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Tetramerization of Phosphoprotein Is Essential for Respiratory Syncytial Virus Budding while Its N-Terminal Region Mediates Direct Interactions with the Matrix Protein

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1 **Tetramerization of Phosphoprotein is essential for Respiratory Syncytial virus**
2 **budding while its N terminal region mediates direct interactions with the Matrix**
3 **protein.**

4
5 **Running title: Identification of P protein regions interacting with M protein**

6
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18
19 **Abstract**

20 It was shown previously that the Matrix (M), Phosphoprotein (P), and the Fusion (F)
21 proteins of Respiratory syncytial virus (RSV) are sufficient to produce virus-like
22 particles (VLPs) that resemble the RSV infection-induced virions. However, the exact
23 mechanism and interactions among the three proteins are not known. This work
24 examines the interaction between P and M during RSV assembly and budding. We
25 show that M interacts with P in the absence of other viral proteins in cells using a

26 Split Nano Luciferase assay. By using recombinant proteins, we demonstrate a
27 direct interaction between M and P. By using Nuclear Magnetic Resonance (NMR)
28 we identify three novel M interaction sites on P, namely site I in the α_{N2} region, site II
29 in the 115-125 region, and the oligomerization domain (OD). We show that the OD,
30 and likely the tetrameric structural organization of P, is required for virus-like filament
31 formation and VLP release. Although sites I and II are not required for VLP
32 formation, they appear to modulate P levels in RSV VLPs.

33

34 **Importance**

35 Human RSV is the commonest cause of infantile bronchiolitis in the developed world
36 and of childhood deaths in resource-poor settings. It is a major unmet target for
37 vaccines and anti-viral drugs. The lack of knowledge of RSV budding mechanism
38 presents a continuing challenge for VLP production for vaccine purpose. We show
39 that direct interaction between P and M modulates RSV VLP budding. This further
40 emphasizes P as a central regulator of RSV life cycle, as an essential actor for
41 transcription and replication early during infection and as a mediator for assembly
42 and budding in the later stages for virus production.

43

44 **Introduction**

45 Human RSV is the most frequent cause of infantile bronchiolitis and pneumonia
46 worldwide (1). In France 460,000 infants are infected each year, of which ~30%
47 develop lower respiratory infections and 4.8% to 6.7% are hospitalized, representing
48 45% of the young children admissions at the hospital (2). The enormous burden of
49 RSV makes it a major unmet target for a vaccine and anti-viral drug therapy.
50 However, despite over 60 years of research since its discovery, there is still no

51 vaccine available, and RSV therapy remains mainly supportive. The current standard
52 of care consists of prophylactic treatment of at-risk infants with Palivizumab
53 (Synagis), a monoclonal antibody. However, its limited efficacy (approximately 50%),
54 and high cost (€5.000 per treatment) limits its use to pre-term infants. As a result,
55 60% of at risk children remain untreated, and no efficient therapy is available to treat
56 the adult population (3). There are currently 39 vaccines under development (4). One
57 of the strategies for RSV vaccine development is based on virus like particles
58 (VLPs). However, all anti-RSV VLP vaccines currently in preclinical development are
59 using foreign viral systems incorporating the RSV glycoproteins (5, 6). This is mostly
60 due to the inefficiency of RSV VLP production (most of the virus is cell-associated in
61 cell culture (7)), and to insufficient understanding of RSV particle assembly and
62 budding. RSV VLPs, if they can be produced at sufficient levels, will accurately
63 mimic the viral morphology and structure. Identification of the minimal players
64 involved in particle assembly and budding is an important step in understanding the
65 mechanism behind RSV particle formation. The knowledge can be then used for
66 large scale VLPs and attenuated virus production for vaccination purpose.

67

68 RSV belongs to the *Pneumoviridae* family in the order *Mononegavirales* (8). It
69 primarily infects epithelial cells of the respiratory tract and replicates in the
70 cytoplasm. It is an enveloped, non-segmented, negative-strand RNA virus. The viral
71 genome is encapsidated by the nucleoprotein (N), forming a ribonucleoprotein (RNP)
72 complex, which constitutes the template for the viral polymerase. It was recently
73 shown that the replication and transcription steps of RSV take place in virus-induced
74 cytoplasmic inclusions called inclusion bodies (IBs), where all the proteins of the
75 polymerase complex, i.e. the viral polymerase (L), its main co-factor the P protein,

76 the RNPs and the transcription factor M2-1 concentrate (9). It is noteworthy that
77 pseudo-IBs, similar to those observed in RSV-infected cells, can be observed upon
78 co-expression of only N and P (10, 11). We recently showed that the formation of
79 these pseudo-IBs depends on a liquid-liquid phase separation induced by the N-P
80 interaction (11). Once neo-synthesized, RNPs have to be exported from IBs to the
81 plasma membrane, where RSV virions assemble and bud, forming elongated
82 membrane filaments (12). According to the common paradigm, RSV assembles on
83 the plasma membrane, and infectious viral particles are mainly filamentous (13, 14).
84 However, recent data suggests that viral filaments are produced and loaded with
85 genomic RNA prior to insertion into the plasma membrane. According to this model,
86 vesicles with RSV glycoproteins recycle from the plasma membrane and merge with
87 intracellular vesicles, called assembly granules, containing the RNPs (15, 16).

88

89 Regardless of the cellular location, the minimal RSV viral proteins required for
90 efficient filament formation and budding of VLPs are P, M, and the F protein, more
91 specifically its cytoplasmic tail (FCT) (17, 18). The atomic structure of the external
92 part of F glycoprotein (excluding the transmembrane and cytoplasmic parts) has
93 been resolved (19-21), but little is known about the FCT structure and its function in
94 RSV assembly. M, a key structural protein, directs assembly and budding probably
95 by interacting with FCT on the one hand, and with P associated to RNP on the other
96 hand (22-24). M is required for filament elongation and maturation and, possibly, for
97 transport of the RNP from IBs to the sites of budding (25). M was shown to localize
98 to IBs where, presumably, the first interaction between M and the RNPs occurs.
99 Some early reports have shown that M localization to IBs is mediated by interaction
100 with M2-1 (26, 27). However, more recent work has demonstrated that M is found in

101 IBs when expressed with the N and P proteins alone (17). As N is not required for
102 RSV virus-like filament formation, M was suggested to interact with P. However, the
103 exact mechanism of interactions between these proteins remains largely unknown.
104 Structural data published previously by our group showed that M forms dimers and
105 that the switch from M dimers to higher order oligomers triggers assembly of viral
106 filaments and virus production (28). Based on M structure, a long patch of positively
107 charged surface spanning the entire monomeric protein was suggested to drive the
108 interaction with a negatively-charged membrane (29).

109

110 Functional and structural data are available for the P protein, which is a
111 multifunctional protein capable of interacting with multiple partners. Recent studies
112 allowed better characterization of its interactions and functions within the viral
113 polymerase complex. P forms tetramers of elongated shape composed of a central
114 oligomerization domain (OD), mapped to residues N131-T151 (30-34), and of N- and
115 C-terminal intrinsically disordered regions (IDRs). Structural study of P in solution by
116 NMR gave insight into the secondary structure propensity of these IDRs, forming
117 almost stable helices in the C-terminal region and extremely transient helices in the
118 N-terminal region (35). Residues 1-29 in the N-terminal region confer a chaperone
119 function to P, by binding monomeric and RNA-free N (N^0) and by maintaining N^0
120 unassembled (36). P C-terminal residues 232-241 were shown to bind RNA-bound N
121 assembled as rings mimicking the RNP (37). Very recently, the structure of the L
122 protein bound to tetrameric P solved by cryo-electron microscopy revealed that each
123 of the four P monomers adopts a distinct conformation upon binding to L, the entire
124 L-binding region on P spanning residues 130-228. This includes the OD and the
125 major part of the C-terminal domain (32, 38, 39). As part of viral transcription

126 regulation, P region spanning residues 98-109 was shown to be the binding site for
127 the RSV transcription anti-termination factor M2-1 (40). This interaction is involved in
128 the recruitment of M2-1 to IBs (41). P also plays a pivotal role in M2-1 de-
129 phosphorylation mediated by the host protein PP1, which binds to P through an
130 RVxF-like motif located at P residues 82-87 (41). Dephosphorylated M2-1 is
131 recruited to specific regions in IBs, called IB associated granules (IBAGs), where
132 viral mRNAs are concentrated, before trafficking back to the cytoplasm. This cycle is
133 essential for RSV transcription and translation (41).

134

135 Recently, a P region encompassing residues 39–57 was found to be critical for VLP
136 formation (42). In this study it was also observed that the OD of P had no significant
137 contribution for VLP assembly and budding. Additionally, phosphomimetic
138 substitutions in P region 39-57 inhibited VLP formation, suggesting that this region
139 needs to be un-phosphorylated for VLP production (42). P region 110-120 was also
140 shown to be required for efficient virus budding, affecting the final step of filament
141 scission and virus release (42, 43). However, until now, no direct interaction between
142 P and M has been shown. The lack of structural information on the putative M-P
143 complex makes it difficult to speculate about the mechanism of budding. Thus,
144 identifying specific RSV M-P protein-protein interactions has the potential to break
145 new ground in our understanding of the mechanism behind RSV particle formation.
146 This can further benefit RSV VLP-based vaccine research.

147

148 **Results**

149 **Specific localization of M depends on expression of F, P, and N proteins.**

150 As shown previously, RSV VLPs can be generated independently of viral infection by
151 transfecting cells with plasmids encoding the RSV M, P, N, and F proteins (18, 28).
152 Although N is not required for RSV filament formation (17), it localizes with M in
153 filaments when present, and is required, together with P, for the formation of pseudo-
154 IBs (18, 28). In a first attempt to study the co-localization of M with N and P proteins
155 in pseudo-IBs and in virus-like filaments at the plasma membrane, and to confirm the
156 minimal requirement for filament formation in our system, we used a transfection-
157 based assay.

158 BEAS-2B cells were transfected to express M, N, P, and F, or various combinations
159 of these four proteins. The intracellular localization of RSV proteins and the
160 formation of pseudo-IBs and virus-like filaments were determined by confocal
161 imaging after staining in parallel with either anti-M and anti-N antibodies (Fig. 1A), or
162 anti-P and anti-N (Fig. 1B). In the presence of M, N, P, and F, the formation of
163 pseudo-IBs and of virus-like filaments was detected by immunostaining of M, N and
164 P. Co-localization of M, N, and P was detected on filaments at the plasma
165 membrane as well as in pseudo-IBs, as shown in zoomed merged images (Fig. 1A
166 and 1B, row 1, positive control). When F was absent, M, P, and N localized in
167 pseudo-IBs (Fig. 1A and 1B, row 2). M, but not P or N, was also found in small
168 spikes, most probably at the plasma membrane. In the absence of P or N, no
169 pseudo-IBs formed, as expected (Fig. 1A and 1B, rows 3 and 4). In the absence of
170 P, M was found only as small spikes, whereas N spread all throughout the cytoplasm
171 (Fig. 1A and 1B, row 3). Finally, as previously reported, large filaments containing M
172 and P formed in the absence of N (Fig. 1A and 1B, row 4). Altogether, our results
173 confirm previous observations showing that only M, P, and F are required for virus-

174 like filaments formation, and that M co-localizes with N and P within pseudo-IBs and
175 in virus-like filaments (17, 18, 26, 28).

176

177 **M interacts with P in cells**

178 As M localization in virus-like filaments seems to depend on P, we then studied
179 whether M can interact with P in cells in the absence of other viral proteins. For that
180 purpose, we used the NanoLUC interaction assay based on the split Nano
181 Luciferase reporter (44). In this system, the 114 or the 11S Nano Luciferase
182 fragments were fused to the C-terminus of viral proteins (Fig. 2A). Analysis of the
183 lysates of transfected 293T cells by Western blotting using anti-P, anti-M, anti-N or
184 anti-M2-1 antibody, confirmed protein expression with the fused NanoLUC fragments
185 (Fig. 2B). In addition to the expected bands, a weak higher migrating band, probably
186 unspecific, was detected in P114 and P-11S samples, whereas a double band was
187 detected in M2-1-11S sample, corresponding most probably to the phosphorylated
188 and unphosphorylated forms of M2-1 (41). To investigate the P-M interaction,
189 combinations of two constructs were transfected into 293T cells. 24 h post
190 transfection cells were lysed, luciferase substrate was added, and the luminescence,
191 proportional to the strength of the interaction, was measured (Fig. 2C). As P is
192 known to form tetramers (30-35), P/P interaction was used as positive control. As
193 shown in Fig. 2C, transfection of P-114/P-11S resulted in a high luminescence
194 signal, indicating a strong interaction. We also used the P-N interaction as a control:
195 when co-expressing P-114 and N-11S, positive but relatively low luminescence was
196 observed. Here, as the NanoLUC 114 subunit was cloned at the C-terminus of P
197 protein, thus blocking the interaction between the C-terminus of P and RNA-bound
198 N, the luminescence signal corresponds to the P-N⁰ interaction, which was

199 previously shown to be rather weak, in the micromolar range (36). When M-114 was
200 co-expressed with P-11S, the luminescence signal was similar to the P-N⁰ signal,
201 suggesting that M interacts with P with similar affinity compared to P-N⁰ interaction.
202 In contrast, expression of M-114 with M2-1-11S did not produce luminescence,
203 suggesting that M and M2-1 did not interact when co-expressed in cells in our
204 system. However, for this negative result, we cannot exclude that the C-terminal tag
205 blocks protein-protein interactions that occur via the C-terminus of one or both
206 proteins.

