

1 Mutation of influenza A virus PA-X decreases pathogenicity in chicken embryos and can
2 increase the yield of reassortant candidate vaccine viruses

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4 Saira Hussain^{a*}, Matthew L. Turnbull^{a†}, Helen M. Wise^{a‡}, Brett W. Jagger^{b,c§}, Philippa M.
5 Beard^{a,d}, Kristina Kovacikova^{a□}, Jeffery K. Taubenberger^c, Lonneke Vervelde^a, Othmar G
6 Engelhardt^e & Paul Digard^{a*}

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8 ^aThe Roslin Institute & Royal (Dick) School of Veterinary Studies, University of Edinburgh,
9 Edinburgh, UK

10 ^bDepartment of Pathology, University of Cambridge, Cambridge, UK

11 ^cNational Institutes of Health, Maryland, USA

12 ^dThe Pirbright Institute, Pirbright, Surrey, UK

13 ^eNational Institute for Biological Standards and Control, South Mimms, Hertfordshire, UK

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15 Running Head: Influenza A virus PA-X and pathogenicity in hens' eggs

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17 #Address correspondence to Paul Digard, paul.digard@roslin.ed.ac.uk

18 Present addresses: *Saira Hussain, The Francis Crick Institute, London, United Kingdom;

19 †Matthew L Turnbull, Glasgow Centre for Virus Research, Glasgow, United Kingdom;

20 ‡Helen M. Wise, Herriot-Watt University, Edinburgh, United Kingdom; §Brett W. Jagger,

21 Department of Medicine, Washington University in St. Louis, St. Louis, USA; □ Kristina

22 Kovacikova, Leiden University Medical Centre, Netherlands.

23

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26 **Abstract**

27

28 The PA-X protein of influenza A virus has roles in host cell shut-off and viral pathogenesis.
29 While most strains are predicted to encode PA-X, strain-dependent variations in activity have
30 been noted. We found that PA-X protein from A/PR/8/34 (PR8) strain had significantly lower
31 repressive activity against cellular gene expression compared with PA-Xs from the avian
32 strains A/turkey/England/50-92/91 (H5N1) (T/E) and A/chicken/Rostock/34 (H7N1). Loss of
33 normal PA-X expression, either by mutation of the frameshift site or by truncating the X-
34 ORF, had little effect on the infectious virus titre of PR8 or PR8 7:1 reassortants with T/E
35 segment 3 grown in embryonated hens' eggs. However, in both virus backgrounds, mutation
36 of PA-X led to decreased embryo mortality and lower overall pathology; effects that were
37 more pronounced in the PR8 strain than the T/E reassortant, despite the low shut-off activity
38 of the PR8 PA-X. Purified PA-X mutant virus particles displayed an increased ratio of HA to
39 NP and M1 compared to their WT counterparts, suggesting altered virion composition. When
40 the PA-X gene was mutated in the background of poorly growing PR8 6:2 vaccine
41 reassortant analogues containing the HA and NA segments from H1N1 2009 pandemic
42 viruses or an avian H7N3 strain, HA yield increased up to 2-fold. This suggests that the PR8
43 PA-X protein may harbour a function unrelated to host cell shut-off and that disruption of the
44 PA-X gene has the potential to improve the HA yield of vaccine viruses.

45

46 **IMPORTANCE** Influenza A virus is a widespread pathogen that affects both man and a
47 variety of animal species, causing regular epidemics and sporadic pandemics with major
48 public health and economic consequences. A better understanding of virus biology is
49 therefore important. The primary control measure is vaccination, which for humans, mostly
50 relies on antigens produced in eggs from PR8-based viruses bearing the glycoprotein genes of

51 interest. However, not all reassortants replicate well enough to supply sufficient virus antigen
52 for demand. The significance of our research lies in identifying that mutation of the PA-X
53 gene in the PR8 strain of virus can improve antigen yield, potentially by decreasing the
54 pathogenicity of the virus in embryonated eggs.

55

56 **Introduction**

57

58 Influenza epidemics occur most years as the viruses undergo antigenic drift. Influenza
59 A viruses (IAV) and influenza B viruses cause seasonal human influenza but IAV poses an
60 additional risk of zoonotic infection, with the potential of a host switch and the generation of
61 pandemic influenza. The 1918 ‘Spanish flu’ pandemic was by far the worst, resulting in 40-
62 100 million deaths worldwide (1), while the 2009 swine flu pandemic caused an estimated
63 200,000 deaths worldwide (2).

64 IAV contains eight genomic segments encoding for at least ten proteins. Six genomic
65 segments (segments 1, 2, 3, 5, 7 and 8) encode the eight core “internal” proteins PB2, PB1,
66 PA, NP, M1, NS1 and NS2, as well as the ion channel M2. These segments can also encode a
67 variety of accessory proteins known to influence pathogenesis and virulence (reviewed in (3,
68 4)). Segments 4 and 6 encode for the two surface glycoproteins haemagglutinin (HA) and
69 neuraminidase (NA) respectively (5, 6) and virus strains are divided into subtypes according
70 to the antigenicity of these proteins.

