on a side, thereby increasing the gaps above and below the  
181 clamps. The helical propagation of the clamps and gaps confers the RSV NC its unique  
182 appearance, accentuated by an inward shift of the densities corresponding to projections of the  
183 most lying subunits in the 2D class averages (Figure 4; Supplementary Figure 6). The variation of  
184 the protomer tilt is visible even on the five protomer-subsection of the helix (Supplementary Figure  
185 1). The numbering of the protomers in the asymmetric unit is done based on the correspondence  
186 between their axial tilts in the double-headed NC, where the first barbed-end subunit is clearly  
187 identified, and in the helical NC (Supplementary Figure 7).

## 188 **Periodic variations of RNA accessibility and the CTD-arm-mediated inter-turn interactions in** 189 **helical RSV NCs**

190 One consequence of this NC organisation is particularly conspicuous: in the configuration  
191 where the lower-turn protomers are lying and shifted inwards and the upper-turn protomers  
192 standing above, the RNA of the lying protomers is hidden inside the clamps; in contrast, in the  
193 standing-protomer configuration the RNA appears exposed (Figure 5). Another striking observation  
194 (Figure 5) is that three consecutive “nearly standing” protomers in the lower turn interact with the  
195 upper turn through their CTD-arms, which are therefore better defined than in the other protomers  
196 where they are not constrained. Indeed, inspection of the CTD-arm densities in the five protomer-  
197 subsection and in the entire asymmetric unit shows that the definition of the CTD-arm of each  
198 protomer N<sub>i</sub> depends on the position and orientation of the protomer(s) located immediately above  
199 (*i.e.* N<sub>i+10</sub>, N<sub>i+11</sub> and N<sub>i+12</sub>) (Figure 5). Although modelling of the CTD-arm after the residue 379  
200 <sup>4</sup> would be unreliable, a rigid body fit into the subsection map indicates that the three “nearly  
201 standing” subunits of the helical RSV NCs do show densities extending beyond. The CTD-arms of  
202 the subunits N<sub>2</sub> and N<sub>3</sub> (equivalent to N<sub>18</sub> and N<sub>19</sub> in Figures 4 and 5) seem to contact almost the  
203 same zones in the subunits N<sub>13</sub> and N<sub>14</sub>, (*i.e.* N<sub>29</sub> and N<sub>30</sub>) respectively, whereas the CTD-arm of the  
204 subunit N<sub>4</sub> (*i.e.* N<sub>20</sub>) falls nearly in between the upper subunits N<sub>14</sub> and N<sub>15</sub> (*i.e.* N<sub>30</sub> and N<sub>31</sub>)  
205 because of the singular helical symmetry of the RSV NC (Figure 5).

## 206 Shortening of the CTD-arm transforms the RSV NCs into paramyxoviral-like canonical 207 helices

208 Since the structure of the helical RSV NC demonstrates the involvement of the CTD-arm in  
209 the longitudinal contacts, we supposed that shortening of this arm may abrogate inter-turn  
210 interactions and thereby transform the non-canonical helix with asymmetric units composed of 16  
211 N arranged in  $\sim 1.5$  turns into a classical helix with one N per asymmetric unit (Supplementary  
212 Figure 6). Considering the previously published data on the major role played by the last 20  
213 residues of N in the RSV polymerase activity and the critical requirement of the N residue L370 for  
214 the stabilisation of the N<sup>0</sup>P complex <sup>26</sup>, we opted for a C-terminally truncated N1-370 construct. The  
215 most glaring differences with the cryo-EM images of the full-length (FL) N-RNA were the  
216 appearance of two new types of filaments - the herringbone-like helices and a major population of  
217 unforeseen rigid stacks of rings (Figure 6; Supplementary Figure 1). The class averages and the  
218 PS of the helical NC formed by the N1-370 mutant are similar to those of paramyxoviral NCs, and  
219 the helical parameters of the resulting 4.3 Å resolution 3D map, 6.58 Å rise and  $-36^\circ$  twist, closely  
220 agree with the ones derived from the FL “super-helix” (Supplementary Figure 6). Moreover, the  
221 mutant manifests no longitudinal contacts (Figure 6a-c), validating the structure-based hypothesis  
222 that it is the CTD-arm of the RSV N that, by periodically linking two successive helical turns,  
223 induces the non-canonical symmetry of the RSV NC and the resulting systematic variations in the  
224 RNA accessibility.

225 The rigid polymers coexisting with these helices are D10-symmetric and formed by  
226 alternating bottom-to-bottom and top-to-top packing of N<sub>10</sub> rings (Figure 6d-f). Thus, these stacks  
227 are very different from those observed for digested mumps N-RNA rings packed top-to-bottom <sup>12</sup>.  
228 The ensuing 2.8 Å resolution cryo-EM map shows that the NTD-NTD stacked units are  
229 indistinguishable from the N<sub>10</sub> double rings of FL N until the end of the  $\alpha$ -helix 344-358 and the  
230 beginning of the CTD-arm. In the rings and helical assemblies of the FL N, the CTD-arm of the  
231 subunit N<sub>i</sub> protrudes straight onto the top of the CTD of N<sub>i+1</sub>. However, in the stacked rings of the  
232 N1-370 mutant, the truncated CTD-arm sharply pivots away and, instead of engaging into a lateral  
233 interaction, tucks into an identical site but on a CTD of the opposite ring in the stack. The pivoting  
234 of all CDT-arms tightly locks the adjacent rings together through their CTDs, such as to generate a  
235 polymer built of layers of inversely oriented N<sub>10</sub> rings engaged both in NTD-NTD and CTD-CTD  
236 contacts; the latter are additionally stabilised by binding between CTD-arms of two opposing  
237 protomers, in particular through a Y365-Y365 stacking (Figure 6g, h).

## 238 Discussion

239 The major finding of this work was the non-canonical helical organisation of the RSV NC,  
240 generated by  $\sim 1.5$ -turn asymmetric units composed of 16 N protomers, which undergo a concerted  
241 variation of their poses while remaining in quasi-equivalent environments. This unique symmetry,  
242 together with the great flexibility of the NCs, complicates their high resolution analysis, and is  
243 totally different from those described for other *Mononegavirales* NCs. Excitingly, the arrangement  
244 of the RSV NC is reminiscent of the one proposed for the Dahlemense strain of tobacco mosaic  
245 virus (TMV), and may be similarly considered in terms of a periodic deformation of a regular helical  
246 structure <sup>27</sup>. In the Dahlemense TMV model, the additional meridional reflexions appear on the  
247 layer lines halfway between those of the common TMV and the asymmetric unit contains exactly  
248 two turns. Likewise, for the RSV NC the maximum on the meridian is observed at two thirds of the

249 layer line of the expected pitch and the asymmetric unit contains ~1.5 turns. The exterior distortion  
250 proposed in the Dahlemense TMV model is explained by the inside and outside sets of inter-turn  
251 interaction being incompatible with the same periodicity, whereas the common TMV does not have  
252 any axial outside interactions. The C-terminally truncated RSV NC mutant also has no inter-turn  
253 interactions and features a canonical helical symmetry with equivalent environments for each N  
254 protomer. In contrast, in the FL NC, the CTD-arms at the filament interior are periodically involved  
255 in axial interactions with the upper turn, which induces a global structural reorganisation leading to  
256 tilting and inwards shifting of certain protomers and manifesting itself as a helical distortion at the  
257 NC exterior. Continuing the parallel, the stability of the observed full-length RSV NC structure  
258 would indicate that “the decrease in the free energy upon forming some additional bonds is greater  
259 than the increase in the free energy required to move the subunits into the slightly different, but  
260 quasi-equivalent positions”<sup>27</sup>.