207

208 **M directly interacts with P via its N-terminal region and OD**

209 Next, we investigated whether M directly interacts with P *in vitro* using recombinant
210 proteins, and determined the P region involved in the interaction. Based on structural
211 data available for isolated P (30, 35), we generated rational deletions of each
212 subdomain of P (Fig. 3A). While P fragments lacking the OD (P_{ΔOD}, P₁₋₁₂₆ and P₁₆₁₋
213 ₂₄₁) were shown to be monomeric by NMR, P fragments containing the OD (full-
214 length P, P_{OD}, P₁₋₁₆₃ and P₁₂₇₋₂₄₁) appeared to be associated via the OD (35). The
215 purity and the size of all the P fragments were controlled by SDS-PAGE (Fig. 3B). M
216 and full-length P or P fragments were co-incubated and the formation of complexes
217 was analysed by native agarose gel electrophoresis. Of note, in native gels proteins
218 migrate according to the combination of their size, shape and charges. This explain
219 why P_{ΔOD} migrates at higher apparent molecular weight than full-length P, as the
220 global charges are -20.7 for monomeric P_{ΔOD} and -86.8 for tetrameric P at pH 7.4.
221 When M was incubated with P fragments, shifts were observed only with full-length P
222 and P₁₋₁₆₃ (Fig. 3C), indicating that the N-terminal domain of P (residues 1-131) and
223 the OD (residues 131-151) are together required for a stable P-M interaction in this

224 system. Additionally, no shift was observed neither for $P_{\Delta OD}$ nor for P_{1-126} when
225 incubated in the presence of M. This suggests that either the M-binding site is at
226 least partly on the OD of P, or that M interaction requires tetrameric P. Since no shift
227 was observed for P_{OD} , $P_{127-241}$ and $P_{161-241}$ when incubated with M, these results
228 showed that neither the OD nor the C-terminal region of P on their own or together
229 were sufficient for a detectable P-M interaction *in vitro*.

230 Based on these results, we next wanted to assess if a P fragment containing the N-
231 terminal region and the OD was sufficient to interact with M and to induce membrane
232 filaments in cells. We used the P_{1-161} fragment, shorter than P_{1-163} by two residues,
233 which are outside of the OD on the C-terminal side. Again, we used the filament
234 formation assay. BEAS-2B cells were transfected to express M, N, F and P, P_{1-161} or
235 P_{1-126} constructs, and the formation of RSV virus-like filaments was determined by
236 confocal imaging after staining with anti-M and anti-P primary antibodies (Fig. 3D).
237 As previously shown (Fig. 1), in the presence of M, N, F, and P, the formation of
238 pseudo-IBs and virus-like filaments was observed, and M co-localized with P in both
239 structures (Fig. 3D, row 1, positive control). Co-localization was not seen in all
240 pseudo-IBs, but this could reflect different maturation states of pseudo-IBs. In the
241 absence of P or when P_{1-126} was expressed, neither IBs nor virus-like filaments were
242 detected, and M was found in small spikes (Fig 3D, row 2 and 4). Virus-like filaments
243 were detected when the P_{1-161} construct was expressed, and M- P_{1-161} co-localization
244 occurred in virus-like filaments (Fig 3D, row 3). These results are in agreement with
245 those obtained with the gel shift assay using recombinant proteins (Fig. 3C), and
246 confirmed that the P_{1-161} fragment is competent for M binding and for filament
247 formation. It is noteworthy that under these conditions no pseudo-IBs were detected,
248 because the interaction between the C-terminus of P (missing in the P_{1-161} construct)

249 and N is critical for their formation (45). Altogether, our results show that the P₁₋₁₆₁
250 fragment is sufficient to interact with M and to induce the formation of virus-like
251 filaments in cells in presence of M, N, and F.

252

253 **Identification of M interaction sites on P by NMR**

254 Next, we sought for a method to identify the M interaction site on P, in a residue–
255 specific manner, without resorting to mutations or internal deletions in potential
256 regions of interest. We have previously used NMR to localize molecular recognition
257 features (MoRFs) on P, taking advantage of the intrinsically disordered nature of this
258 protein (46). Perturbations in amide ¹H-¹⁵N correlation spectra, either of intensities
259 (Fig. 4A and 5) or chemical shifts, were used to map regions that sense direct
260 interaction and/or conformational changes due to binding of a partner.

261 To control the oligomerization state of M, we used the M-Y229A mutant, which was
262 shown to form dimers, but is less prone to form higher order oligomers as compared
263 to WT M (28). No aggregation or self-assembly of M-Y229A was observed, as the
264 samples stayed clear and M-specific signal was detected in ¹H NMR spectra, also in
265 the presence of P (Fig. 4B). Of note, since M-Y229A is a dimeric 2x29 kDa folded
266 protein, M signals are much broader than P signals that stem from the intrinsically
267 disordered N-terminal region of P. This was also verified by SEC analysis made after
268 NMR measurements (data not shown). A buffer with reduced ionic strength (150 mM
269 NaCl as compared to 300 mM, the usual M storage buffer (13)) was used to enhance
270 potential electrostatic interactions between M and P. The temperature was set to 288
271 K to increase the stability of M in this buffer, and also to reduce NMR signal
272 broadening of solvent-exposed amide protons due to exchange with water. The final
273 concentration of M was set to 100 μM. Concentrations and molar ratios are given for

274 protomers, independently of the oligomerization state of the proteins. Since the C-
275 terminal part of P seemed to be dispensable for interaction with M and for virus-like
276 filament formation (Fig. 3), we performed experiments with ^{15}N -labeled P fragments
277 devoid of this part rather than with full-length P: this reduces signal overlap in ^1H - ^{15}N
278 correlation spectra.

279 Incubation of ^{15}N -labeled P_{1-163} with M-Y229A at a P:M molar ratio of 1:4 (25 μM P
280 and 100 μM M) resulted in significant intensity decrease of several P_{1-163} amide
281 signals in 2D ^1H - ^{15}N correlation NMR spectra (Fig. 4A). These perturbations took
282 place in two proximal regions located immediately upstream of the OD: in the $\alpha_{\text{N}2}$
283 region (residues 98-111) and in a stretch spanning residues 115-125 (Fig. 4C). The
284 first corresponds to the extremely transient helix $\alpha_{\text{N}2}$, previously identified as the
285 binding site of RSV M2-1 protein (46, 47). Due to specific dynamics in the α -helical
286 coiled-coil OD, amide ^1H - ^{15}N signals are broadened out beyond detection at 288 K
287 for P fragments that contain the OD flanked by N- and/or C-terminal extensions (46).
288 Hence, M-binding to the OD could not be assessed using P_{1-163} .

289 A concentration effect for P_{1-163} was evidenced by comparing the intensity ration I/I_0
290 measured with two different P:M molar ratios (Fig. 4C). A maximal effect, where
291 signals disappear completely, could not be reached, because M could not be
292 concentrated above 100 μM without starting to aggregate and because the
293 concentration of ^{15}N -labeled P had to be kept >10 μM due to the sensitivity limitation
294 of NMR. Altogether, the NMR experiments with P_{1-163} show that an interaction takes
295 place between P_{1-163} and M-Y229A, which involves a region immediately upstream of
296 the OD. This interaction is rather weak. A set of NMR data with P_{1-163} and M-Y229A
297 was also acquired in a buffer with 300 mM NaCl and compared to 150 mM NaCl
298 (Fig. 4D). Overall intensity perturbation is larger with lower salt concentration,

299 indicating that M-Y229A binding is stronger under these conditions and suggesting
300 that there may be an electrostatic contribution to the P-M interaction.

301 To assess the role of the OD, we used the P₁₋₁₂₆ fragment, which is devoid of it.
302 When incubated with 4 molar equivalents of M-Y229A, no significant effect was
303 observed in the intensities of the ¹H-¹⁵N correlation spectrum (Fig. 5A and 5D, upper
304 panel), indicating that the OD is required for M binding. These results correlate well
305 with the band shift experiments for the M/P complex using native gel analysis (Fig.
306 3C). To further characterize the binding site on P₁₋₁₆₃, we performed NMR
307 interactions experiments using the shorter P₁₁₃₋₁₆₃ fragment, which contains only the
308 OD flanked by residues 115-130 and 152-163 (Fig. 5B and 5D, lower panel). In the
309 presence of M-Y229A, NMR signal intensities decreased by 30 % upstream and
310 downstream of the OD. A stronger effect was again observed for residues 115-125
311 (Fig. 5B and 5D, lower panel), suggesting that they play a particular role in M
312 binding. Like for P₁₋₁₆₃, the OD could not be observed for P₁₁₃₋₁₆₃ fragment at 288 K.
313 However, these signals become visible at higher temperature for P₁₁₃₋₁₆₃ (Fig. 5B
314 and 5D, lower panel). The intensity perturbation pattern of P₁₁₃₋₁₆₃ at 298 K suggests
315 that the OD region is affected by the presence of M-Y229A. For P₁₂₀₋₁₆₀, the smallest
316 fragment used in this study, all amide signals can be observed, even at 288 K (Fig.
317 5C). M-Y229A induces a weak global reduction in intensity that may point to weak
318 binding to P₁₂₀₋₁₆₀. Taken together, these results suggest that M binding to P is
319 achieved through multiple contact sites that are located in α_{N2} (site I), in the 115-125
320 region (site II) and potentially in the OD. Binding to any of these sites appears be
321 rather weak, and M binding to the two N-terminal P regions requires tetrameric P.

322

323 **Validation of M-binding sites on P using P deletion mutants**

324 In order to validate the NMR data showing multiple M binding sites, we then
325 performed band shift assays with different fragments of P. We generated P deletions
326 using P₁₋₁₆₃ as a template and deleted a different part of the N-terminal domain for
327 each construct. The OD was present in all constructs to keep the P protein as a
328 tetramer (Fig. 6A). Each construct contained different M-binding domains. P₄₀₋₁₆₃
329 contained the region spanning residues 39-57, previously reported to affect RSV
330 assembly and potentially being involved in M interaction (42), as well as all binding
331 sites identified by NMR (Fig. 4 and 5). P₈₀₋₁₆₃ contained the two potential binding
332 sites I and II identified by NMR. P₁₁₃₋₁₆₃ contained only site II, and P₁₂₀₋₁₆₀ lacked both
333 sites, containing only the OD. We used both WT M and M-Y229A in order to
334 ascertain that the NMR results were not biased by the Y229A M mutation. M
335 proteins, full-length P and P fragments were purified as previously and analysed by
336 SDS-PAGE (Fig. 6B). M and P constructs were co-incubated before analysis of
337 complexes by native agarose gel electrophoresis (Fig. 6C). Shifts were observed
338 with full-length P and P₁₋₁₆₃, as shown above in Fig. 3C. P₄₀₋₁₆₃ and P₈₀₋₁₆₃ fragments
339 (containing both sites I and II and OD) also induced a shift. No band shift was
340 detected when M was incubated with P₁₁₃₋₁₆₃ (containing only site II and OD) or with
341 P₁₂₀₋₁₆₀. Similar shifts were observed for M WT and M Y229A. This is in line with our
342 NMR results (Fig. 4 and 5), indicating that M binding by P is achieved through
343 multiple sites. Interactions seen by NMR with P₁₁₃₋₁₂₆ or P₁₂₀₋₁₆₀ were not strong
344 enough to induce a band shift when analyzed by native gel electrophoresis.