71 Vaccination is the primary public health measure to reduce the impact of influenza
72 epidemics and pandemics, principally using inactivated viruses chosen to antigenically match
73 the currently circulating virus strains or newly emerging viruses of pandemic concern.
74 However, before efficient vaccine production can commence, high-yielding candidate
75 vaccine viruses (CVVs) need to be prepared. Seasonal CVVs are widely produced by

76 classical reassortment. This process involves co-infecting embryonated hens' eggs with the
77 vaccine virus along with a high yielding "donor" virus adapted to growth in eggs (most
78 commonly the A/Puerto Rico/8/34 strain, or "PR8"). The highest yielding viruses that contain
79 the glycoproteins of the vaccine virus are then selected. Recombinant influenza viruses are
80 also made by reverse genetics (RG) (7-9), which relies on the transfection of cells with
81 plasmids engineered to express both viral genomic RNA and proteins from each of the eight
82 segments and hence initiate virus production; the resultant virus is subsequently amplified in
83 eggs. When making RG CVVs, typically the six segments encoding core proteins (backbone)
84 are derived from the donor strain whereas the two segments encoding the antigens are
85 derived from the vaccine virus. Classical reassortment has the advantage that it allows for the
86 fittest natural variant to be selected but it can be time consuming. In the case of a pandemic,
87 large quantities of vaccine must be made available quickly. Moreover, RG is the only viable
88 method for production of CVVs for potentially pandemic highly pathogenic avian influenza
89 viruses, since it allows for removal of genetic determinants of high pathogenicity in the virus
90 genome, as vaccines are manufactured in biosafety level 2 laboratories. A limited number of
91 donor strains for IAV vaccine manufacture currently exist. Although PR8 is widely used,
92 reassortant viruses based on it do not always grow sufficiently well for efficient vaccine
93 manufacture. In the case of the 2009 H1N1 pandemic (pdm09), vaccine viruses grew poorly
94 in eggs compared with those for previous seasonal H1N1 isolates (10), resulting in
95 manufacturers struggling to meet demand. Thus, there is a clear need for new reagents and
96 methods for IAV production, particularly for pandemic response.

97 In recent years, several approaches have been employed to improve antigen yield of
98 candidate vaccine viruses made by reverse genetics. These have involved empirical testing
99 and selection of PR8 variants (11, 12), as well as targeted approaches such as making
100 chimeric genes containing promoter and packaging signal regions of PR8 while encoding the

101 ectodomain of the CVV glycoprotein genes (13-21), or introducing a wild-type (WT) virus-
102 derived segment 2 (21-29). Our approach was to manipulate expression of an accessory
103 protein virulence factor, PA-X (30). Segment 3, encoding PA as the primary gene product,
104 also expresses PA-X by low-level ribosomal shifting into a +1 open reading frame (ORF)
105 termed the X ORF (Fig 1A) (30). PA-X is a 29 kDa protein that contains the N-terminal
106 endonuclease domain of PA, and in most isolates, a 61 amino acid C-terminus from the X
107 ORF (30-32). It has roles in shutting off host cell protein synthesis and, at the whole animal
108 level, modulating the immune response (30, 33). Loss of PA-X expression has been shown to
109 be associated with increased virulence in mice for 1918 H1N1, H5N1 and also pdm09 and
110 classical swine influenza H1N1 strains, as well as in chickens and ducks infected with a
111 highly pathogenic H5N1 virus (30, 34-40). However, in other circumstances, such as avian
112 H9N2 viruses (40) or, in some cases, A(H1N1)pdm09 viruses (37, 41), mutation of PA-X
113 resulted in reduced pathogenicity in mice. Similarly, a swine influenza H1N2 virus (42)
114 lacking PA-X showed reduced pathogenicity in pigs. Moreover, PA-X activity in repressing
115 cellular gene expression is strain dependent (33, 34, 40, 43), with laboratory-adapted viruses
116 such as A/WSN/33 showing lower levels of activity (33). Here, we show that although the
117 PR8 PA-X polypeptide has low shut-off activity, removing its expression decreases the
118 pathogenicity of the virus in the chick embryo model. Moreover, we found that, for certain
119 poor growing CVV mimics, ablating PA-X expression improved HA yield from embryonated
120 eggs up to 2-fold. In no case did loss of PA-X appear to be detrimental to the growth of
121 CVVs, making it a potential candidate mutation for incorporation into the PR8 CVV donor
122 backbone.