261 The helical NC, together with the RNA polymerase L, its phosphoprotein cofactor P and the  
262 transcription factor M2-1, form the RSV RNA synthesis complex that constitutes the minimal  
263 infectious unit of the virus. P acts as a central hub by tethering L to the NC template, chaperoning  
264 neosynthesised N such as to keep it monomeric and RNA-free (N<sup>0</sup>) for specific nascent RNA  
265 encapsidation, and recruiting M2-1<sup>28</sup>. The matrix protein M is thought to direct RSV assembly and  
266 budding by interacting both with the NC-bound P and with the envelope glycoprotein F<sup>29,30</sup>. Recent  
267 cryo-ET analysis of filamentous RSV virions demonstrated that M is organised in a helical array  
268 that would coordinate helical ordering of glycoprotein spikes<sup>19</sup>. In addition, in *Paramyxoviridae*, M  
269 was shown to directly bind the CTD-arm of N<sup>31,32</sup>. Thus, RSV M may potentially also influence the  
270 helical parameters of the NC upon the viral cycle via a direct or a P-mediated interaction.  
271 Noteworthy, the binding pocket of P on the NC helix is situated far both from the CTD-arm itself  
272 and from its binding site to the upper helical turn<sup>33,34</sup>.

273 In order to bind M or another viral or host factor, the end of the CTD-arm of N would need to  
274 escape outside the NC through an interstice between two turns, as shown for *Paramyxo-* and  
275 *Filoviridae*, which however have a much longer CTD extension. Here we showed that the non-  
276 canonical helical organisation of the RSV NC is engendered by the last 20 residues of the CTD-  
277 arm of N. Considering high similarity between RSV and hMPV N, we suppose that all  
278 *Pneumoviridae* NCs adopt an analogous arrangement. Our structures and the structural homology  
279 between RSV and hMPV N<sup>0</sup>P complexes inferred from biochemical studies<sup>26</sup> suggest that,  
280 similarly to the situation in hMPV<sup>22</sup>, binding of the N-terminal peptide of RSV P to N<sup>0</sup> would hamper  
281 its self-oligomerisation by preventing the CTD-arm from subdomain swapping and flipping it  
282 downwards along the core of N such as to block the RNA binding<sup>22</sup>. In contrast, in the stacked N1-  
283 370 rings, the truncated CTD-arm rotates upwards to dock into dedicated subdomain swapping site  
284 on the opposite N protomer from the ring above. Thus, comparison of these structures indicates  
285 that the CTD-arm is able to explore a large angular space (Figure 6i).

286 While the truncation of the CTD-arm is not supposed to occur *in vivo*, the high rotational  
287 freedom of the CTD-arm hints to a possibility of its reorientation upon interaction with viral or host  
288 factors. One may therefore envision a temporary capping of the pointed end of the NC helix by an  
289 N-RNA ring or a second NC through a CTD-CTD interaction, such as to protect the 3' end of the  
290 RNA from host antiviral responses. This would however imply an uncapping of the 3' end in order  
291 to initiate transcription or replication from the respective promoters that reside at the pointed end of  
292 the NC, by either P or L or another factor. The 5' end, in its turn, would be protected inside the



294 double-helical and 5' ring-capped NCs formed through NTD-NDT interactions. Such top-to-top  
295 and/or bottom-to-bottom assemblies of RSV NCs would be consistent with the observations in the  
296 RSV virion<sup>19,25</sup> and in the infected cells<sup>35</sup>.

297 The most obvious consequence of the non-canonical RSV NC structure is the periodic  
298 variation of the RNA accessibility, the RNA binding groove of N being severely obstructed in the  
299 inwards shifted lying protomers and exposed in the standing ones. While this difference in access  
300 to the RNA should inevitably influence pneumoviral synthesis by L during its gliding along the NC,  
301 the exact mechanistic implications of the observed variations are difficult to conceptualise. Indeed,  
302 because of the limited long-range order of the RSV NC helices, any prediction of the protomer  
303 poses based on the numbering in the clam and semi-clam structures as adopted here can only be  
304 reliable for the protomers located very close to the barbed, 5'-end. Even an attempt to estimate the  
305 tilt of the protomer containing the most 5'-end proximal gene start (GS), at the onset of the gene of  
306 L, would be too error-prone. In addition, considering that the CTD-arm is required both for the inter-  
307 turn interactions, responsible for the non-canonical NC organisation, and for the prevention of the  
308 premature RNA encapsidation by N<sup>0</sup>, the mechanisms behind the strong inhibition of the RSV RNA  
309 polymerase activity by mutations and truncation of the CTD-arm<sup>26</sup> are certainly convoluted.

310 Finally, it is essential to keep in mind that *in cellula* the RSV RNA synthesis occurs in virally  
311 induced cytoplasmic inclusions considered as active viral factories and formed by liquid-liquid  
312 phase separation<sup>36-38</sup>. In line with the structural polymorphism of the purified NCs obtained by  
313 heterologous expression described here, we hypothesise that particular functional states of the  
314 NCs can be enriched in VFs depending on the progress of the viral cycle or the status of certain  
315 cellular pathways. The material properties of biomolecular condensates may also influence the NC  
316 structures. Thus, in future it is essential to combine cryo-EM investigation of the structure-function  
317 relationships of the RSV synthesis machinery *in vitro* with its cryo-ET analysis in the cellular  
318 context.

## 319 **Methods**

### 320 **Plasmids and baculoviruses**

321 The codon-optimized sequence coding for the wild type (WT) N (strain Long) was syn-  
322 thetised (GenScript) and cloned in the pFastBac Dual vector under the control of the polyhedrin  
323 promoter at BamHI and Sall sites. A stop codon was inserted after amino acid residue 370 by site  
324 directed mutagenesis using Q5 Site-Directed Mutagenesis Kit (NEB), in order to express the trun-  
325 cated N1-370 construct. Recombinant baculoviruses were recovered using the Bac-to-Bac bac-  
326 ulovirus expression system (Invitrogen). N WT or N1-370 bacmids were obtained after transforma-  
327 tion of DH10EMBacY bacteria (Geneva Biotech). Recombinant baculoviruses were recovered after  
328 transfection of High Five cells using Cellfectin reagent (ThermoFisher Scientific) and amplification.

329 The plasmid pGEX-PCT (C-terminal residues 161-241 of RSV P protein), used for bacterial  
330 expression of the recombinant GST-PCT, has been described previously<sup>39,40</sup>. Plasmids for  
331 minigenome assay expressing hRSV N, P, M2-1, and L are designated pN, pP, pM2-1 and pL, and  
332 have been described previously<sup>41,42</sup>. The pM/Luc subgenomic minigenome which encodes the  
333 firefly luciferase (Luc) reporter gene under the control of the M/SH gene start sequence has also  
334 been described<sup>43</sup>. The plasmids encoding N mutants pNH100E, pNH100E-R101D, pNH100E-

335 E122R, and pN1-370 were generated using Q5 Site-Directed Mutagenesis Kit (NEB). Primers are  
336 shown in Supplementary Table 1.