345 In order to verify which of the identified specific M/P binding regions is relevant for
346 interaction between M and P in cells, we used again the NanoLUC interaction assay
347 based on the split Nano Luciferase reporter. The 114 or the 11S Nano Luciferase
348 fragments were fused to the C-terminus of M and P proteins (Fig. 2A). P_{WT} and four

349 P deletion mutants were analysed: P_{Δ39-57} (deleted of the region previously reported
350 to be involved in M/P interaction (42)), P_{Δ93-126} (lacking sites I and II as identified by
351 NMR), P_{Δ39-57,Δ93-126} (lacking the three regions), and P_{ΔOD}. Combinations of two
352 proteins were transfected into 293T cells. 24 h post transfection cells were lysed,
353 luciferase substrate was added, and the luminescence, proportional to the strength
354 of the interaction, was measured (Fig. 6D). P/P interaction was again used as
355 positive control. As shown in Fig. 6D, transfection of P-114/P-11S resulted in high
356 luminescence signal, indicating a strong interaction. Transfection of M-114 and P-
357 11S resulted in positive signal (comparable to Fig. 2C). Transfection of P_{Δ39-57}-11S,
358 P_{Δ93-126}-11S, or P_{Δ39-57, Δ93-126}-11S and M-114, all resulted in luminescence
359 comparable to transfection when P-11S was used. In contrast, when M-114 was
360 transfected with P_{ΔOD}-11S, luminescence signal was almost completely lost. These
361 results indicate that, in transfected cells, P OD deletion, and as a consequence
362 abrogated tetramerization, resulted in loss of interaction with M, whereas deletion of
363 amino acids 39-57, 93-126 or both regions did not. Of note, analysis of the lysates of
364 transfected 293T cells by Western blotting using anti-P or anti-M antibody confirmed
365 correct protein expression with the fused NanoLUC fragments (Fig. 6E).

366

367 **Functional implications of P regions displaying direct M-binding**

368 Next, we asked whether the identified M-binding domains on P were of functional
369 significance. We analysed virus-like filament formation using full-length P or deletion
370 constructs based on NMR results and on previous publications. Specifically, we
371 checked full-length P, P₁₋₁₆₁, P_{ΔOD}, P_{Δ39-57}, P_{Δ93-110} (deleted of M-binding site I), P_{Δ113-}
372 ₁₂₆ (deleted of M-binding site II), and P_{Δ93-126} (deleted of both sites I and II as
373 identified by NMR) (Fig. 7A). BEAS-2B cells were transfected to express M, N, F and

374 P_{WT} or various P deletion constructs, and the formation of RSV virus-like filaments
375 was determined by confocal imaging and immuno-fluorescence after staining with
376 anti-M and anti-P primary antibodies (Fig. 7B). Transfecting M, N, F and P resulted in
377 pseudo-IBs and filament formation, and co-localization of M and P proteins in both
378 (positive control, Fig. 7B, upper left). Transfecting M, N, F and $P_{\Delta OD}$ did not produce
379 any virus-like filaments or pseudo-IBs, and M and P did not co-localize (Fig. 7B,
380 lower left). Cells expressing $P_{\Delta 39-57}$ failed to produce virus-like filaments, and
381 although P was found in pseudo-IBs, M was not recruited to IBs (Fig. 7B, upper
382 right). Transfection of $P_{\Delta 93-126}$ resulted in virus-like filament formation (Fig. 7B, lower
383 right), and both M and P proteins were found in virus-like filaments, similar to P_{WT} ,
384 showing that sites I and II are not critical for these processes.

385 Finally, we asked whether P deletion mutants could negatively affect VLP release.
386 HEP-2 cells were transfected to express M, F, and various P constructs. The N
387 protein was not included in the assay, since it is not required for VLP production (Fig.
388 1 and (17)). Cell lysates (soluble fractions) and the VLPs released into the media
389 were analysed by Western blotting using anti-P and anti-M antibodies (Fig. 7C,
390 upper panel). All P mutants were correctly expressed and were well detected by the
391 anti-P antibody, as shown by the bands in the cell lysates. Quantification of relative
392 M and P levels in the VLPs, revealed by WB, is shown in Fig. 7C, lower panel. When
393 P_{WT} was expressed, VLP release was validated using anti-M antibodies, and P was
394 detected in VLPs using anti-P antibodies (positive control). The absence of P
395 prevented VLP release (negative control). The P_{1-161} construct resulted in VLP
396 release comparable to P_{WT} . Expression of $P_{\Delta OD}$ greatly reduced VLP release, as
397 almost no M could be detected. $P_{\Delta 39-57}$ abolished VLP release, as no M and P were
398 detected. This is in agreement with our virus-like filament formation assay (Fig. 7B),

399 where neither $P_{\Delta OD}$ nor $P_{\Delta 39-57}$ induced filament formation. Transfection of $P_{\Delta 93-110}$,
400 $P_{\Delta 113-126}$ or $P_{\Delta 93-126}$ mutants did not abolish VLP release, as M was detected in the
401 VLP fraction at comparable levels to P_{WT} with $P_{\Delta 93-126}$, or slightly reduced with $P_{\Delta 93-}$
402 110 and $P_{\Delta 113-126}$. $P_{\Delta 93-110}$ incorporation into released VLPs was comparable to that of
403 P_{1-161} , i.e. with a 25-30% reduction relative to P_{WT} . In contrast, transfection of $P_{\Delta 113-}$
404 126 and even more of $P_{\Delta 93-126}$ greatly reduced detection of these mutants in released
405 VLPs. This suggests that $P_{\Delta 113-126}$ and $P_{\Delta 93-126}$ mutants still supported VLP formation,
406 in contrast to $P_{\Delta OD}$ and $P_{\Delta 39-57}$, but could be more easily displaced from VLPs than
407 $P_{\Delta 93-110}$, P_{1-161} or P_{WT} . This effect appears to be linked to the deletion of site II. It was
408 not evidenced in the virus-like filament formation assay carried out with $P_{\Delta 93-126}$ (Fig.
409 7D), most likely because of milder conditions.

410

411 Discussion

412 RSV M makes a direct interaction with RSV P

413 It was reported earlier that the three proteins, RSV P, M and F were sufficient for
414 virus-like filament formation and VLP release (17). Our results confirm this minimal
415 requirement (Fig. 1). A question that remained open was which interactions M
416 needed to be involved in to promote assembly and budding of viral particles. For
417 *Paramyxoviridae* it was shown that M proteins organize viral assembly by bridging
418 between the glycoproteins and the RNPs, and that specific M-N interactions were
419 required for the RNP to be packed into viral pseudo-particles (48). The interaction
420 between the RNP and M proteins occurs most probably first in IBs and/or assembly
421 granules (16), where M recruits the RNP, before they are transported to viral
422 filaments formed on the cellular membrane.

423 Here we showed that M co-localized with P in pseudo-IBs, which are formed when N
424 and P are present, as well as in virus-like filaments, even in the absence of N (Fig.
425 1). Our NanoLUC results showed that M could interact with P in the absence of other
426 viral proteins in cells (Fig. 2). Gel shift assays performed with purified recombinant M
427 and P fragments (Fig. 3C) confirmed M and P could directly interact. Moreover, our
428 data indicated that the P domain responsible for this M interaction was located in
429 fragment P₁₋₁₆₃, comprising the N terminal domain and the OD of P (Fig. 3C and Fig.
430 6C). When M was co-expressed with P₁₋₁₆₁, no pseudo-IBs were formed, as
431 expected, since P₁₋₁₆₁ lacks the C-terminus of P needed for interaction with RNA-N
432 complexes and formation of IBs (11, 37, 45). However, virus-like filaments were
433 formed, where M and P₁₋₁₆₁ co-localized. This argues against the requirement of
434 preliminary IB formation before virus-like filament assembly, as these were observed
435 in cells transfected with RSV M, P, and F only. Furthermore, an interaction between
436 RSV M and P strongly suggests that for RSV assembly, bridging between M and the
437 RNP might be mediated by P.

438

439 **P displays multiple direct contact sites for M**

440 According to our NMR data (Fig. 4 and 5), M binding to P could be achieved through
441 multiple contact sites that are located within three regions, α_{N2} region (site I), the
442 115-125 region (site II) and the OD (Fig. 5). It must be noted that NMR experiments
443 were carried out with the M-Y229A mutant, which stays dimeric in solution. The
444 interactions observed by NMR therefore represent a P-M complex formed with
445 dimeric M. The NMR results were corroborated *in vitro* by native gel interaction
446 assays using both M WT and M-Y229A and N-terminally truncated P fragments (Fig.
447 3 and 6). Interestingly, neither sites I and II together but without the OD (P₁₋₁₂₆ as

448 compared to P₈₀₋₁₆₃ or P₁₋₁₆₃) nor the OD without sites I and II (P₁₂₀₋₁₆₀ as compared
449 to P₈₀₋₁₆₃) seemed to be sufficient to bind M (Fig. 3C, 4, 5 and 6C). The split
450 luciferase assay shed another light on the complexity of M-P interactions. The
451 deletion of the internal N-terminal region spanning site I and II did not impair M
452 binding *in cellula*, in contrast to the internal deletion of the OD, which completely
453 impaired the M-P interaction under the same conditions (Fig. 6D). This could indicate
454 that other short intrinsically disordered N-terminal motifs, similar in sequence to sites
455 I or II, could complement the M-binding site, as they are closer to the OD than in P_{WT}
456 due the 30-residue long truncation, or that cellular proteins help stabilize the M-P
457 complex. Interestingly, as illustrated by the P_{Δ39-57} deletion mutant, highlighting the
458 essential role of the 39-57 region (42), the ability to form an M-P complex *in cellula* is
459 not correlated with virus-like filament formation or P-M co-localization on these virus-
460 like filaments (Fig. 6D and 7B).

461 Although P OD is not sufficient to bind M, it seems to play a major role, possibly
462 because of the higher level of structural organization provided by tetramerization. We
463 previously showed that transient intra-protomer interactions take place in P (35), and
464 the cryo-EM structures of *Pneumoviridae* L-P complexes show that the four
465 protomers can engage each in different interaction in a single complex (32, 38, 39).
466 This suggests that the P-M interactions are complex and probably cooperative, and
467 that M could recognize one or several sites only formed when four P protomers are
468 present.

469 As can be seen from *Pneumoviridae* P protein sequence alignment (Fig. 8), the N-
470 terminal P region contains conserved motifs, in particular the N⁰-binding motif, the
471 RVxF-like motif, which is the binding site for cellular PP1 phosphatase (41), and α_{N2},
472 which is the binding site for M2-1 protein. This region also contains conserved

473 clusters of negatively charged residues, notably just upstream and inside binding site
474 II. As indicated by reduced M-P binding in high salt buffer, these residues could be
475 important for interaction with M, which displays a large positively charged surface
476 patch (29). Finally, the differences observed between *in vitro* and *in cellula*
477 experiments could also depend on post-translational modifications of the proteins.

478

479 **Functional relevance of direct binding between M and P**

480 Our virus-like filament formation and VLP assays shed light on the functional
481 implications of these interactions. Whereas the OD, and likely tetrameric
482 organization provided by the OD, was clearly needed for VLP formation, deletion of
483 sites I or II individually, or both at the same time, did not prevent virus-like filament
484 production (Fig. 7B). However, reduced levels of P were detected in released VLPs,
485 when both sites I and II were simultaneously deleted (Fig. 7C). This could be
486 attributed to detachment of mutated P from released VLPs rather than defective
487 recruitment to virus-like filaments, since co-localization was observed in these
488 structures (Fig. 7B). This could also rise the question about the integrity of these
489 VLPs.

490 M binding sites I and II thus rather appear to modulate P binding to M and to affect
491 the final organization of the VLPs. The newly identified sites I and II also stand in
492 contrast to the previously reported functional importance of the P region spanning
493 residues 39-57 (42). In agreement with previously published data, we confirm that
494 P_{Δ39-57} completely prevented filament formation and VLP budding (Fig. 7B and 7C).
495 However, no perturbations were detected in the NMR signals in the 39-57 region of
496 P in presence of M-Y229A (Fig. 4). The P-M interaction was still detected when
497 residues 39-57 were deleted in transfected cells (Fig. 6D), indicating that this is

498 probably not a direct binding region for M, at least not in its dimeric form.
499 Dephosphorylation of the Ser/Thr-rich 39-57 region of P was reported to be required
500 for VLP formation (42). This was the case for recombinant P protein produced in *E.*
501 *coli*. As shown by our IF confocal microscopy experiments (Fig. 7B), M failed to co-
502 localize to pseudo-IBs when P_{Δ39-57} was expressed. This could indicate that a third,
503 possibly cellular factor is required for M recruitment to IBs. Some early reports have
504 shown that M localization to IBs was mediated by an interaction with M2-1 (26, 27),
505 and M interaction with M2-1 in cells was shown by co-IP (49). Cryo-EM analysis of
506 RSV filamentous particles showed that M2-1 is located between M layer and RNP
507 (14, 50, 51). However, our work here shows that M2-1 is not required for M
508 localization into pseudo-IBs (Fig. 1). Moreover M and M2-1 did not interact in our
509 NanoLUC assay (Fig. 2).

510 In conclusion, our virus-like filament formation and VLP assays show a strong
511 functional relevance of P region 39-57, which is essential for M localization to IBs
512 and budding process, but most probably not due to a direct P-M binding. In addition,
513 we show the functional importance of the tetramerisation of P, occurring via the short
514 OD region. The OD is probably part of the M interaction site with P, but must be
515 complemented by the N-terminal region of P to bind M (Fig. 7).