123

124 **Results**

125

126 **The PR8 virus strain PA-X has relatively low shut-off activity.**

127 Previous work has noted variation in apparent activity of PA-X proteins from
128 different strains of virus, with the laboratory adapted-strain WSN showing lower activity than
129 many other strains (33). Re-examination of evidence concerning a postulated proteolytic
130 activity of PA (43) suggested that lower PA-X activity might also be a feature of the PR8
131 strain. To test this, the ability of PR8 segment 3 gene products to inhibit cellular gene
132 expression was compared to that of two avian virus-derived PA segments (from
133 A/chicken/Rostock/34 [H7N1; FPV] and A/turkey/England/50-92/91 [H5N1; T/E]. Avian
134 QT-35 (Japanese quail fibrosarcoma) cells were transfected with a consistent amount of a
135 plasmid encoding luciferase under the control of a constitutive RNA polymerase II promoter
136 (pRL) and increasing amounts of the IAV cDNAs (in pHW2000-based RG plasmids), or as a
137 negative control, the maximum amount of the empty pHW2000 vector. Luciferase expression
138 was measured 48 h later and expressed as a % of the amount obtained from pRL-only
139 transfections. Transfection of a 4-fold excess of empty pHW2000 vector over the luciferase
140 reporter plasmid had no significant effect on luciferase expression, whereas co-transfection of
141 the same amount of either the FPV or T/E segments suppressed activity to around 10% of the
142 control (Fig 1B). Titration of the FPV and T/E plasmids gave a clear dose-response
143 relationship, giving estimated EC₅₀ values of 24 ± 1.1 ng and 32 ± 1.1 ng respectively. In
144 contrast, the maximum amount of the PR8 plasmid only inhibited luciferase expression by
145 around 30% and an EC₅₀ value could not be calculated, indicating a lower ability to repress
146 cellular gene expression. Similarly low inhibitory activity of the PR8 segment 3 was seen in a
147 variety of other mammalian cell lines (data not shown), suggesting it was an intrinsic feature
148 of the viral gene, rather than a host- or cell type-specific outcome.

149 Several studies have shown the X-ORF to be important in host cell shut-off function
150 and virulence of PA-X (37, 44-46). To further explore the influence of X-ORF sequences on

151 virus strain-specific host cell shut-off, mutations were constructed in segment 3 in which PA-
152 X expression was either hindered (*via* mutation of the frameshift site [FS]) or altered by the
153 insertion of premature termination codons (PTCs 1-4; silent in the PA ORF) such that C-
154 terminally truncated forms of PA-X would be expressed (Figure 1A). QT-35 cells were co-
155 transfected with the pRL plasmid and a fixed amount of WT, FS or PTC plasmids and
156 luciferase expression measured 48 h later. As before, the WT FPV and T/E PA-Xs reduced
157 luciferase activity by approximately 5-10 fold, while WT PR8 PA-X had no significant effect
158 (Figure 1C). Introducing the FS mutation into both PR8 and T/E segment 3 significantly
159 increased luciferase activity relative to the WT construct. Truncation of the PR8 PA-X to 225
160 AA or less (PTC mutations 1-3) significantly improved shut-off activity, although not to the
161 levels seen with the WT avian virus polypeptides, while the PTC4 truncation had no effect. In
162 contrast, none of the PTC mutations significantly affected activity of the T/E PA-X, although
163 there was a trend towards increased activity from the PTC2, 3 and 4 truncations

164 Low activity could be due to decreased expression and/or decreased activity of PA-X.
165 To examine this, expression of the low activity PR8 and high activity FPV PA-X constructs
166 were compared by *in vitro* translation reactions in rabbit reticulocyte lysate. Translation of
167 segment 3 from both PR8 and FPV produced both full length PA and similar quantities of a
168 minor polypeptide species of the expected size for PA-X whose abundance decreased after
169 addition of the FS mutation or whose electrophoretic mobility was altered in stepwise fashion
170 after C-terminal truncation with the PTC1-4 mutations (Figure 1D). This suggested that
171 differences in shut-off potential were not linked to intrinsic differences in PA-X protein
172 synthesis. To confirm the identity of the PR8 *in vitro* translated polypeptides,
173 immunoprecipitation of IVT products with sera raised either against the N-terminal domain
174 of PA, or an X-ORF derived polypeptide or pre-immune sera (30) were performed (Figure
175 1E). WT PA-X was clearly visible in samples immunoprecipitated with anti-PA-X and anti-

176 PA-N but not the pre-immune serum, where it co-migrated with the product from the
177 delC598 plasmid, a construct in which cytosine 598 of segment 3 (the nucleotide skipped
178 during the PA-X frameshifting event (47)) had been deleted to put the X-ORF into the same
179 reading frame as the N-terminal PA domain (Figure 1E, lanes 2 and 7). In contrast, only
180 background amounts of protein were precipitated from the FS IVT (lane 3). Faster migrating
181 polypeptide products from the PTC3 and 4 plasmids showed similar reactivities to WT PA-X
182 (lanes 5 and 6) whereas the product of the PTC1 plasmid was only precipitated by anti-PA-N
183 (lane 4), as expected because of the loss of the epitope used to raise the PA-X antiserum
184 (Figure 1A). Overall therefore, the PR8 PA-X polypeptide possessed lower shut-off activity
185 than two avian virus PA-X polypeptides despite comparable expression *in vitro*, and its
186 activity could be modulated by mutation of the X-ORF.