### 337 **Protein expression and purification**

338 Recombinant GST-PCT was used for purification of recombinant N expressed in insect  
339 cells. Briefly, *Escherichia coli* BL21 (DE3) bacteria (Novagen) transformed with the pGEX-PCT  
340 plasmid were grown at 37 °C, in 2xYT medium with 100 µg/ml ampicillin. After 7 h, an equal  
341 volume of 2xYT medium with 100 µg/ml ampicillin and 80 µg/ml of isopropyl β-d-1-  
342 thiogalactopyranoside (IPTG) was added to induce protein expression, before overnight incubation  
343 of the culture at 28 °C. Bacteria were harvested by centrifugation at 3000 g for 30 min at 4 °C,  
344 resuspended for 30 min in lysis buffer (Tris 50 mM, NaCl 60 mM, EDTA 1 mM, 0.1 % Triton X-100,  
345 DTT 2 mM, pH 7.8, anti-proteases (Roche)), and sonicated on ice. Benzonase was then added to  
346 the lysate, followed by a 30 min incubation at room temperature. After centrifugation at 10 000 g for  
347 30 min at 4 °C, the supernatant was incubated with Glutathione-Sepharose 4B beads (Cytiva) for 3  
348 h at 4°C. The beads were washed once in the lysis buffer and twice in PBS 1X buffer. Beads were  
349 resuspended in PBS 1X and stored at 4 °C.

350 For expression of N, High Five cells (ThermoFisher Scientific) were infected at a multiplicity  
351 of infection of 2 for 72 h with the baculovirus coding either for the WT RSV N or the N1-370  
352 construct. Cells were washed in TEN buffer (50 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, pH 7.4)  
353 and centrifuged at 3000 g for 5 min. The cells were resuspended in 10 ml of lysis buffer (TEN  
354 buffer, NP-40 at 0.6 % (v/v), anti-proteases/phosphatases (Roche), RNase (200 µg/ml, Invitrogen),  
355 DNase (5 units/ml, Promega)) and incubated 40 min at 37 °C. The lysate was clarified by  
356 centrifugation at 14 000 g for 15 min at 4 °C, then incubated for 3 h at 4 °C with the GST-PCT  
357 beads previously rinsed in TEN buffer. The GST-PCT beads were then washed once in lysis buffer  
358 and twice in TEN buffer and then incubated in TEN buffer in the presence of thrombin (Sigma), for  
359 72 h at 4 °C. The supernatant was collected and concentrated using a column with a MWCO of  
360 100 kDa (Sartorius).

### 361 **Minigenome assay**

362 BSRT7/5 cells, a cell line derived from the BHK-21 cells, constitutively expressing the T7  
363 RNA polymerase<sup>44</sup>, were used for the minigenome assay. Cells were grown in Dulbecco's modified  
364 Eagle's medium (Lonza) with 2 mM L-glutamine, antibiotics and 10 % fetal bovine serum. Cells at  
365 90 % confluence in 96-well plate were transfected using Lipofectamine 2000 (Invitrogen) according  
366 to manufacturer's instructions with the following plasmid mixture: 62.5 ng of pM/Luc, pP and pN  
367 (WT or N mutants), 31.3 ng of p L, 15.6 ng of pM2-1, and 15.6 ng of pRSV-β-galactosidase  
368 (Promega) for transfection efficiency normalisation. After 24 h, the cells were lysed in luciferase  
369 lysis buffer (30 mM Tris pH 7.9, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 1% Triton X-100, and 15% glycerol).  
370 After addition of the substrate (Luciferase assay system, Promega), the luciferase activities were  
371 determined for each cell lysate with an Infinite 200 Pro (Tecan, Männedorf, Switzerland) and  
372 normalised based on β-galactosidase (β-Gal) expression. Four replicates were carried out and  
373 mean values were calculated. The analysis was done using Excel (Microsoft) and Prism 9  
374 (GraphPad). Expression of WT and mutant N was assessed by Western blot using a rabbit anti-N  
375 antiserum<sup>39</sup> and a mouse monoclonal anti-tubulin antibody (Sigma), revealed by incubation with  
376 anti-rabbit and anti-mouse antibodies coupled to HRP (SeraCare).

### 377 **Cryo-EM data collection**

378 3  $\mu\text{L}$  of the purified FL or truncated RSV NC sample were applied to a glow-discharged  
379 R2/1 300 mesh holey carbon copper grid (Quantifoil Micro Tools GmbH) and plunge-frozen in liquid  
380 ethane using a Vitrobot Mark IV (FEI) operated at 100% humidity at room temperature. Datasets  
381 were recorded at the EM platform of the IBS Grenoble, on a Glacios microscope (Thermo  
382 Scientific) equipped with a K2 summit direct electron detector (Gatan) operated in counting mode.  
383 A summary of cryo-EM data collection parameters can be found in Supplementary Table 2. Movies  
384 were acquired with a total dose of  $42 \text{ e}^-/\text{\AA}^2$  and a defocus range of  $-0.7$  to  $-2.4 \mu\text{m}$ , at  $1.145 \text{ \AA}/\text{pixel}$   
385 at the specimen level. Cryo-EM data on the N1-370 mutant was acquired with using beam-tilt  
386 induced image-shift protocol (9 images for each stage movement). Micrographs were manually  
387 screened based on the presence of particles, amount of contamination and apparent beam-  
388 induced movement, resulting in a total of 11,386 selected micrographs for the FL NCs and 6,312  
389 selected micrographs for N1-370 NCs. A visual inspection of the full-length NC dataset showed the  
390 presence of at least four types of assemblies – helical NCs, double-headed NCs, ring-capped NCs  
391 and double rings – which were processed separately. Similarly, the helical NCs and the stacks  
392 formed by the N1-370 mutant were also processed separately.

### 393 **Image analysis of the non-canonical helical FL RSV NCs**

394 For the helical NCs, an initial manual picking of 800 filaments from a subset of micrographs  
395 was performed with EMAN2 e2heliboxer.py<sup>45</sup> and used to create a training dataset for crYOLO<sup>46</sup>,  
396 which was subsequently used for the picking on all micrographs, resulting in 97,280 filaments  
397 traced. The filament coordinates were then used for particle extraction in RELION<sup>47</sup> with a binning  
398 to a boxsize of 128 pixels (corresponding to  $3.04 \text{ \AA}/\text{pixel}$ ) and a  $15 \text{ \AA}$  distance between boxes. A  
399 total of 1,406,835 helical segments were picked and iteratively classified in RELION, keeping the  
400 straightest 2D class averages at each round, which resulted in 544,972 selected segments. The  
401 sum of PS of the aligned segments corresponding to 27 classes selected based on estimated  
402 resolution and number of particles was calculated with RELION and inspected with bshow<sup>48</sup>,  
403 showing a similar pattern for all selected classes. Subsequent processing steps were carried out in  
404 cryoSPARC<sup>49</sup>. After estimating the helical rise to  $\sim 100 \text{ \AA}$  from the PS (Supplementary Figure 6),  
405 the helical twist was determined on the imported binned segment selection whereby multiple  
406 helical 3D refinements were run in parallel using as initial symmetry parameters a fixed  $100 \text{ \AA}$  rise  
407 and varying the twist from  $60$  to  $180^\circ$  with a  $10^\circ$  step. The crystal structure of the RSV N-RNA ring  
408<sup>4</sup> was used to validate the 3D maps after refinement, choose the best one for further analysis and  
409 impose the correct handedness. An examination of this intermediate map and of its refined  
410 symmetry parameters ( $\sim 105 \text{ \AA}$  rise and  $\sim 150^\circ$  twist) enabled us to understand that the helical RSV  
411 NC in our cryo-EM images was in fact a right-handed “super-helix”, with an asymmetric unit  
412 corresponding to a left-handed helix composed of  $\sim 16$  adjacent N protomers. Since the rise of this  
413 super-helix was larger than the initially defined distance between successive segments, a new  
414 template-based picking with the projections of this intermediate map was done in cryoSPARC,  
415 using  $105 \text{ \AA}$  as a distance between segments. The picking yielded 769,699 helical segments that  
416 were cleaned down to 546,489 particles by further 2D classification. This cleaned particle set was  
417 further processed in two different ways. First, it was subjected to a heterogeneous refinement  
418 using 5 classes, with no symmetry imposed, in order to select the better resolved and most regular