516

517 **Possible role for RSV P as a switch between transcription and budding**

518 Although the functional relevance of binding site I (α_{N2} region) could not be fully
519 assessed in the framework of this study, it is striking that it completely overlaps with
520 the binding site of transcription anti-termination factor M2-1, which is an essential
521 factor for efficient RSV transcription (52) (Fig. 8). It is likely that RNA synthesis
522 processes are frozen just before viral budding. However, such mechanism has not

523 been clearly investigated to our knowledge. It is established that P plays a critical
524 role in viral transcription, viral RNA synthesis and budding. In particular, interaction
525 of P with M2-1 is critical for M2-1 recruitment to IBs (53). The overlap of binding sites
526 for two essential proteins driving transcription (M2-1) on the one hand and virus
527 assembly (M) on the other hand, could be part of a switch mechanism, where both
528 proteins compete for this site. Interestingly, binding sites I and II contain four
529 reported phosphorylation sites on T108 (54), S116, S117 and S119 (43), which are
530 conserved among RSV P proteins (Fig. 8). Previously published data showed that P
531 phosphorylation on residue T108 abolished P-M2-1 interaction (54). Phosphomimetic
532 mutants of P serines 116, 117 and 119 (inside binding site II) were reported to
533 significantly prevent virus budding (43). Moreover, P lacking residues 110-120
534 reduced budding, which was completely restored when these residues were added
535 to P (42). P is known to be highly phosphorylated in purified virions, and the timing of
536 appearance of phosphorylated P was shown to correspond to the release of RSV
537 virions (55). We therefore suggest that the phosphorylation state of P sites I and II
538 could regulate the switch between RSV transcription/replication and assembly.
539 In summary, our work further confirms that RSV P protein is a multifunctional protein
540 playing different roles depending on its interactions with other viral proteins. We
541 confirm here that P plays a key role in RSV assembly. We also bring evidence for a
542 direct interaction between M and P, P OD being required for M-P interaction and
543 sites I and II modulating P binding to M in VLPs by mechanisms yet to uncover.

544

545 **MATERIALS AND METHODS**

546 **Plasmid constructs**

547 pcDNA3.1 codon-optimized plasmids for mammalian expression encoding the RSV
548 A2 M, P, N, F, and M2-1 proteins were a gift from Marty Moore, Emory University
549 (56). Commercially made pciNanoLUC 114 and 11S vectors (GeneCust) were used
550 to clone the RSV A2 codon-optimized M, P, N, and M2-1 constructs using standard
551 PCR, digestion and ligation techniques. pcDNA P₁₋₁₂₆ and P₁₋₁₆₁ deletion mutants of
552 P were obtained by introducing stop codons at the appropriate site in the coding
553 sequence. All pcDNA3.1 P deletion mutants in the full-length construct were
554 generated by using the Q5 site-directed mutagenesis kit (New England BioLabs),
555 following the manufacturer recommendations. pciNanoLUC 11S P_{Δ39-57}, P_{Δ93-126}, P<sub>Δ39-
556 57, Δ93-126</sub>, and P_{ΔOD} mutants were obtained by re-cloning the relevant constructs from
557 pcDNA vector using standard PCR, digestion and ligation techniques. For
558 expression and purification of recombinant P proteins, the previously described
559 pGEX-P, and pGEX-P₁₋₁₂₆, pGEX-P₁₋₁₆₃, pGEX-P_{ΔOD}, pGEX-P₁₂₇₋₂₄₁ and pGEX-P₁₆₁₋₂₄₁
560 plasmids were used (36). pGEX-P_{OD}, pGEX-P₄₀₋₁₆₃, pGEX-P₈₀₋₁₆₃, pGEX-P₁₁₃₋₁₆₃, and
561 pGEX-P₁₂₀₋₁₆₀ were generated using standard PCR, digestion and ligation techniques
562 and introducing codons at the appropriate site in the coding sequence. It is important
563 to note that pcDNA P₁₋₁₆₁ and pGEX P₁₋₁₆₃ were used for cellular expression or for
564 bacterial expression respectively. For expression and purification of recombinant M
565 protein, the previously described pCDF-M and pCDF-M_{Y229A} were used (28).

566

567 **Cell culture**

568 HEp-2 (ATCC CCL-23TM) and 293T cells were maintained in Dulbecco modified
569 Eagle medium (eurobio) supplemented with 10% fetal calf serum (FCS; eurobio), 1%
570 L-glutamine, and 1 % penicillin streptomycin. The transformed human bronchial
571 epithelial cell line (BEAS-2B) (ATCC CRL-9609) was maintained in RPMI 1640

572 medium (eurobio) supplemented with 10% fetal calf serum (FCS; eurobio), 1% L-
573 glutamine, and 1% penicillin-streptomycin. The cells were grown at 37°C in 5% CO₂.

574

575 **Bacteria expression and purification of recombinant proteins**

576 For M expression (WT and Y229A mutant), *E. coli* Rosetta 2 bacteria transformed
577 with the pCDF-M plasmid were grown from fresh starter cultures in Luria-Bertani (LB)
578 broth for 5 h at 32°C, followed by induction with 0.4 mM isopropylthi-galactoside
579 (IPTG) for 4 h at 25°C. Cells were lysed by sonication (4 times for 20 s each time)
580 and lysozyme (1 mg/ml; Sigma) in 50 mM NaH₂PO₄-Na₂HPO₄, 300 mM NaCl, pH
581 7.4, plus protease inhibitors (Roche), RNase (12 g/ml, Sigma), and 0.25% CHAPS
582 {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, . Lysates were
583 clarified by centrifugation (23,425 g, 30 min, 4°C), and the soluble His₆-M protein
584 was purified on an Nickel sepharose column (HiTrap™ 5 ml IMAC HP; GE
585 Healthcare). The bound protein was washed extensively with loading buffer plus 25
586 mM imidazole and eluted with a 25 to 250 mM imidazole gradient. M was
587 concentrated to 2 ml using Vivaspin20 columns (SartoriusStedimBiotec) and
588 purified on a HiLoad 10/600 Superdex S200 column (GE Healthcare) in 50 mM
589 NaH₂PO₄-Na₂HPO₄, 300 mM NaCl, pH 7.4. The M peak was concentrated to 3
590 mg/ml using Vivaspin4 columns. The quality of protein samples was assessed by
591 SDS-PAGE. Protein concentration was determined by measuring absorbance at 280
592 nm. For NMR interaction experiments a fresh preparation of M was dialyzed into
593 NMR buffer.

594 For P expression, *E.coli* BL21 (DE3) bacteria transformed with pGEX-P derived
595 plasmids were grown at 37°C for 8 h in LB. Protein expression was induced by
596 adding one volume of fresh LB medium, 0.4 mM IPTG for 16 h at 28°C. Bacterial

597 pellets were resuspended in lysis buffer (20 mM Tris/HCl pH 7.4, 60 mM NaCl, 1 mM
598 EDTA, 1 mg/mL lysozyme, 1 mM DTT, 0,1% Triton X-100) supplemented with
599 complete protease inhibitor cocktail (Roche) for 1 h on ice. Benzonase (Millipore)
600 was then added and the lysate was incubated for 1 h at RT under rotation. The
601 lysates were centrifuged at 4°C for 30 min at 10,000g. Glutathione-Sepharose 4B
602 beads (GE Healthcare) were added to the clarified supernatants and the mixtures
603 were incubated overnight at 4°C under rotation. The beads were washed with lysis
604 buffer, three times with 1X phosphate-buffered saline (PBS) and then stored at 4°C
605 in an equal volume of PBS.

606 For ¹⁵N-labeled P expression, bacteria were grown in minimal medium supplemented
607 with ¹⁵NH₄Cl (Eurisotop) as a ¹⁵N source. The GST tag was removed by thrombin
608 (Millipore) cleavage and the cleaved product exchanged into NMR buffer (50 mM Na
609 phosphate at pH 6.7, 150 mM NaCl). The quality of protein samples was assessed
610 by SDS-PAGE. Protein concentration was determined by Bradford assay (Biorad)
611 and checked by measuring absorbance at 280 nm for fragments containing tyrosine
612 residues. It is given as protomer concentration in the case of P tetramers.

613

614 **NanoLUC interaction assay**

615 Constructs expressing the NanoLUC subunits 114S and 11S were used (44). 293T
616 cells were seeded at a concentration of 3x10⁴ cells per well in 48-well plate. After 24
617 h, cells were co-transfected in triplicate with 0.4 µg of total DNA (0.2 µg of each
618 plasmid) using Lipofectamine 2000 (Invitrogen). 24 h post transfection cells were
619 washed with PBS, and lysed for 1 h in room temperature using 50 µl NanoLUC lysis
620 buffer (Promega). NanoLUC enzymatic activity was measured using the NanoLUC
621 substrate (Promega). For each pair of plasmids, three normalized luminescence

622 ratios (NLRs) were calculated as follows: the luminescence activity measured in cells
623 transfected with the two plasmids (each viral protein fused to a different NanoLUC
624 subunit) was divided by the sum of the luminescence activities measured in both
625 control samples (each NanoLUC fused viral protein transfected with an plasmid
626 expressing only the NanoLUC subunit) Data represents the mean \pm SD of 4
627 independent experiments, each done in triplicate. Luminescence was measured
628 using Infinite 200 Pro (Tecan, Männedorf, Switzerland).

629

630 **NMR spectroscopy**

631 For NMR experiments, P and M solutions were mixed to obtain the desired molar
632 ratio and concentrated to reach a concentration of 100 μ M for M (WT and Y229A
633 mutant).

634 NMR measurements were carried out in a Bruker Avance III spectrometer at a
635 magnetic field of 18.8 T (800 MHz 1 H frequency) equipped with a cryogenic TCI
636 probe. The magnetic field was locked with 7 % 2 H $_2$ O. The temperature was set to
637 288 K or 298 K. 1 H- 15 N correlation spectra were acquired with a BEST-TROSY
638 version. Spectra were processed with Topspin 4.0 (Bruker Biospin) and analyzed
639 with CCPNMR 2.4 (57) software.

640 1 H and 15 N amide chemical shift assignment of full-length P and P $_{1-126}$ and P $_{1-163}$
641 fragments was done previously (46). Amide chemical shift assignment of 15 N-labeled
642 P $_{113-160}$ and of 15 N-labeled P $_{120-160}$ was done by recording a 15 N-separated NOESY-
643 HSQC spectrum with 80 ms mixing time. Sequential information was retrieved
644 through H $_{Ni}$ -H $_{Ni-1}$, H $_{Ni}$ -H $_{Ni+1}$ and H $_{Ni}$ -H $_{\alpha i-1}$ correlations.

645

646 **Virus-like filament/particle formation**

647 Over night cultures of BEAS-2B cells seeded at 4×10^5 cells/well in 6-well plates (on a
648 16-mm micro-cover glass for immunostaining) were transfected with pcDNA3.1
649 codon-optimized plasmids (0.4 μ g each) carrying the RSV A2 WT or deletion/mutant
650 P protein along with pcDNA3.1 codon-optimized plasmids carrying RSV A2 M, N,
651 and F using Lipofectamine 2000 (Invitrogen) according to the manufacturer's
652 recommendations. Cells were fixed at 24 h post transfection, immunostained, and
653 imaged as described below. For VLP formation, over-night cultures of HEp-2 cells
654 seeded at 4×10^5 cells/well in 6-well plates were transfected as described above.
655 Released VLPs were harvested from the supernatant; the supernatant was clarified
656 of cell debris by centrifugation (1,300 g, 10 min, 4°C) and pelleted through a 20%
657 sucrose cushion (13,500 g, 90 min, 4°C). Cells were lysed in radio immune
658 precipitation assay (RIPA) buffer. Cellular lysates and VLP pellets were dissolved in
659 Laemmli buffer and subjected to Western analysis.

660

661 **Immunostaining and imaging**

662 Cells were fixed with 4% paraformaldehyde in PBS for 10 min, blocked with 3% BSA
663 in 0.2% Triton X-100–PBS for 10 min, and immunostained with monoclonal anti-M
664 (1:200; a gift from Mariethe Ehnlund, Karolinska Institute, Sweden), polyclonal anti-N
665 (1:5000; (30)), polyclonal anti-P (1:500; (30)) or monoclonal anti-P (1:100; a gift from
666 Jose A. Melero, Madrid, Spain) antibodies, followed by species-specific secondary
667 antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 568 (1: 1,000; Invitrogen).
668 Images were obtained using the White Light laser SP8 (Leica Microsystems,
669 Wetzlar, Germany) confocal microscope at a nominal magnification of 63. Images
670 were acquired using the Leica Application Suite X (LAS X) software.