187

188 **Loss of PA-X expression results in significantly less pathogenicity in chick embryos**
189 **without affecting virus replication**

190

191 In order to further characterise the role of PA-X as a virulence determinant, we tested
192 the panel of high and low activity mutants in the chicken embryo pathogenicity model.
193 Embryonated hens' eggs were infected with PR8-based viruses containing either PR8 or T/E
194 WT or mutant segment 3s and embryo viability was monitored at 2 days post infection (p.i.)
195 by candling. Both WT PR8 and the WT 7:1 reassortant with the T/E segment 3 viruses had
196 killed over 50% of the embryos by this point (Figures 2A and B). Truncation of PA-X by the
197 PTC mutations led to small improvements in embryo survival, although none of the
198 differences were statistically significant. However, embryo lethality was significantly
199 reduced to below 20% following infection with the PR8 FS virus compared with PR8 WT
200 virus. A similar reduction in lethality was seen for the T/E FS virus, although the difference

201 was not statistically significant. This reduction in embryo pathogenicity following ablation of
202 PA-X expression suggested potential utility as a targeted mutation in the PR8 backbone used
203 to make CVVs. Accordingly, to characterise the effects of mutating PR8 PA-X over the
204 period used for vaccine manufacture, embryo survival was monitored daily for 72 h. Eggs
205 infected with WT PR8 showed 45% embryo survival at 2 days p.i. and all were dead by day 3
206 (Figure 2C). However, the PR8 FS infected eggs showed a statistically significant
207 improvement in survival compared to WT, with 80% and 30% survival at days 2 and 3,
208 respectively. Embryos infected with PR8 expressing the C-terminally truncated PTC1 form
209 of PA-X showed an intermediate survival phenotype with 60% and 20% survival at days 2
210 and 3, respectively.

211 To further assess the effects of mutating PA-X, the chicken embryos were examined
212 for gross pathology. WT PR8 infection resulted in smaller, more fragile embryos with diffuse
213 reddening, interpreted as haemorrhages (Figure 2D). In comparison, the PA-X null FS
214 mutant-infected embryos remained intact, were visibly larger and less red. To quantitate these
215 observations, embryos were scored blind for gross pathology. Taking uninfected embryos as
216 a baseline, it was clear that WT PR8 virus as well as the PA-X truncation mutants induced
217 severe changes to the embryos (Figure 2E). In contrast, the PA-X null FS mutant caused
218 significantly less pathology. The WT 7:1 T/E reassortant virus gave less overt pathology than
219 WT PR8 but again, reducing PA-X expression through the FS mutation further reduced
220 damage to the embryos (Figure 2E). Similar trends in pathology were also seen with 7:1 PR8
221 reassortant viruses containing either WT or FS mutant versions of FPV segment 3 (data not
222 shown).

223 Examination of haematoxylin and eosin (H&E) stained sections through the embryos
224 revealed pathology in numerous organs including the brain, liver and kidney (Figure 3). In
225 the brain of embryos infected with WT virus there was marked rarefaction of the neuropil,

226 few neurons were identifiable, and there was accumulation of red blood cells (Figure 3C). In
227 the liver of embryos infected with WT virus the hepatic cords were disorganised, and the
228 hepatocytes were often separated by large pools of red blood cells (Figure 3D). In the kidney
229 of embryos infected with WT virus, tubules were often lined by degenerate epithelial cells
230 (characterised by loss of cellular detail). In all cases the pathology noted in WT virus-infected
231 embryos was also present in the FS virus-infected embryos but at a reduced severity. Thus
232 overall, disruption of PA-X expression in PR8 resulted in significantly less pathogenicity in
233 chick embryos.

234 Reduced pathogenicity *in vivo* following loss of PA-X expression could be due to a
235 replication deficiency of the virus, although the viruses replicated equivalently in mammalian
236 MDCK cells (data not shown). To test if replication did differ *in ovo*, infectious virus titres
237 were obtained (by plaque titration on MDCK cells) from the allantoic fluid of embryonated
238 hens' eggs infected with the panels of PR8 and T/E viruses at 2 days p.i.. However, there
239 were no significant differences in titres between either PR8 or T/E WT and PA-X mutant
240 viruses (Figures 4A, B). Since the reduced pathogenicity phenotype *in ovo* on loss of PA-X
241 expression was more pronounced for viruses with PR8 segment 3 than the T/E gene, embryos
242 from PR8 WT and segment 3 mutant-infected eggs were harvested at 2 days p.i., washed,
243 macerated and virus titres from the homogenates determined. Titres from embryos infected
244 with the PR8 FS and PTC4 viruses were slightly (less than 2-fold) reduced compared to
245 embryos infected with PR8 WT virus (Figure 4C), but overall there were no significant
246 differences in titres between the viruses. To see if there were differences in virus localisation
247 in tissues between PR8 WT and FS viruses, immunohistochemistry was performed on chick
248 embryo sections to detect viral NP as a marker of infected cells. NP positive cells were seen
249 in blood vessels throughout the head and body of both PR8 WT and FS-infected embryos;
250 liver, heart and kidney are shown as representatives (Figure 4D), indicating that the

251 circulatory system had been infected. However, there were no clear differences in virus
252 localisation between embryos infected with WT and FS viruses.