419 particles. The three most similar classes were combined into a final set of 389,540 segments that  
420 was used for a final helical refinement to an average resolution of 6.2 Å (Fourier Shell Correlation  
421 (FSC) at 0.143), which was sharpened with a B-factor of  $-470 \text{ \AA}^2$  for visualisation and rigid body fit  
422 of the crystal structure. This map reflects the non-canonical helical RSV NC. Second, because of  
423 the flexibility of the super-helix, we decided to focus on one asymmetric unit and performed a  
424 refinement with a mask enclosing 16 N protomers created from the helical map. This resulted in a  
425 final map, with a subsection of five consecutive well-defined protomers at its centre that had an  
426 average resolution of 3.5 Å (FSC at 0.143). The five protomer-subsection map, sharpened with a  
427 B-factor of  $-94 \text{ \AA}^2$ , was used for subsequent model building and structural analysis.

428 The variation of the axial shifts between two consecutive protomers and their distances  
429 from the helical axis (Figure 4c) was calculated based on the coordinates of the centers of gravity  
430 of each protomer obtained from the rigid body-fitted crystal structure. To estimate the tilt of each  
431 protomer relative to the helical axis, two C $\alpha$  atoms (from G106 and H274) were picked on the long  
432 inertia axis and the angle between the vector formed by these two atoms and the helical axis was  
433 computed.

#### 434 **Image analysis of the double-headed helical NCs and ring-capped NCs**

435 Inspection of the cryoSPARC analysis of the 2D classification from the template-based  
436 picking of helical segments, allowed us to identify a 2D class average, corresponding to a class of  
437 10,718 particles, that showed features of a double-headed helix (potentially mixed with ring-capped  
438 helices). This class was used for template-based picking, giving a set of 176,614 particles that was  
439 iteratively cleaned by 2D classification down to 12,699 particles, mostly by removing double ring  
440 side views. From these particles, an *ab initio* reconstruction was calculated, followed by non-  
441 uniform refinement, which gave a  $\sim 7 \text{ \AA}$  resolution map used to create templates to re-pick the  
442 entire dataset. The resulting over 200,000 particles were classified down to 52,127 particles, with a  
443 mixture of classes showing double-headed features and classes showing ring-capped helices.  
444 Because the separation between double-headed helices and ring-capped helices by 2D  
445 classification only did not seem entirely satisfactory, we imported the particles into RELION and  
446 iteratively performed 3D classifications until obtainment of two stable particle subsets (and one  
447 subset discarded as junk particles). This procedure resulted in 22,162 particles of ring-capped  
448 helices and 25,338 particles of double-head helices. These were imported back into cryoSPARC  
449 and used for final non-uniform refinements using the RELION 3D class averages as initial  
450 references. The average resolutions (FSC at 0.143) of the resulting 3D reconstructions of the ring-  
451 capped and the double-headed helices were 3.9 Å and 3.8 Å respectively; finally, the maps were  
452 respectively sharpened with a B-factor of  $-35 \text{ \AA}^2$  and  $-54 \text{ \AA}^2$ .

453 In order to establish the correspondence between the protomers in the double-headed NCs  
454 and in the non-canonical helical NCs (and thereby to allow the numbering of the protomers in the  
455 asymmetric unit of the non-canonical helix as in Figure 4), we matched each of the 16 rigid body-  
456 fitted protomers of the non-canonical NC onto the rigid body-fitted protomers of the double-headed  
457 NCs (alignment done on one protomer) and calculated the RMSD between the two structures over  
458 five consecutive protomers. The lowest RMSD value (3.39 Å) indicated the register of the N  
459 protomers in the double-headed NCs compared to the helical NC.

#### 460 **Image analysis of the RSV N-RNA double rings**

461 In cryoSPARC, manual picking of 50 side views of double rings followed by 2D  
462 classification was used to create a 2D template for automatic picking, giving 23,704 particles after  
463 cleaning by 2D classification. These particles were used for *ab initio* 3D reconstruction followed by  
464 refinement and new particle picking using the refined map projections. Iterative 2D classification  
465 gave a set of 57,896 side views. Ring-like top views were not considered because they could  
466 potentially correspond to single rings or short helices instead of double rings. The selected side  
467 views were cleaned down to 47,212 particles by an additional 3D classification step in order to  
468 remove undetected potential C11-symmetric rings or short helices mixed with the C10-symmetric  
469 double ring side views. A final non-uniform refinement led to a 3D reconstruction at an average  
470 resolution of 2.9 Å (FSC at 0.143), sharpened with a B-factor of -96 Å<sup>2</sup>.

#### 471 **Image analysis of the canonical helices formed by the N1-370 mutant**

472 In cryoSPARC, manual picking of 200 helical segments followed by 2D classification was  
473 used to prepare templates for the filament tracer job. Two rounds of 2D classification and re-  
474 picking were then performed to yield a set of 471,549 helical segments used for initial  
475 reconstruction. Different helical symmetries ranging from 9 to 11 subunits per turn with a starting  
476 pitch of 60 Å were tested by running multiple helical refinements, and only the refinement starting  
477 at 10 subunits per turn gave an interpretable map with visible secondary structures. This map was  
478 used to generate 2D projections for a final template-based filament tracer job, which gave, after  
479 keeping only the straightest helical segments by iterative 2D classification steps, a final set of  
480 329,706 segments. The last refinement was run with a mask enclosing 30% of the segment length,  
481 giving a reconstruction at an average resolution of 4.3 Å (FSC at 0.143), sharpened with a B-factor  
482 of -155 Å<sup>2</sup>. The refined helical parameters - a -36° twist and a 6.58 Å rise – are very similar to the  
483 ones calculated from the helical parameters of the non-canonical helical NC formed by the FL N,  
484 assuming one protomer per asymmetric unit (-35.7° twist and 6.58 Å rise).

#### 485 **Image analysis of the N1-370 mutant stack**

486 A 2D class average corresponding to stacked rings derived from an automatic picking of  
487 helical segments in cryoSPARC was used for template-based particle picking, followed by 2D  
488 classification and re-picking until a stable subset of 81,918 particles was obtained. *Ab initio* 3D  
489 reconstruction followed by refinement with imposed D10 symmetry as well as defocus refinement  
490 gave a final 3D map at an average resolution of 2.8 Å (FSC at 0.143), which was sharpened with a  
491 B-factor of -99 Å<sup>2</sup>.