671

672 **SDS-PAGE and Western analysis**

673 Protein samples were separated by electrophoresis on 12% polyacrylamide gels in
674 Tris-glycine buffer. All samples were boiled for 3 min prior to electrophoresis.
675 Proteins were then transferred to a nitrocellulose membrane (RocheDiagnostics).
676 The blots were blocked with 5% non fat milk in Tris-buffered saline (pH 7.4), followed
677 by incubation with rabbit anti-P antiserum (1:5,000) (30), rabbit anti N antiserum
678 (1:5,000) (30), rabbit anti M antiserum (1:1,000), or rabbit anti M2-1 antiserum
679 (1:2,000) (30), and horseradish peroxidase (HRP)-conjugated donkey anti-rabbit
680 (1:5,000) antibodies (P.A.R.I.S.). Western blots were developed using freshly
681 prepared chemiluminescent substrate (100 mM TrisHCl, pH 8.8, 1.25 mM luminol,
682 0.2 mM p-coumaric acid, 0.05% H₂O₂) and exposed using BIO-RAD ChemiDoc™
683 Touch Imaging System.

684

685 **Generation of M antiserum**

686 Polyclonal anti M serum was prepared by immunizing a rabbit three times at 2 week
687 intervals using purified His- fusion proteins (100 mg) for each immunization. The first
688 and second immunizations were administered subcutaneously in 1 ml Freund's
689 complete and Freund's incomplete adjuvant (Difco), respectively. The third
690 immunization was done intramuscularly in Freund's incomplete adjuvant. Animals
691 were bled 10 days after the third immunization.

692

693 **Native gel**

694 Protein samples were separated by electrophoresis on 1% agarose gel in TBE (Tris
695 Borate EDTA) buffer. 50 µg M and x 10 µg P were incubated in PBS buffer (pH 7.4)

696 for 20 min in RT. Samples were mixed with 50% sucrose and run for 2 h at 80 V,
697 following by staining with Amido Black.

698

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890

891

892 **Figure legends**

893 **Fig.1: Specific localization of M depends on expression of F, P, and N proteins.**

894 BEAS-2B cells were co-transfected with pcDNA3.1 plasmids expressing RSV M, P,
895 N, and F or a different combination of three proteins. 24 h post-transfection, cells
896 were fixed, and immunostained with **(A)** anti-M (green) and anti-N (red) or **(B)** anti-P
897 (green) and anti-N (red) primary antibodies followed by Alexa Fluor secondary
898 antibodies, and were analyzed by confocal microscopy. Scale bars represent 10µm.
899 Merged images are zoomed in 3X.

900

901

902 **Fig. 2: M interacts with P in cells.** Protein-protein interactions were measured
903 using the NanoLuc assay. **(A)** Scheme of the RSV protein constructs fused with
904 NanoLUC 114 or 11S subunit and pair combinations used in (C). **(B)** 293T cells were
905 transfected with plasmids encoding P, N, M, or M2-1 fused to 114 or 11S NanoLUC
906 subunit. Cells were lysed 24 h post transfection, and cell lysates were then subjected
907 to Western analysis using anti-P, anti-M, anti-N or anti-M2-1 polyclonal antibody.
908 Size markers are shown on the left side of each gel. **(C)** 293T cells were transfected
909 with pairs of constructs, combined as shown in the graph. P/P and P/N were used as
910 positive controls. Cells were lysed 24 h post transfection, and luminescence was
911 measured using a Tecan Infinite 200 plate reader. The NLR is the ratio between
912 actual read and negative controls (each protein with the empty NanoLUC vector).
913 The graph is representative of four independent experiments, each done in three
914 technical repeats. Data represents the means and error bars represent standard
915 deviation across 4 independent biological replicates.

916

917 **Fig. 3: M directly interacts with P via its N-terminal region and OD.** **A.** Scheme
918 of the secondary structure of P protein (α_{N1} , α_{N2} , α_{C1} and α_{C2} denote transient helices
919 detected in isolated P (35)) and the P constructs used. P₁₋₁₆₃ and P₁₋₁₆₁ constructs
920 were used for bacterial and mammalian expression, respectively. **B.** SDS-PAGE and
921 Coomassie blue staining of purified recombinant M, P and P fragments. **C.** M protein
922 was co-incubated with P or P fragments for 30 min prior analysis of formation of
923 complexes by band shift on native agarose gel. Arrows indicate complex formation.
924 **D.** BEAS-2B cells were co-transfected with pcDNA3.1 plasmids expressing RSV M,
925 F, N, and P WT or P deletion mutants. Cells were fixed, and immunostained with
926 anti-M (green) and anti-P (red) antibodies followed by Alexa Fluor secondary

927 antibodies, and were analyzed by confocal microscopy. Scale bars represent 10 μ m.
928 Merged images are zoomed in 3X.

929

930 **Fig. 4: Observation of a direct interaction between RSV P1-163 and M-Y229A**

931 **mutant by NMR. A.** Superimposed 2D ^1H - ^{15}N BEST-TROSY HSQC spectra and **B.** 1D

932 ^1H spectra with water suppression of 25 μM ^{15}N -labeled P₁₋₁₆₃ alone (red) and with 4

933 molar equivalents of M-Y229A (black). Acquisition was done in 50 mM Na phosphate

934 pH 6.7, 150 mM NaCl buffer at 800 MHz ^1H frequency and a temperature of 288 K.

935 Residue-specific assignment of each 2D peak is indicated by the P residue number and

936 the amino acid type. Signals with significant intensity decrease are annotated in colour.

937 Arrows indicate specific M ^1H NMR signals, in methyl (-1-1 ppm) and aromatic (6-7

938 ppm) proton regions. **C.** Intensity ratios (I/I_0), represented as bar diagrams, were

939 measured for each peak in the HSQC spectra of 25 μM ^{15}N -labeled P₁₋₁₆₃ in the

940 absence and in presence of M-Y229A, at P:M molar ratios of 1:2 and 1:4. Signals from

941 the α -helical oligomerization domain (α_{OD} , hatched area) are broadened beyond

942 detection. Coloured background indicates the localization of P specific regions:

943 transient helices α_{N1} , α_{N2} (also termed site I), extended region β_{N} and site II upstream of

944 α_{OD} . **D.** Intensity ratios (I/I_0) measured from HSQC spectra of 40 μM P₁₋₁₆₃ in the

945 absence and in the presence of 150 μM M-Y229A, using two different salt

946 concentrations, 150 mM and 300 mM, as indicated.

947

948 **Fig. 5: Localisation of RSV M-Y229A interaction regions on P by NMR using**

949 **fragments of P₁₋₁₆₃.** Superimposed 2D ^1H - ^{15}N BEST-TROSY HSQC spectra of ^{15}N -

950 labeled N-terminal P fragments. Samples were in 50 mM Na phosphate pH 6.7 150

951 mM NaCl buffer. Spectra were recorded at 800 MHz ^1H frequency. **A.** 25 μM ^{15}N -

952 labeled P₁₋₁₂₆ alone (green) and after addition of 4 molar equivalents of M-Y229A
953 (black) at a temperature of 288 K, **B.** 50 μ M ¹⁵N-labeled P₁₁₃₋₁₆₃ alone (pink) and with
954 2 molar equivalents of M-Y229A (black), at 288 K and 298 K, **C.** 25 μ M ¹⁵N-labeled
955 P₁₂₀₋₁₆₀ alone (blue) and in the presence of 2 molar equivalents of M-Y229A (black),
956 at 288 K. **D.** Intensity ratios (I/I₀) were determined from the HSQC spectra of P₁₋₁₂₆
957 and P₁₁₃₋₁₆₃ with and without M-Y229A. Signals from the OD (α_{OD} , hatched area),
958 which are broadened beyond detection at 288K for P₁₁₃₋₁₆₃, become visible at 298 K.
959 Other areas are highlighted using the same colour code as in Fig. 4.

960

961 **Fig. 6: Validation of novel M-binding sites on P using P deletion mutants. A.**
962 Scheme of the P protein secondary structure and the P constructs used. **B.** His-
963 tagged and size-exclusion chromatography purified M, P or P fragments purified on
964 glutathione beads were analysed using SDS-PAGE and Coomassie Blue staining. **C.**
965 Purified M WT (upper panel) or M Y229A (lower panel), and P or P deletion
966 constructs were incubated 30 min prior analysis of formation of complexes by band
967 shift on native agarose gel. Arrows indicate complex formation. **D.** 293T cells were
968 transfected with pairs of M and P constructs fused to 114 or 11S NanoLUC subunit,
969 combined as shown in the graph. P/P was used as positive control. Cells were lysed
970 24 h post transfection, and luminescence was measured using a Tecan Infinite 200
971 plate reader. The NLR is the ratio between actual read and negative controls (each
972 protein with the empty NanoLUC vector). The graph is representative of four
973 independent experiments, each done in three technical repeats. Data represents the
974 means and error bars represent standard deviation across 4 independent biological
975 replicates.

976 **E.** Same cellular lysates used in D. were then subjected to Western analysis using
977 anti-P or anti-M polyclonal antibody. Size markers are shown on the left side of each
978 gel.

979

980 **Fig. 7: Functional analysis of P regions displaying direct M-binding. A.** Scheme
981 of the P protein secondary structure and the P constructs used. **B.** BEAS-2B cells
982 were co-transfected with pcDNA3.1 plasmids expressing RSV M, N, F and P deletion
983 mutants. Cells were fixed, permeabilized at 24 h post transfection, immunostained
984 with anti-M and anti-P primary antibodies followed by Alexa Fluor secondary
985 antibodies, and were analysed by confocal microscopy. Scale bars represent 10 μ m.
986 Merged images are zoomed in 3X. **C.** HEp-2 cells were co-transfected with
987 pcDNA3.1 plasmids expressing RSV M, F and P_{WT} (lane 1, positive control) or
988 pcDNA3.1 plasmids carrying RSV M, F and an empty pcDNA3.1 vector (lane 2,
989 negative control) or with the indicated RSV P mutant constructs (lanes 3 to 8). At 48
990 h post transfection, VLPs (top) were isolated from the supernatant by pelleting of the
991 clean supernatant through a sucrose cushion. Cell lysates (bottom) were generated
992 using RIPA buffer. VLPs and cell lysates were then subjected to Western analysis
993 using anti-P or anti-M polyclonal antibody. The amount of M and P protein in VLPs
994 was quantified using the ImageJ software, and is presented as percentage (%) of M
995 and P protein released when P_{WT} was used (100%). The graph is representative of
996 four independent experiments. Data represents the % means and error bars
997 represent standard deviation across 4 independent biological replicates.

998

999 **Fig. 8: Sequence alignment of the central part of *Pneumoviridae***
1000 **phosphoproteins.** Sequence alignment for human (strains A and B), bovine and

1001 ovine respiratory syncytial virus, murine pneumonia virus, human and avian
1002 metapneumovirus P proteins was generated using T-coffee suite of program (58).
1003 Uniprot accession numbers are indicated next to each sequence and residue
1004 numbers are given for each sequence. Complete conservation is indicated with a
1005 white font on red background, and relative conservation is indicated by a red font.
1006 Conserved motifs and structure elements are annotated for hRSV P and boxed
1007 throughout the aligned sequences. The boundaries of the OD are from the cryo-EM
1008 structures of L-P complexes of hRSV and hMPV (32, 59). The “RVxF”-like motif that
1009 binds PP1 and the α_{N2} M2-1 binding site (41) are boxed in green and magenta,
1010 respectively. M binding sites I and M binding site II, as identified by NMR, are
1011 indicated or boxed in blue, respectively. Identified phosphorylation sites in the hRSV
1012 P (43, 54) are indicated by a star above the sequence.
1013