253 Overall therefore, the loss of PA-X expression reduced IAV pathogenicity in chick
254 embryos, as assessed by mortality curves and both gross and histopathological examination
255 of embryo bodies. This reduced pathogenicity did not appear to correlate with reduced
256 replication or altered distribution of the virus *in ovo*.

257

258 **Ablating PA-X expression alters virion composition**

259 Other viruses encode host-control proteins with mRNA endonuclease activity,
260 including the SOX protein of murine gammaherpesvirus MHV68 whose expression has been
261 shown to also modulate virion composition (48). Also, egg-grown IAV titre and HA yield do
262 not always exactly match, with certain problematic candidate vaccine viruses (CVVs)
263 containing lower amounts of HA per virion (16, 49, 50). Accordingly, we compared the
264 relative quantities of virion structural proteins between PA-X expressing and PA-X null
265 viruses. Two pairs of viruses were tested: either an entirely PR8-based virus, or a 7:1
266 reassortant of PR8 with FPV segment 3, both with or without the FS mutation. Viruses were
267 grown in eggs as before and purified from allantoic fluid by density gradient
268 ultracentrifugation before polypeptides were separated by SDS-PAGE and visualised by
269 staining with Coomassie blue. To ensure that overall differences in protein loading did not
270 bias the results, 3-fold dilutions of the samples were analysed. From the gels, the major virion
271 components of both WT and FS virus preparations could be distinguished: NP, the two
272 cleaved forms of haemagglutinin, HA1 and HA2, the matrix protein, M1 and in lower
273 abundance, the polymerase proteins (Figures 5A, B, lanes 4-9). In contrast, only trace
274 polypeptides were present in similarly purified samples from uninfected allantoic fluid (lanes
275 1-3). Densitometry was used to assess the relative viral protein contents of the viruses. The

276 two most heavily loaded lanes (where band intensities were sufficient for accurate
277 measurement) were quantified and average HA1:NP and HA2:M1 ratios calculated. When
278 the data from three independent experiments were examined in aggregate by scatter plot
279 (Figures 5C and D), a statistically significant increase in the average quantity of HA1 relative
280 to NP was evident for both PR8 and the FPV reassortant FS viruses of ~1.4-fold and ~1.6-
281 fold respectively compared to WT (Figures 5 C, D). The ratio of HA2:M1 was also
282 significantly increased in the PR8 FS virus (~1.2- fold greater for WT) and a similar but non-
283 significant increase was seen for the FPV virus pair. These data are consistent with the
284 hypothesis that PA-X expression modulates virion composition.

285

286 **Ablating PA-X expression increases HA yield of CVVs bearing pdm2009** 287 **glycoproteins**

288 The reduced pathogenicity and corresponding longer embryo survival time induced
289 by the PR8 FS mutant *in ovo* coupled with evident modulation of virion composition in
290 favour of HA content suggested a strategy to increase overall antigen yields for PR8-based
291 CVVs. Therefore, the effect of incorporating the PA-X FS mutation into CVV mimics
292 containing glycoproteins of different IAV subtypes was examined. Reasoning that a benefit
293 might be most apparent for a poor-yielding strain, 6:2 CVV mimics containing the
294 glycoprotein genes from the A(H1N1)pdm09 vaccine strain, A/California/07/2009 (Cal7)
295 with the six internal genes from PR8, with or without the FS mutation in segment 3, were
296 generated. Growth of these viruses in embryonated hens' eggs was then assessed by
297 inoculating eggs with either 100, 1,000 or 10,000 PFU per egg (modelling the empirical
298 approach used in vaccine manufacture to find the optimal inoculation dose) and measuring
299 HA titre at 3 days p.i.. Both viruses grew best at an inoculation dose of 100 PFU/egg, but
300 final yield was both relatively low (as expected, ~ 64 HAU/50 µl) and insensitive to input

301 dose, with average titres varying less than 2-fold across the 100-fold range of inocula (Figure
302 6A). However, at each dose, the 6:2 FS virus gave a higher titre (on average, 1.6-fold) than
303 the parental 6:2 reassortant. In order to assess HA yield between the WT and FS viruses on a
304 larger scale, comparable to that used by WHO Essential Regulatory Laboratories (ERLs)
305 such as the National Institute for Biological Standards and Control, UK, 20 eggs per virus
306 were infected at a single inoculation dose. In this experiment, the average HA titre of the FS
307 virus was over 3 times higher than the WT 6:2 virus (Figure 6B). To further determine the
308 consistency of these results, HA titre yields were assessed from two independently rescued
309 reverse genetics stocks of the Cal7 6:2 CVV mimics with or without the PR8 PA-X gene as
310 well as another 6:2 CVV mimic bearing the glycoproteins from the A/England/195/2009
311 (Eng195) A(H1N1)pdm09 strain. HA yield from different stocks was assessed in independent
312 repeats of both small- (5 eggs for each of three different inoculation doses, taking data from
313 the dose that gave maximum yield) and large-scale (20 eggs per single dose of virus)
314 experiments. Examination of the average HA titres showed considerable variation between
315 independent experiments (Figure 6C). However, when plotted as paired data points, it was
316 obvious that in every experiment, the FS viruses gave a higher yield than the parental 6:2
317 reassortant and on average, there were 2.7- and 3.8-fold higher HA titres with the segment 3
318 FS mutation for Cal7 and Eng195 respectively (Table 1).