#### 492 **Map visualisation, local resolution calculation, model building and refinement**

493 For all final maps, the local resolution was calculated in cryoSPARC. The most interpretable  
494 maps were used for further structural analysis. The crystal structure of the N-RNA monomer (PDB:  
495 2WJ8, chain S) was rigid-body fitted in the maps with Chimera<sup>50</sup>. Where appropriate, refinement  
496 was performed using the Phenix software package<sup>51</sup> and manual correction in Coot<sup>52</sup>. At different  
497 processing stages, the structures were inspected with Chimera and bsoft<sup>48</sup>, and figures were done  
498 using ChimeraX<sup>53</sup>.

## 499 **Data Availability**

500 All data presented in this study are included in the published article and its supplementary  
501 information, and are available from the corresponding author on request.

## 502 **Statistics and reproducibility**

503 Data was collected on independent experiments. Statistics details are presented in the  
504 Methods section and in the figure legends where appropriate.

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## 627 **Acknowledgements**

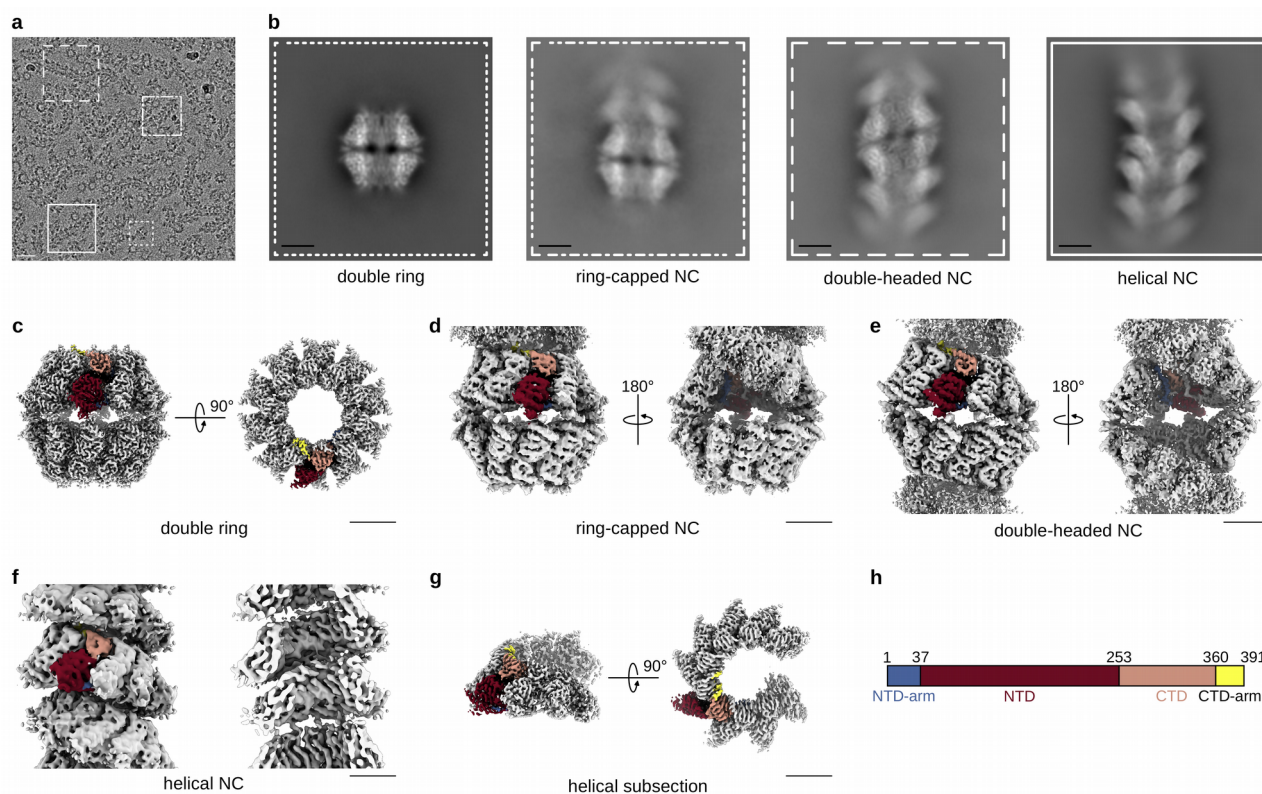
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## 641 **Author contributions**

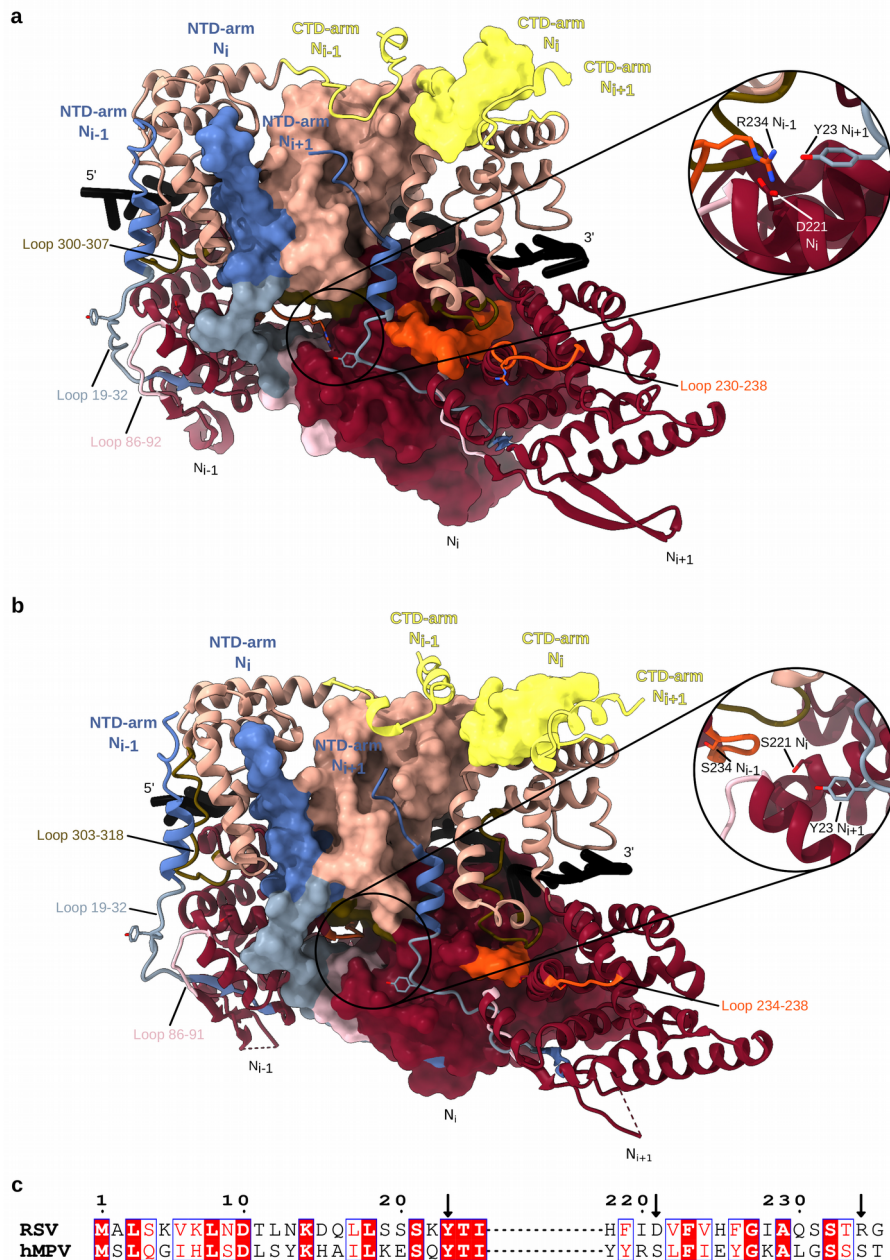
642 LG, MBV, DC, MG and IG performed experiments, LG, AD and IG analysed the cryo-EM  
643 data. LG, JFE and MG analysed the biological data. IG and JFE designed the overall study. IG,  
644 AD, JFE and MG supervised the project. IG wrote the manuscript with contributions of LG, AD, MG  
645 and JFE. LG, AD and IG prepared the figures. All authors read the manuscript prior to submission.