Fig. 1A

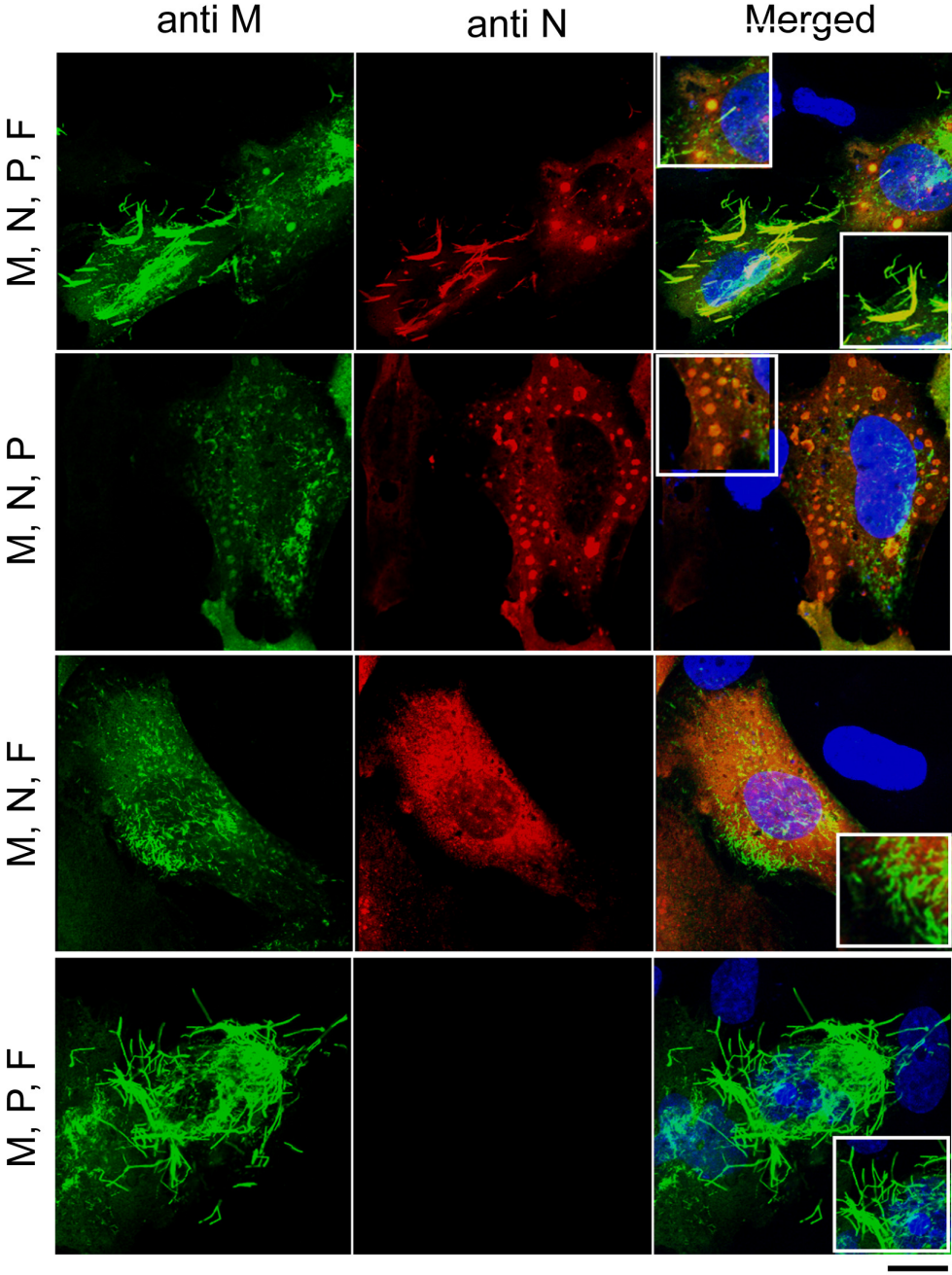


Fig. 1B

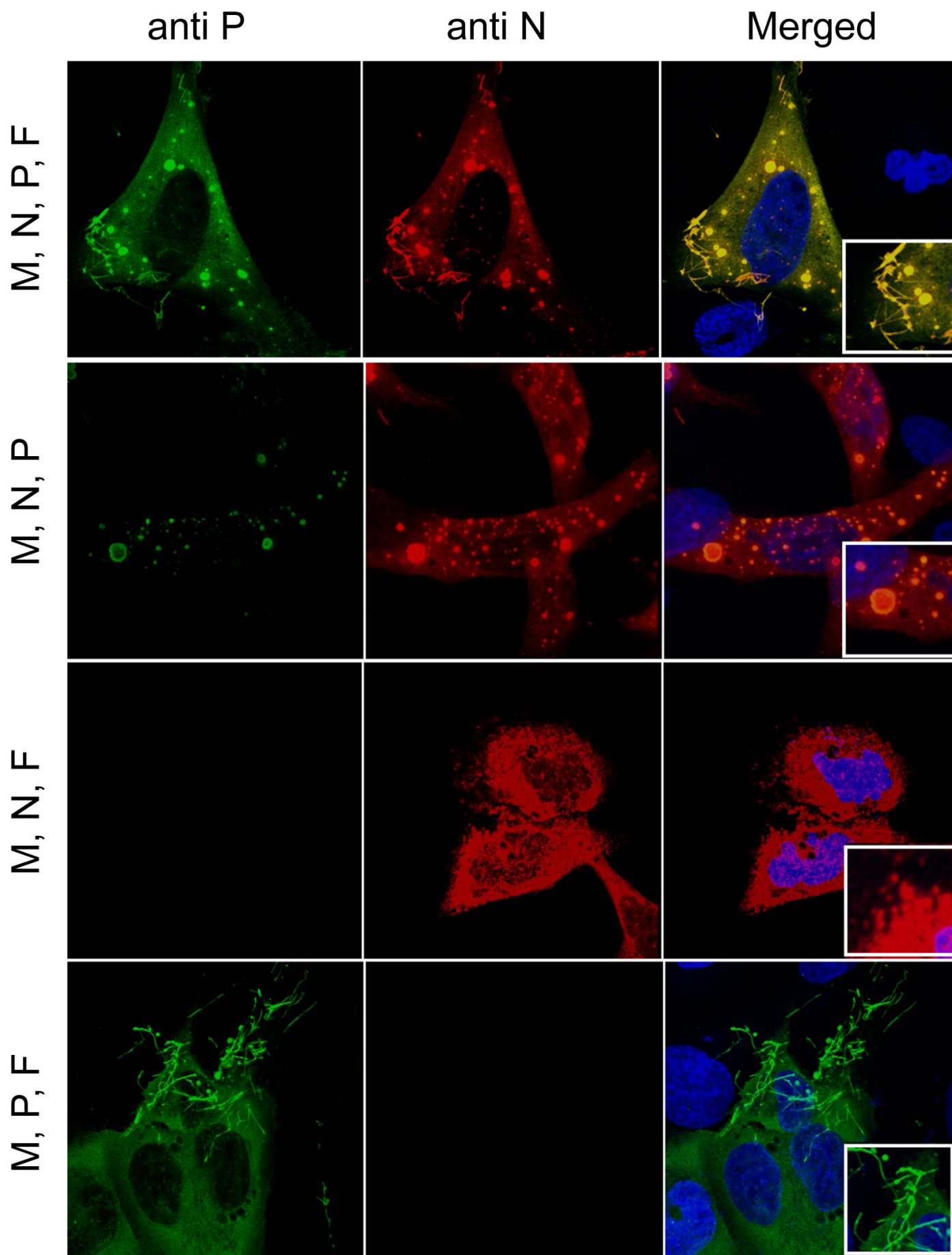
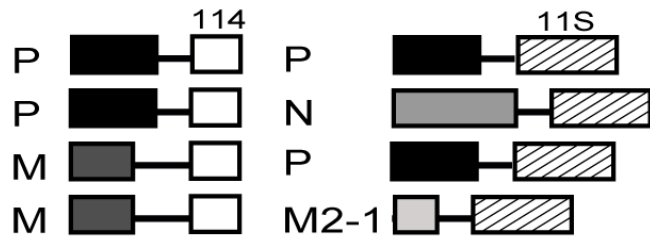
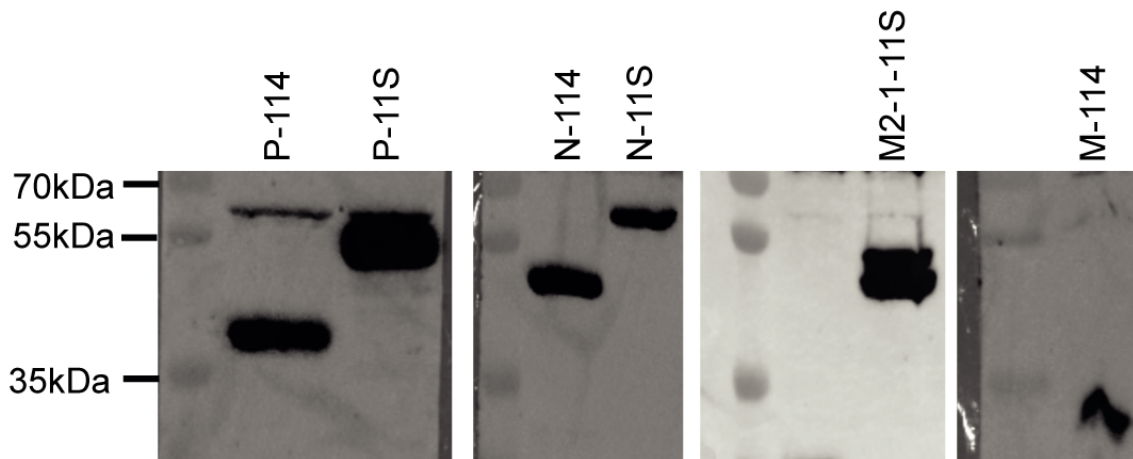


Fig. 2

A.



B.



C.

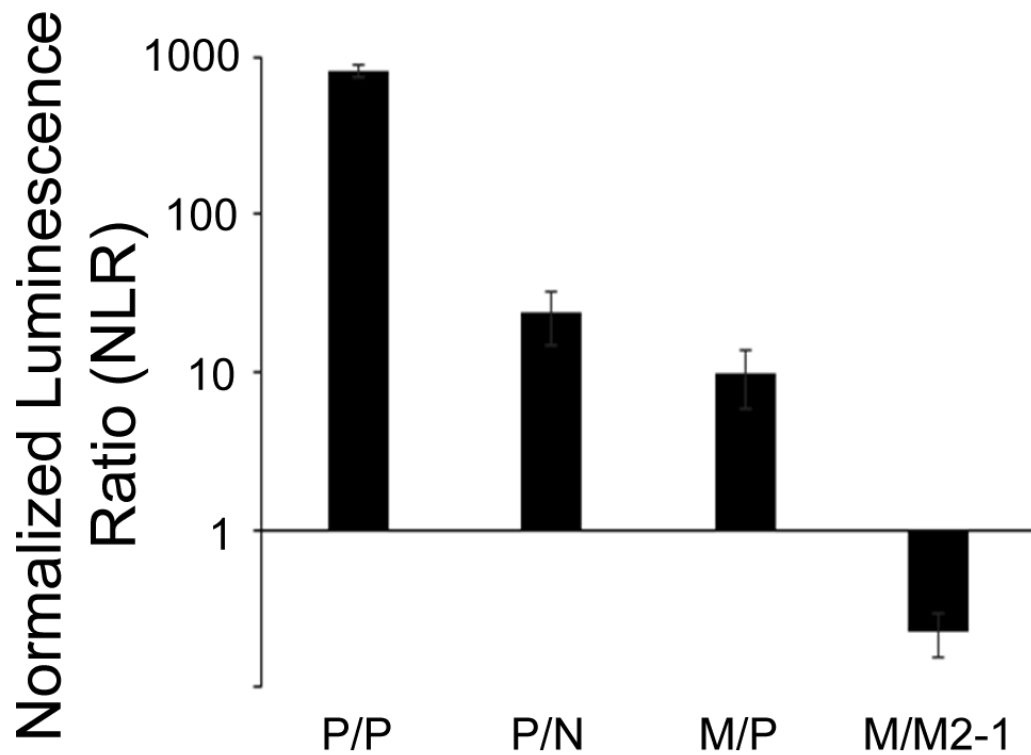
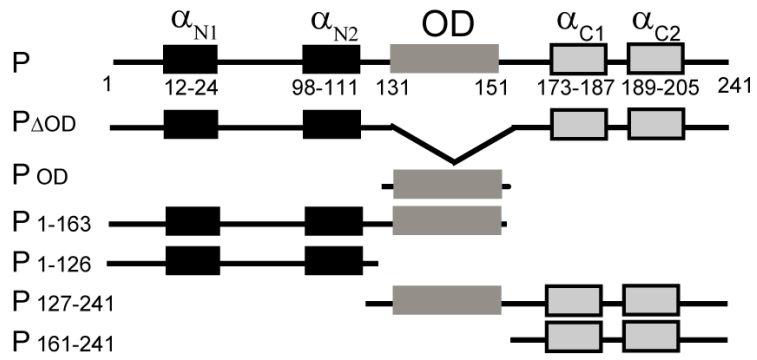
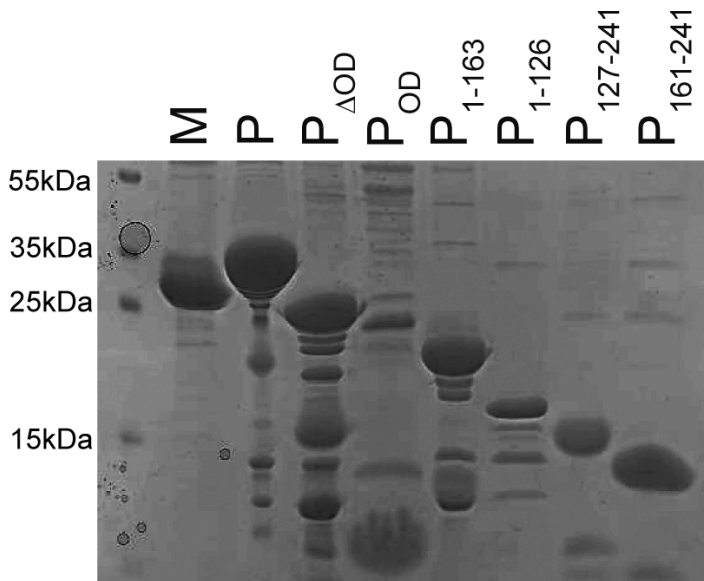


Fig. 3

A.