319 To directly assess HA protein yield, viruses were partially purified by
320 ultracentrifugation of pooled allantoic fluid through 30% sucrose cushions. Protein content
321 was analysed by SDS-PAGE and Coomassie staining, either before or after treatment with N-
322 glycosidase F (PNGaseF) to remove glycosylation from HA and NA. Both virus preparations
323 gave polypeptide profiles that were clearly different from uninfected allantoic fluid processed
324 in parallel, with obvious NP and M1 staining, as well other polypeptide species of less certain
325 origin (Figure 6D). Overall protein recovery was higher in the FS virus than the WT

326 reassortant virus (compare lanes 3 and 4 with 5 and 6), but the poor yields of these viruses
327 made unambiguous identification of the HA polypeptide difficult. However, PNGaseF
328 treatment led to the appearance of a defined protein band migrating at around 40 kDa that
329 probably represented de-glycosylated HA1, and this was present in appreciably higher
330 quantities in the 6:2 FS preparation (compare lanes 4 and 6). Therefore, equivalent amounts
331 of glycosylated or de-glycosylated samples from the Cal7 WT and FS reassortants were
332 analysed by SDS-PAGE and western blotting using anti-pdm09 HA sera. The western blot
333 gave a clear readout for HA1 content, confirmed the mobility shift upon de-glycosylation and
334 showed increased amounts of HA1 in the 6:2 FS samples (Figure 6D lower panel).
335 Quantitative measurements of the de-glycosylated samples showed that the 6:2 FS virus gave
336 1.9-fold greater HA1 yield than the WT reassortant. To test the reproducibility of this finding,
337 HA1 yield was assessed by densitometry of de-glycosylated HA1 following SDS-PAGE and
338 western blot for partially purified virus from 9 independent experiments with the Cal7 and
339 Eng195 reassortants. When examined as paired observations, it was evident that in 8 of the 9
340 experiments, the FS viruses gave greater HA yields than the parental virus, with only one
341 experiment producing a lower amount (Figure 6E). In one large-scale experiment, the HA1
342 yield of 6:2 FS was approximately 20-fold higher compared to its 6:2 counterpart. However,
343 in all other experiments, the 6:2 FS virus gave between 1.5 and 3-fold increases in HA1 yield
344 when compared with the 6:2 virus. When the outlier was discounted (as possibly resulting
345 from an artefactually low recovery for the WT sample), average HA1 yield from the other 8
346 experiments showed 1.9- and 2.4-fold improvements with the segment 3 FS mutation for
347 Cal7 and Eng195 respectively (Table 1).

348 The HA yield of CVVs with pdm09 glycoproteins has been shown to be improved by
349 engineering chimeric HA genes which contain signal peptide and transmembrane
350 domain/cytoplasmic tail sequences from PR8 HA and the antigenic region of the HA gene

351 from Cal7 (19, 20). To test if these gains were additive with those seen with the FS mutation,
352 we introduced the NIBRG-119 construct, which is a segment 4 with the ectodomain coding
353 region of Cal7 HA and all other sequences (3'- and 5'-noncoding regions, signal peptide,
354 transmembrane domain, and cytoplasmic tail) from PR8 (19) into 6:2 CVV mimics with the
355 WT A(H1N1)pdm09 NA gene and a PR8 backbone with or without the PA-X mutation.
356 Viruses bearing the NIBRG-119 HA did not agglutinate chicken red blood cells (data not
357 shown) so HA yield from eggs was assessed by SDS-PAGE and western blot of partially
358 purified virus. Chimeric HA viruses containing the FS backbone showed an average HA
359 yield improvement of 1.54-fold compared to the WT backbone counterpart, across
360 independent small- and large-scale experiments (Table 1). Thus, the FS mutation is
361 compatible with other rational strategies for increasing egg-grown reverse genetics vaccines.