646 **Competing Interests:** The authors declare that there are no competing interests.

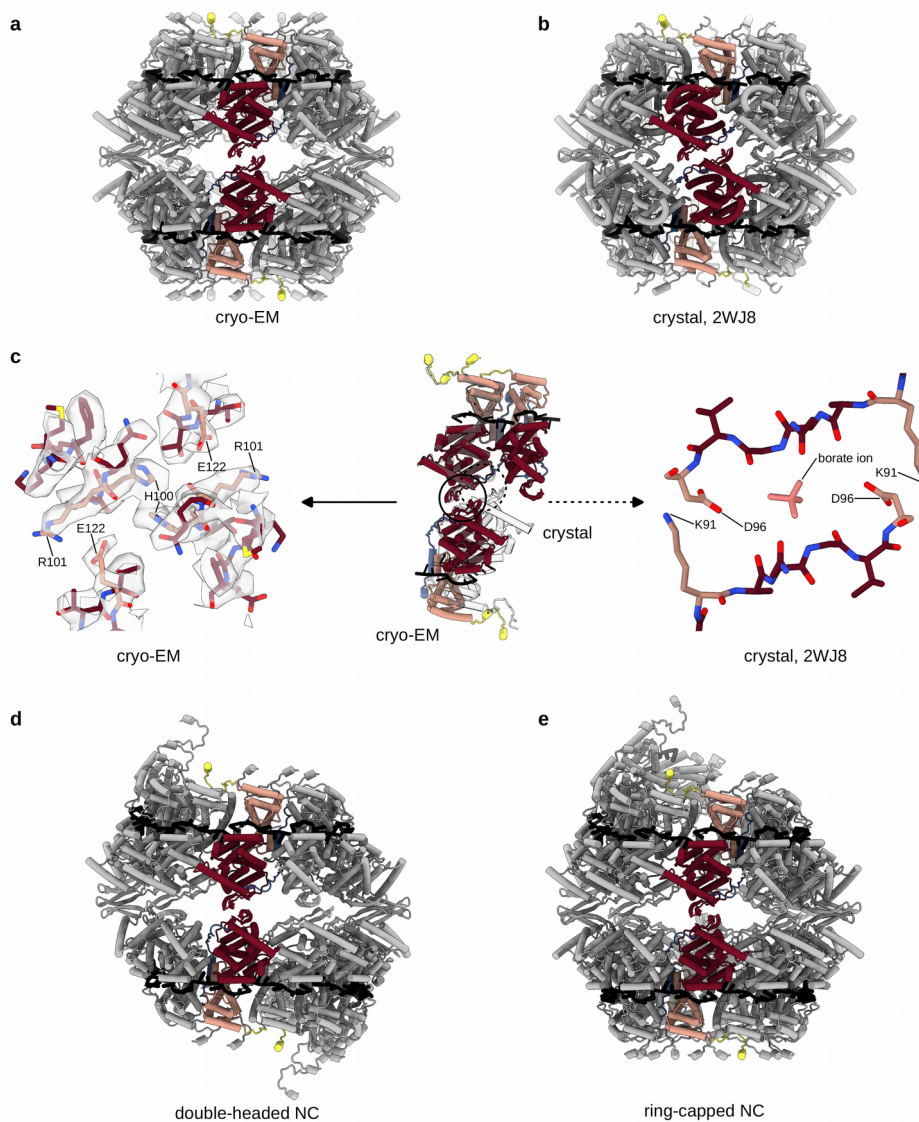
## 647 Figures and Legends



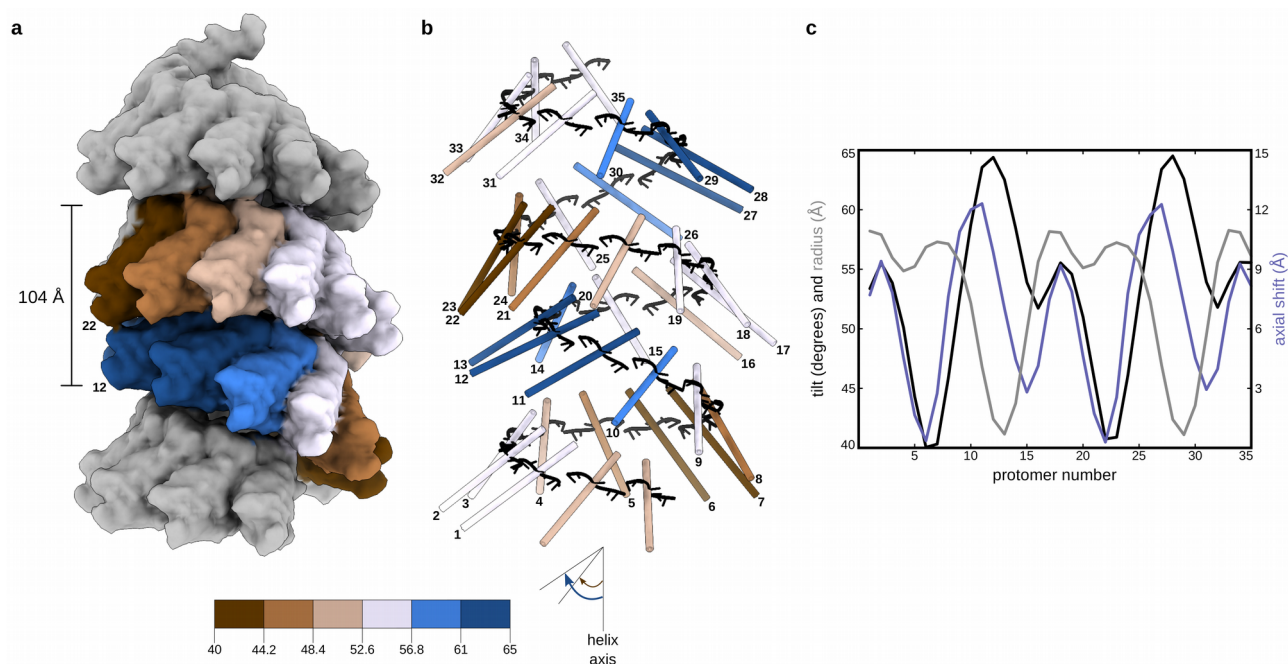
648 **Figure 1: Cryo-EM analysis of RSV NCs.** (a) A representative micrograph of RSV NCs purified  
 649 from insect cells and featuring double rings, ring-capped NCs, double-headed NCs and helical  
 650 NCs. Particles are boxed as an illustration, scale bar 200 Å. (b) Representative 2D classes with the  
 651 outline matching the particles highlighted in (a). (c) Cryo-EM map of the N<sub>10</sub> double ring (side and  
 652 top view). (d) Cryo-EM map of the ring-capped NC (front and back view). (e) Cryo-EM map of the  
 653 double-headed NC (front and back view). (f) Cryo-EM map of the helical NC (front and cut-through  
 654 view). (g) Helical subsection (side and top view). Scale bar, 50 Å in (b-g). (f) Schematic of the RSV  
 655 N sequence divided into an NTD-arm (blue-gray), NTD (rosewood), CTD (old rose) and CTD-arm  
 656 (yellow). In cryo-EM maps in (c-g), one protomer is coloured according to this schematic, with the  
 657 RNA in black.