B.



C.

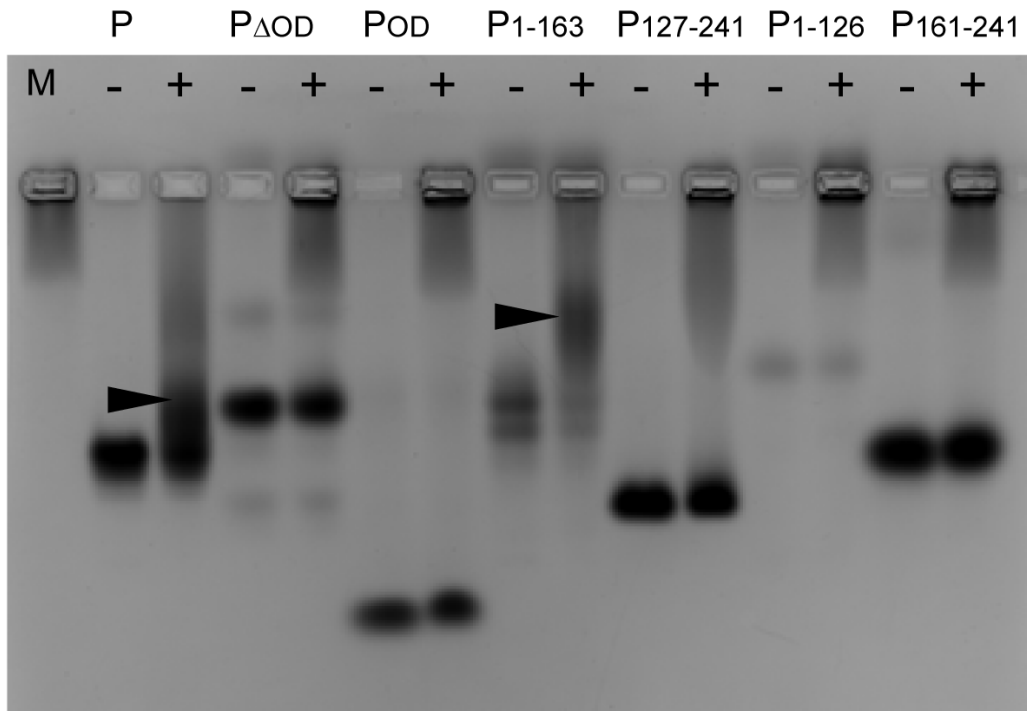


Fig. 3D

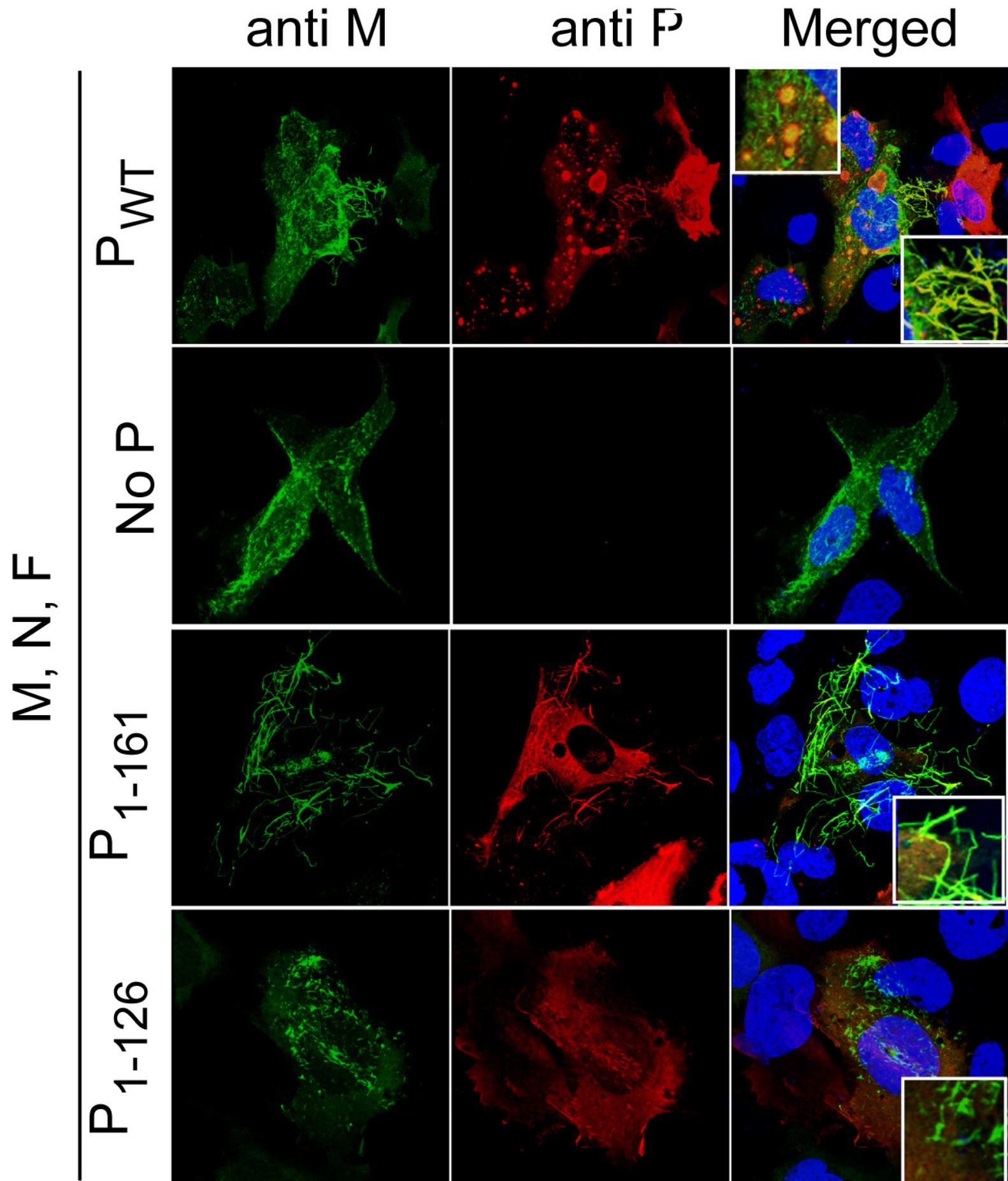


Fig. 4

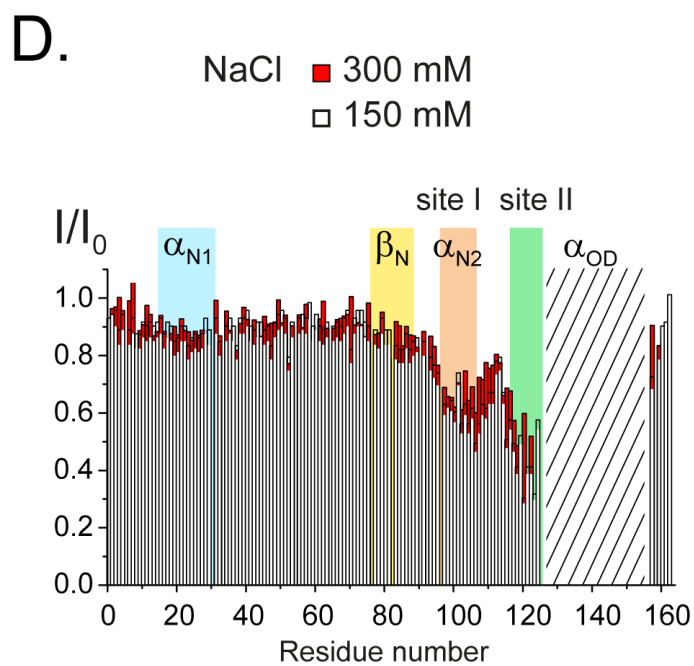
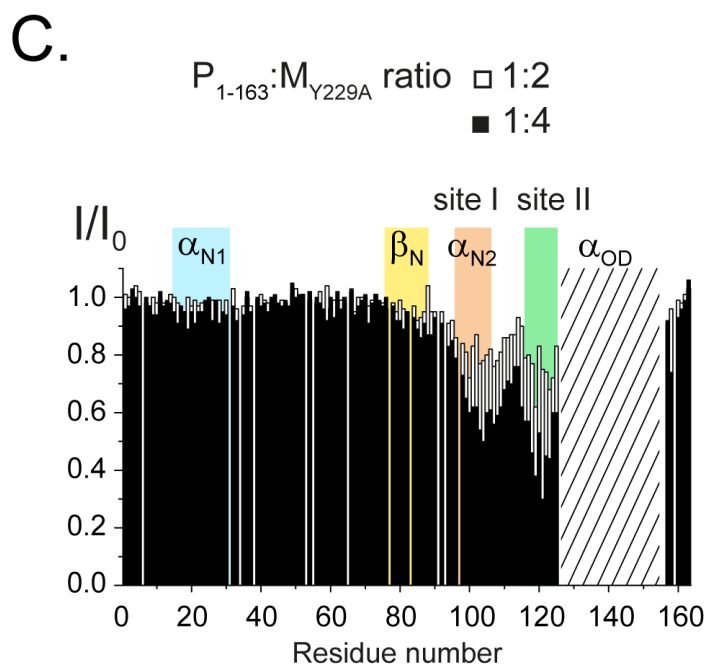
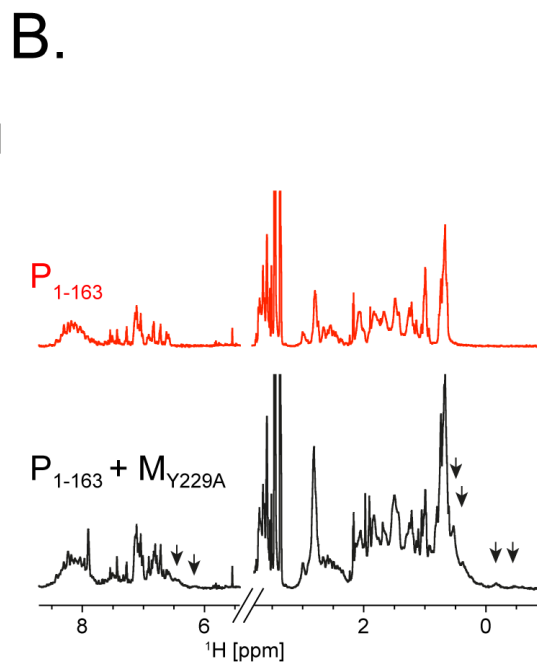
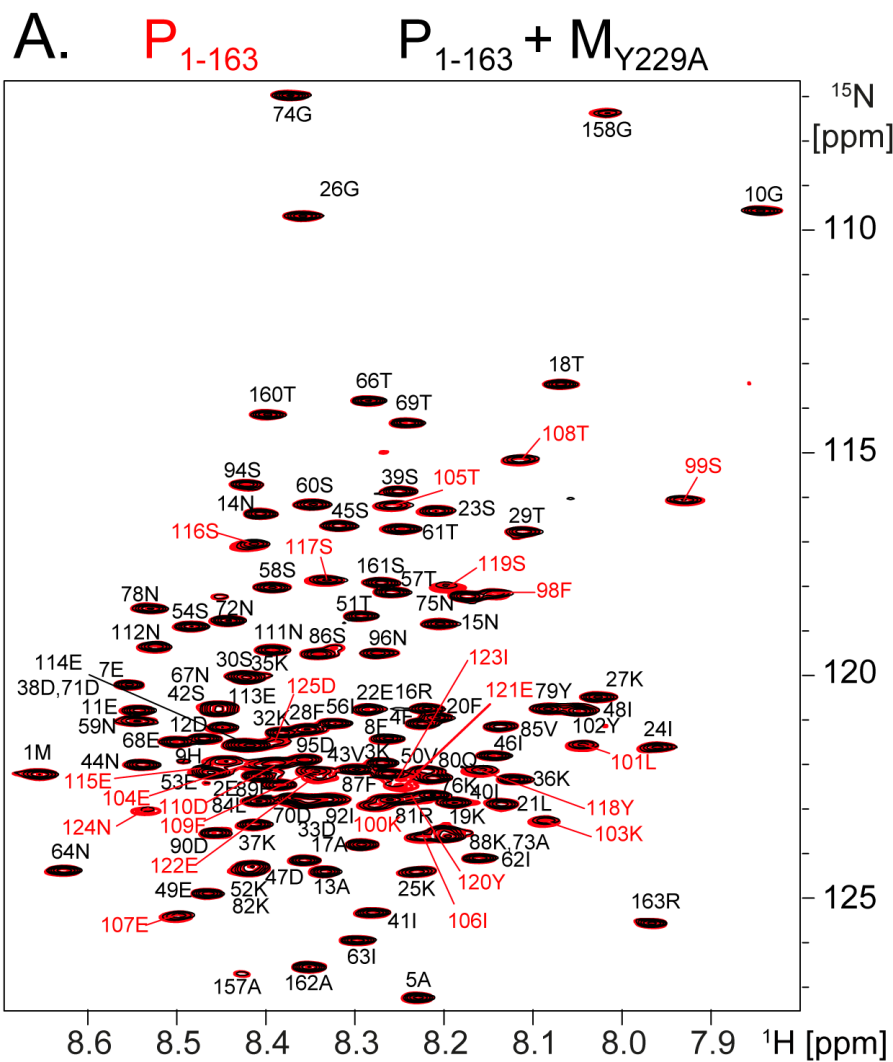