362 Following on from this, several pairs of CVV mimics were made with glycoproteins
363 from different IAV strains with either WT or FS mutant PR8 segment 3. These included
364 viruses with glycoproteins of potentially pandemic strains such as highly pathogenic avian
365 virus A/turkey/Turkey/1/2005 (H5N1), as well as low pathogenic avian strains
366 A/mallard/Netherlands/12/2000 (H7N3), A/chicken/Pakistan/UDL-01/2008 (H9N2) and
367 A/mallard/Netherlands/10/99 (H1N1), as well as the human H3N2 strain, A/Hong Kong/1/68,
368 and an early seasonal H3N2 isolate, A/Udorn/307/72 (Table 1). HA yield in eggs was
369 assessed from both the small-scale and large-scale experimental conditions described earlier,
370 by measuring HA titre and HA1 yield from partially purified virus particles. In general, the
371 two techniques were in agreement (Table 1). Ablating PA-X expression moderately improved
372 HA1 yields of some of the CVVs tested: 1.5-fold for the avian H7N3 strain,
373 A/mallard/Netherlands/12/2000 and 1.3-fold for the human H3N2 A/Udorn/307/72 strain.
374 Other CVVs showed lesser or effectively no increases. However, in no case, did ablation of
375 PA-X appear to be detrimental to the growth of CVVs.

376

377 **Discussion**

378 Here we show that ablating expression of PA-X resulted in reduced pathogenicity in
379 the chicken embryo model despite the PR8 PA-X protein having relatively low host cell shut-
380 off activity compared to PA-X from other IAV strains. Although loss of PA-X expression had
381 no effect on infectious titres in eggs, subtle differences in virion composition were observed,
382 and more importantly, the HA yield from poor growing 6:2 reassortant vaccine analogues
383 containing the HA and NA segments from A(H1N1) pdm09 strains was significantly
384 improved.

385 The majority of studies examining the effect of loss of PA-X expression on IAV
386 pathogenicity have used mice as the experimental system. As discussed above, in most cases,
387 the outcome has been increased virulence (30, 34-40), but several studies have found the
388 opposite effect, with PA-X deficiency reducing pathogenicity in mice (37, 41, 42). In adult
389 bird challenge systems using chickens and ducks infected with a highly pathogenic H5N1
390 virus, abrogating PA-X expression caused increased virulence (35). In our infection model of
391 embryonated hens' eggs, loss of PA-X expression markedly reduced the pathogenicity in
392 chick embryos. Thus like PB1-F2, another *trans*-frame encoded IAV accessory protein (51),
393 the impact of PA-X expression on viral pathogenicity seems to vary according to both host
394 and virus strain, but not in a fashion that can simply be correlated with mammalian or avian
395 settings.

396 In previous studies, changes in virulence phenotypes following loss of PA-X
397 expression have been associated with its host cell shut-off function. In the virus strains used,
398 whether from high pathogenicity or low pathogenicity IAV strains, the PA-X polypeptides
399 were shown to significantly affect host cell gene expression. Here, despite PR8 PA-X failing
400 to repress cellular gene expression, a strong phenotypic effect was seen in chicken embryos

401 following loss of PA-X expression. Furthermore, these effects on pathogenicity were more
402 pronounced in an otherwise WT PR8 virus than in a 7:1 reassortant with segment 3 from the
403 highly pathogenic H5N1 avian influenza T/E strain which encodes a PA-X with strong host
404 cell shut-off activity. This lack of correlation between repression of cellular gene expression
405 in avian cells and phenotypic effects in chicken embryos suggests that the PR8 PA-X protein
406 may harbour a function unrelated to host cell shut-off. The PR8 PA-X protein has been
407 proposed to inhibit stress granule formation, but via a mechanism linked to its endonuclease
408 activity and therefore presumably reflecting shut-off activity (52). Alternatively, it could be
409 that the PR8 PA-X polypeptide only exhibits repressive function in specific cell types, such
410 as those of the chorioallantoic membrane (the primary site of virus replication in eggs) or the
411 chick embryo itself. However, since we found low shut-off activity from it in a variety of
412 cells from different species and conversely, no great cell specificity of high activity PA-X
413 polypeptides (data not shown), we do not favour this hypothesis.

414 Several studies have found that sequences in the X-ORF make positive contributions
415 to the shut-off activity of PA-X (30, 37, 39, 45, 46). In contrast, here we found that for both
416 PR8 and T/E strains of the polypeptide, removal of X-ORF sequences actually increased
417 shut-off activity compared to the WT polypeptide. The effect was relatively modest and in
418 the case of PR8, did not confer equivalent activity to the full-length avian virus PA-X
419 polypeptides (Figure 1C). A similar outcome of greater inhibition from a truncated PA-X
420 polypeptide was seen with a triple reassortant swine influenza virus (42), suggesting that the
421 X-ORF can harbour negative as well as positive regulatory polymorphisms.