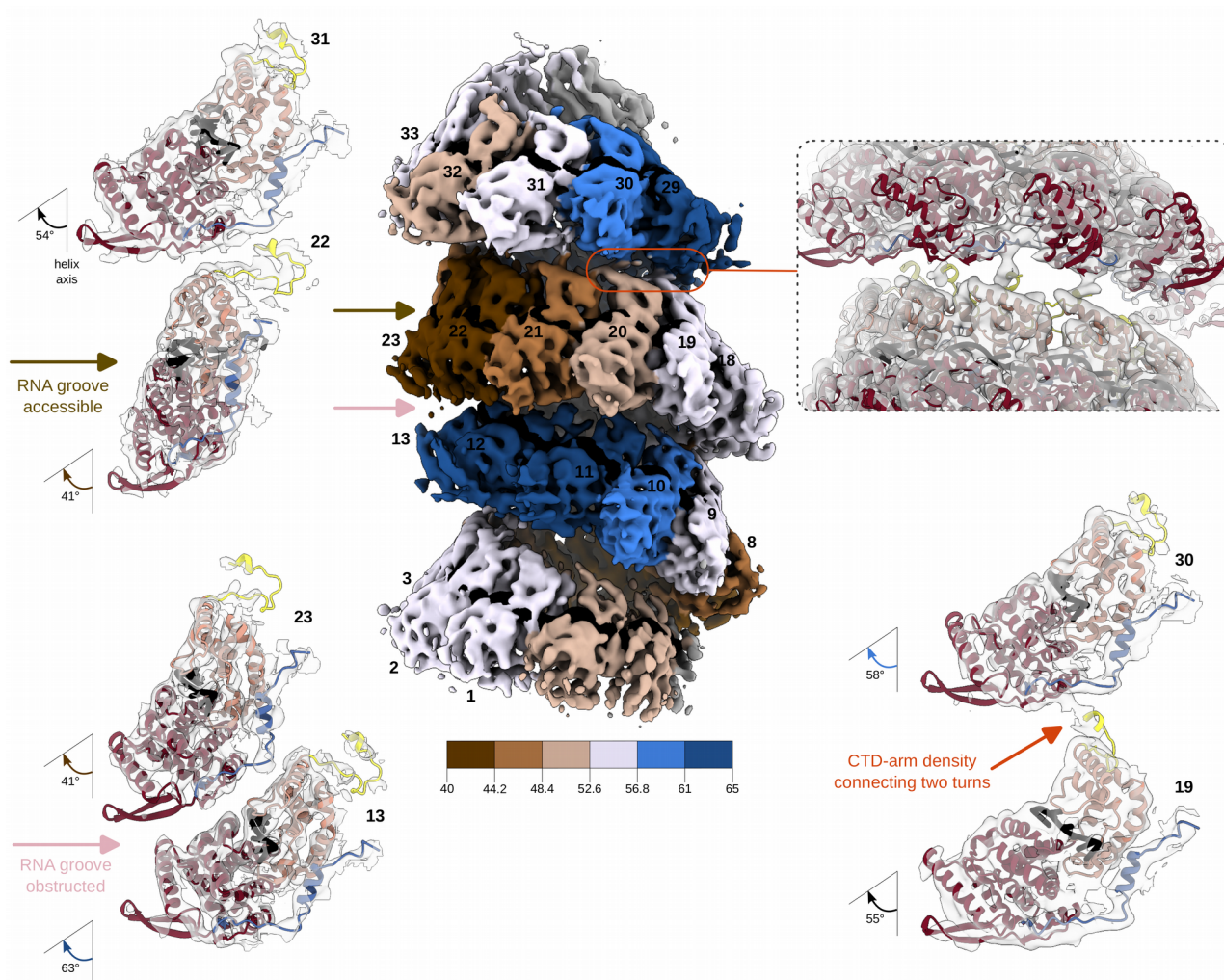
658 **Figure 2: Lateral interactions between N protomers in RSV and hMPV N<sub>10</sub> double rings.** (a)  
 659 Atomic models of three consecutive RSV N protomers are shown, the middle one as a surface and  
 660 the edge ones as ribbons. NTD-arm, NTD, CTD and CTD-arm coloured as in Figure 1, loop 19-32  
 661 in powder blue, loop 86-92 in mimi pink, loop 230-238 in orange and loop 300-307 in olive. The  
 662 close-up of the N-hole of the middle protomer shows the tripartite Y23-D221-R234 interaction. (b)  
 663 Same as (a) but for three protomers from the hMPV N-RNA ring crystal structure (PDB: 5FVC).  
 664 Colouring as in Figure 1 and in (a), loop 19-32 in powder blue, loop 86-91 in mimi pink, loop 234-  
 665 238 in orange and loop 303-318 loop in olive. The close-up shows the absence of a tripartite  
 666 interaction in the N-hole. (c). Pairwise sequence alignment of RSV and hMPV N around the  
 667 residues involved in the tripartite interaction in the RSV N oligomer. Conserved residues in red  
 668 boxes; arrows pointing at residues 23, 221 and 234.