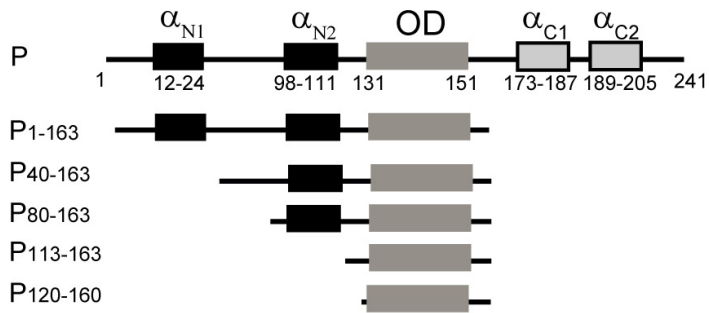
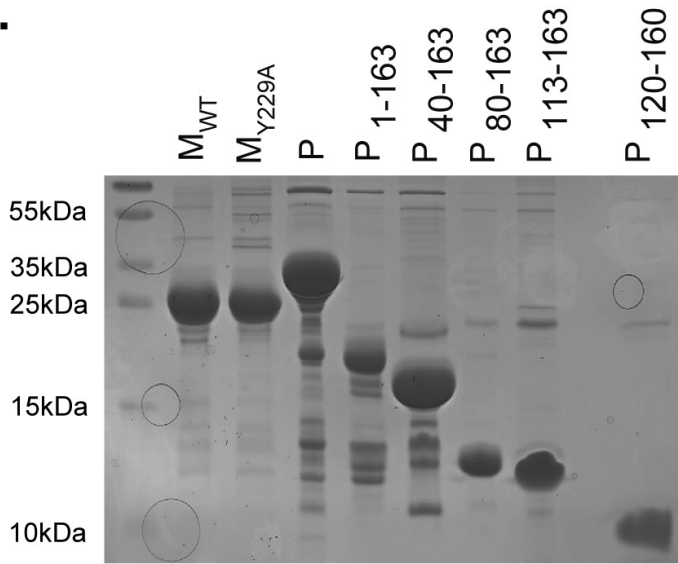
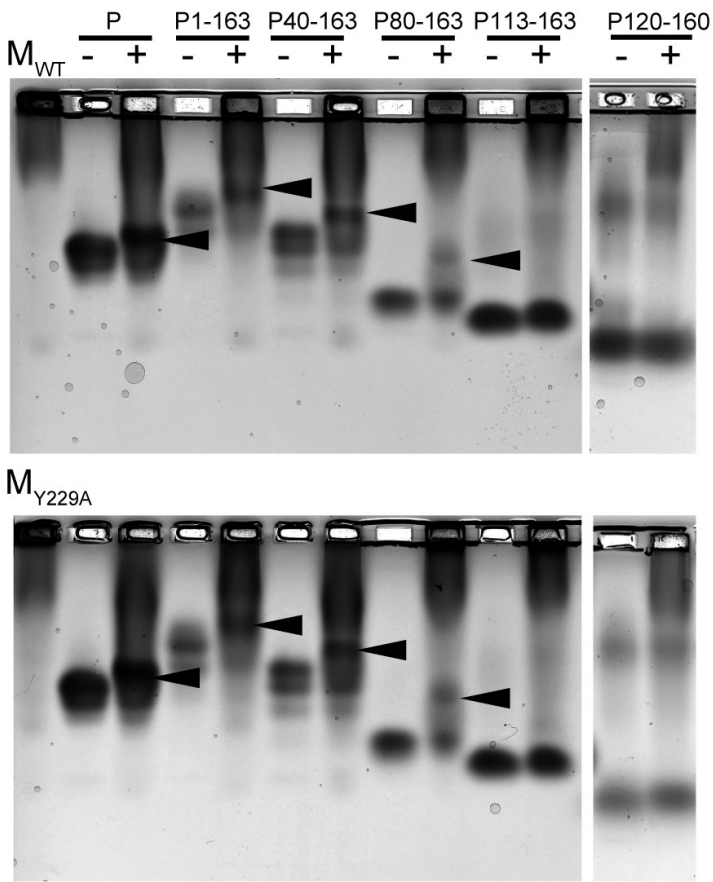
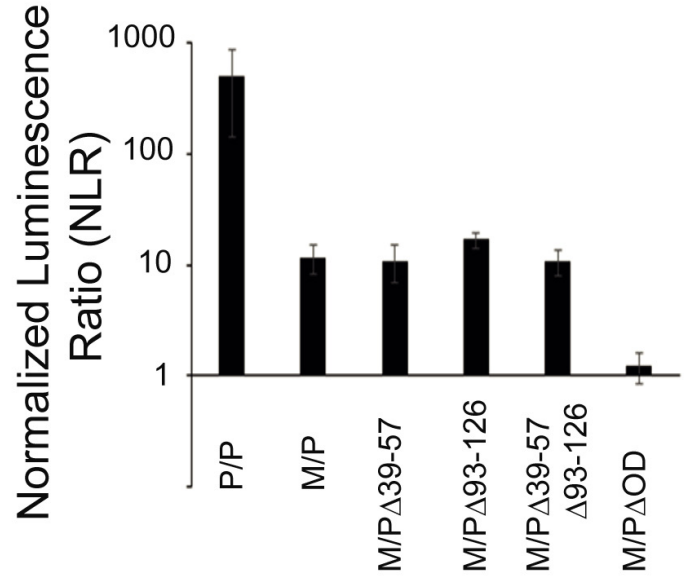
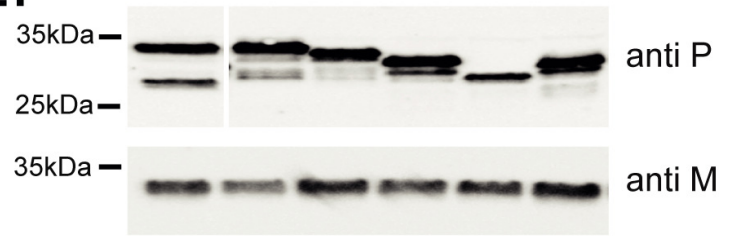
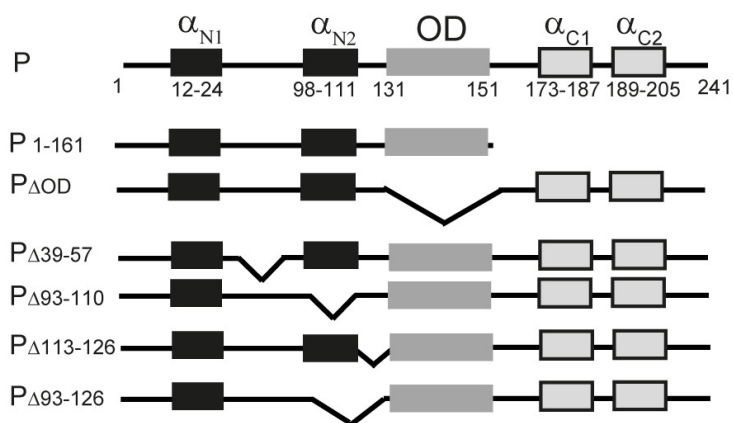
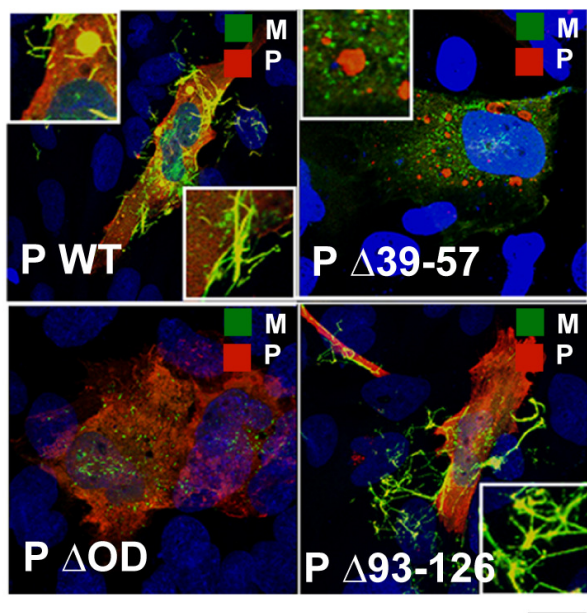
Fig. 6**A.****B.****C.****D.****E.**

Fig. 7

A.



B.



C.

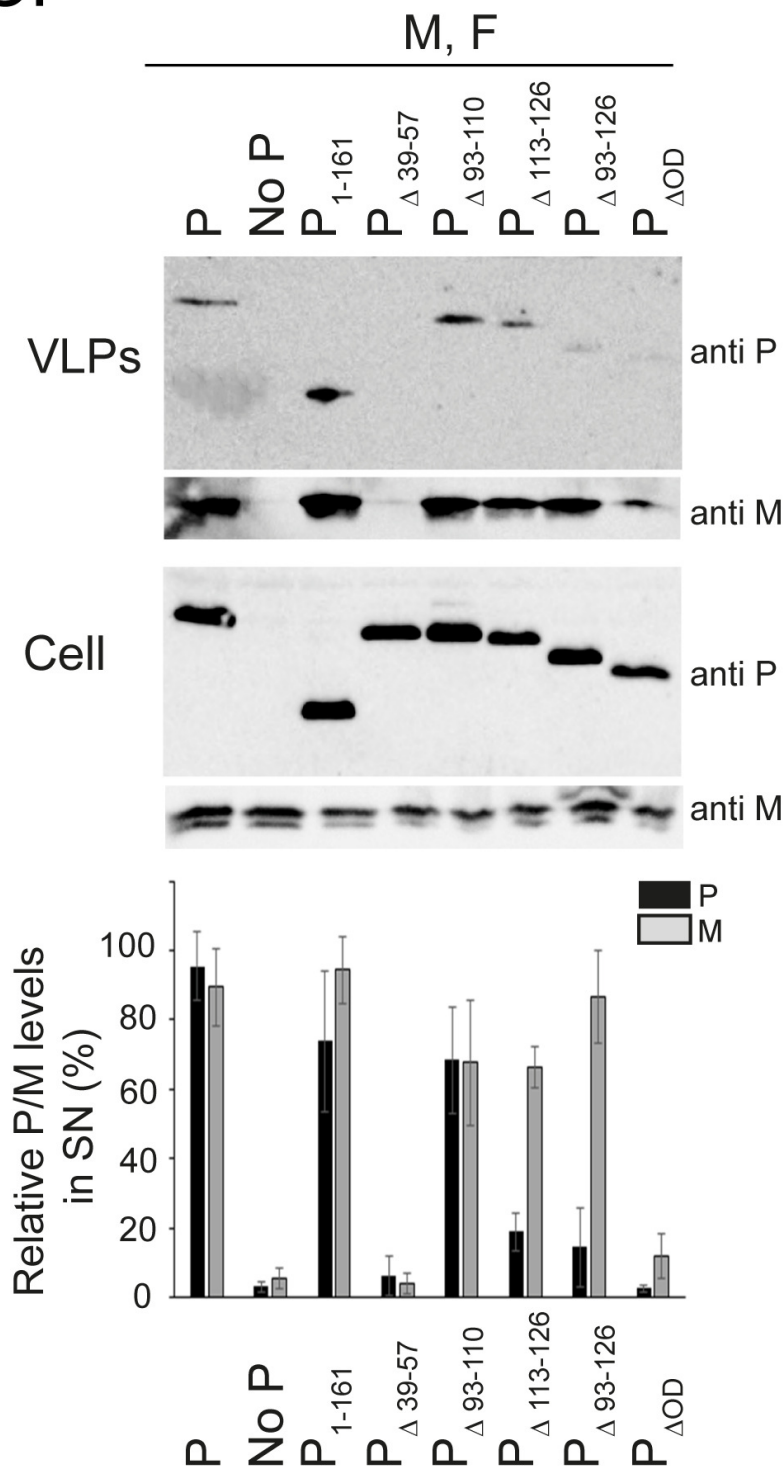


Fig. 8