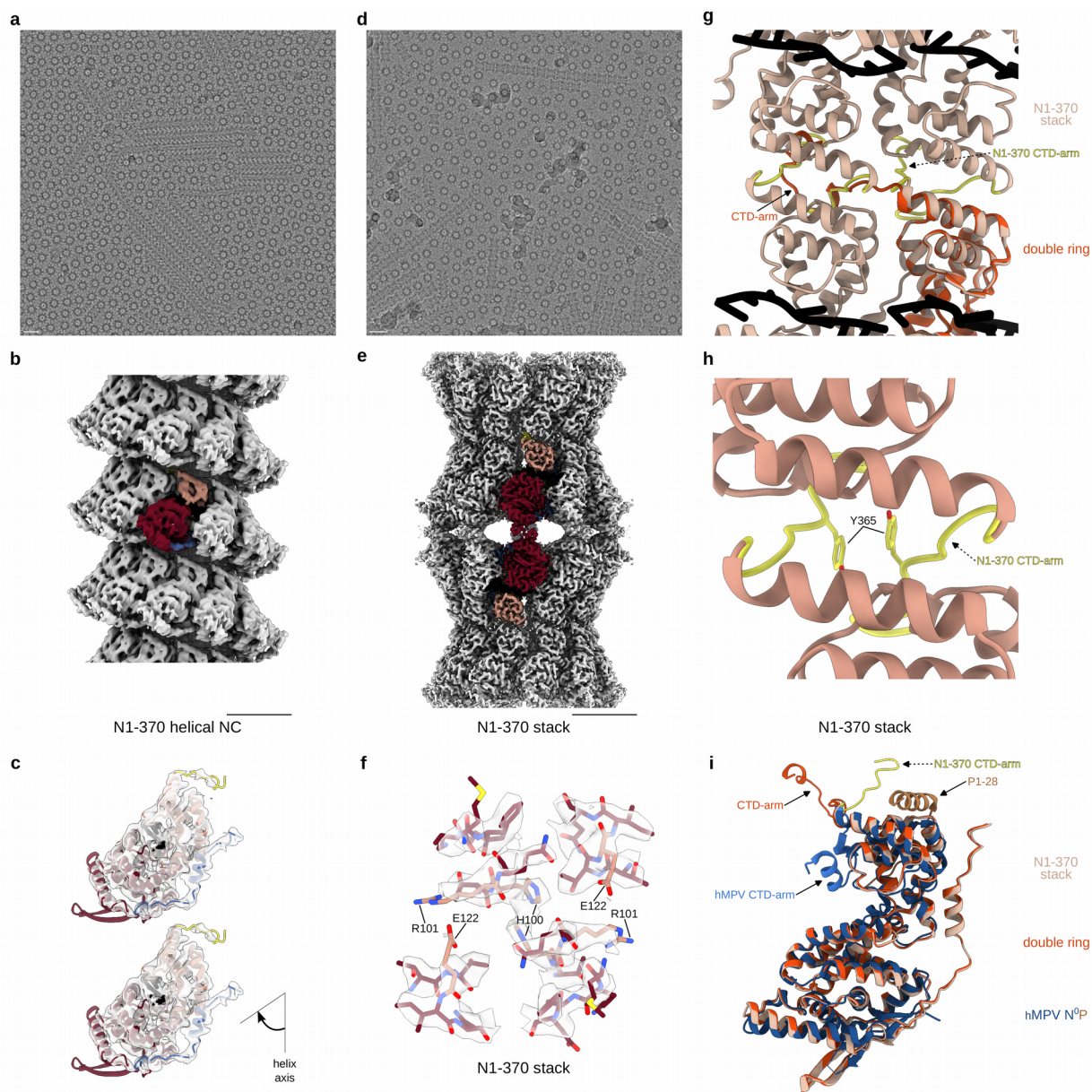
669 **Figure 3. Longitudinal NTD-NTD interactions conserved between RSV N<sub>10</sub> double ring,**  
670 **double-headed and ring-capped NCs but different from the crystal structure of the RSV N<sub>10</sub>**  
671 **double ring.** In each panel, two opposite protomers are coloured as in Figure 1. (a) Atomic model  
672 of the RSV N<sub>10</sub> double ring derived from the cryo-EM map shown as cartoon. (b) Atomic model of  
673 the RSV N<sub>10</sub> double ring crystal structure (PDB: 2WJ8). (c) Alignment of the top rings of the cryo-  
674 EM and crystal structure-based models reveals a rotation between the bottom rings. Two top-ring  
675 protomers and one opposing bottom-ring protomer are shown in the middle of the panel, with the  
676 cryo-EM-based structure coloured as in Figure 1 and the crystal structure in white. A close-up of  
677 the cryo-EM map and the atomic model highlighting the NTD-NTD interactions is on the left, a  
678 close-up of the NTD-NTD interactions in the crystal structure is on the right. The difference  
679 between the crystallographic and the solution inter-ring interfaces may be related to the presence  
680 of a borate ion in the interaction site in PDB: 2WJ8, possibly embarked during the electrophoretic  
681 separation of decameric and undecameric RSV N-RNA rings prior to crystallisation. (d) Atomic  
682 model of the double-headed NC. (e) Atomic model of the ring-capped NC.



683 **Figure 4. Non-canonical helical symmetry of the RSV NC.** (a). Atomic model of the NC is  
684 filtered to 10 Å resolution and displayed as surface. Protomers in one asymmetric unit are coloured  
685 dependent on their axial tilt following the colour code shown at the schematic underneath, the rest  
686 of the protomers are coloured in grey. (b). Protomers of the model in (a) are shown as sticks  
687 coloured dependent on the protomer axial tilt and numbered 1 to 35. (c) Plot showing the axial tilt  
688 (black), the radial position (grey) and the relative axial shift of each protomer.



689 **Figure 5.** RNA accessibility and CTD-arm-mediated inter-turn interactions in the helical NC. Cryo-  
690 EM map of the helical NC is shown in the middle, coloured as in Figure 4 and as reminded in the  
691 schematic underneath the map, with protomers numbered as in Figure 4. On the left, close-up  
692 views of two sets of opposing protomers from two successive helical turns are shown to illustrate  
693 the difference in the RNA accessibility, with the cryo-EM density in transparent grey and the atomic  
694 model represented as a ribbon and coloured as in Figure 1. On the top right, a similarly-coloured  
695 view of the inter-turn interaction is shown to highlight the densities corresponding to the CTD-arm,  
696 with a corresponding two-protomer close-up underneath. RNA is in black.



697 **Figure 6. Canonical helical NCs and stacked assemblies formed by the N1-370 mutant.** (a) A  
698 representative micrograph of the N1-370 NCs featuring mostly helical NCs and rings, scale bar 200  
699 Å. (b) Cryo-EM map of the canonical N1-370 helical NC, with one protomer coloured as in Figure  
700 1, scale bar 50 Å. (c) Close-up view of protomers from two successive helical turns are shown to  
701 illustrate the position of the CTD-arm and the absence of inter-turn interactions, with the cryo-EM  
702 map in transparent grey and the atomic model represented as a ribbon and coloured as in Figure  
703 1. (d) A representative micrograph of the N1-370 NCs featuring mostly stacks and rings, scale bar  
704 200 Å. (e) Cryo-EM map of the N1-370 stack, with one protomer coloured as in Figure 1, scale bar  
705 50 Å. (f) Close-up of the cryo-EM map of the N1-370 stack and the atomic model highlighting the  
706 NTD-NTD interactions (similar to the ones in the N<sub>10</sub> double ring shown in Figure 3c). (g) Alignment  
707 of the atomic models of the N<sub>10</sub> double ring and the N1-370 stack illustrating the difference in the  
708 orientation of the CTD-arms. One protomer of the N<sub>10</sub> double ring is shown in the bottom-right and  
709 coloured in orange, 4 protomers of the N1-370 stack are shown and colored in beige, with the

710 CTD-arms in yellow. RNA is in black. (h) Close-up of the atomic model of the N1-370 stack  
711 highlighting the CTD-CTD interactions. (i) Alignment of N protomeres of the N<sub>10</sub> double ring  
712 (orange), the N1-370 stack (beige) and the hMPV N<sup>0</sup>P crystal structure (N<sup>0</sup> in blue and P1-28 in  
713 brown) (PDB: 5FVD). Positions of the CDT-arms are indicated.