422 In some but not all studies, effects of PA-X mutations on viral pathogenicity have
423 been associated with differences in virus replication *in vivo*. While Jagger et al., (30) did not
424 attribute the increased virulence in mice upon loss of 1918 H1N1 PA-X to virus replication,
425 Gao and colleagues found that increased virulence in mice on loss of H5N1 PA-X was

426 associated with increased titres of Δ PA-X viruses in the lungs, brains and blood of infected
427 mice (34, 39). Similarly, Hu et al. found that increased virulence in chicken, ducks and mice
428 of a Δ PA-X H5N1 virus was associated with increased virus titres in the host (35). Given the
429 postulated role of PA-X-mediated repression of cellular gene expression in controlling host
430 responses to infection, it is reasonable to hypothesise that these differing outcomes reflect the
431 variable interplay between host and virus that is well known to tip in favour of one or other
432 depending on exact circumstance (53). Our present study, where loss of a PA-X with little
433 apparent ability to modulate host gene expression had no significant effect on virus titres in
434 allantoic fluid or the chick embryos themselves, but nevertheless reduced pathogenicity, do
435 not support this hypothesis. However, differences in progeny virion composition in the form
436 of altered ratios of HA to NP and M1 between WT and FS viruses were seen. This may
437 differentially affect their ability to infect specific cell types, as the amount of virus receptor
438 varies between different tissue types and is a known determinant of tissue tropism of
439 influenza viruses (reviewed in (54, 55)).

440 Our findings have direct implications for HA yield of vaccine viruses in eggs.
441 Ablating PA-X expression did not affect yield from eggs of high growth viruses such as PR8
442 or 6:2 reassortant CVV mimics containing glycoproteins of human H3N2 strains, or
443 potentially pandemic low pathogenicity avian H9N2 or H1N1 viruses. However, mutation of
444 the PR8 PA-X gene in the background of a CVV analogue containing the HA and NA
445 segments from poor growing strains, such as A(H1N1)pdm09 viruses or a potentially
446 pandemic avian H7N3 isolate, increased HA yield by around 2-fold. The mechanism of
447 improved yield of certain virus subtypes but not others on loss of PA-X expression is unclear.
448 Others have found that mutating the FS site of PR8 PA-X has subtle effects on viral protein
449 expression *in vitro*, including lower levels of M1 (45), perhaps explaining the changes in HA
450 to M1 ratio we see. Beneficial outcomes to HA yield may only be apparent in low-yielding

451 strains where perhaps viral rather than cellular factors are limiting. Alternatively, changes in
452 virion composition between WT and FS viruses could result in subtype/strain-specific effects
453 depending on the balance between HA and NA activities (56). Whatever the mechanism, in
454 no case was loss of PA-X expression detrimental to yield of CVVs, when assessing HA yield
455 of a wide range of different influenza A subtypes/strains. This approach of modifying the
456 PR8 donor backbone therefore potentially supplies a ‘universal’ approach that can be applied
457 to all CVVs that is additive with, but without the need for, generation and validation of,
458 subtype/strain-specific constructs, as is required for strategies based on altering the
459 glycoprotein genes. This could be beneficial to improve antigen yield in a pandemic setting
460 where manufacturers are required to produce large amounts of vaccine quickly.

461

462 **Materials and methods**

463

464 **Cell lines and plasmids**

465 Human embryonic kidney (293T) cells, canine kidney Madin-Darby canine kidney epithelial
466 cells (MDCK) and MDCK-SIAT1 (stably transfected with the cDNA of human 2,6-
467 sialtransferase; (57)) cells were obtained from the Crick Worldwide Influenza Centre, The
468 Francis Crick Institute, London. QT-35 (Japanese quail fibrosarcoma; (58)) cells were
469 obtained from Dr Laurence Tiley, University of Cambridge. Cells were cultured in DMEM
470 (Sigma) containing 10% (v/v) FBS, 100 U/mL penicillin/streptomycin and 100 U/mL
471 GlutaMAX with 1 mg/ml Geneticin as a selection marker for the SIAT cells. Infection was
472 carried out in serum-free DMEM containing 100 U/mL penicillin/streptomycin, 100 U/mL
473 GlutaMAX and 0.14% (w/v) BSA. Cells were incubated at 37°C, 5% CO₂. Reverse genetics
474 plasmids were kindly provided by Professor Ron Fouchier (A/Puerto Rico/8/34; (59)),
475 Professor Wendy Barclay (A/England/195/2009 (60) and A/turkey/England/50-92/91 (61)),

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478 Professor Earl Brown (A/Hong Kong/1/68 (65)) and Professor Munir Iqbal
479 (A/chicken/Pakistan/UDL-01/2008 (66)). RG plasmids for A/mallard/Netherlands/12/2000
480 (NIBRG-60) and A/turkey/Turkey/1/2005 (NIBRG-23; with the multi-basic cleavage site
481 removed (67)) were made by amplifying HA and NA genes by PCR from cDNA clones
482 available within NIBSC and cloning into pHW2000 vector using BsmB1 restriction sites. A
483 plasmid containing the *Renilla* luciferase gene behind the simian virus 40 early promoter
484 (pRL) was supplied by Promega Ltd.

485

486 **Antibodies and sera**

487 Primary antibodies used were: rabbit polyclonal antibody anti-HA for swine H1 (Ab91641,
488 AbCam), rabbit polyclonal anti-HA for H7N7 A/chicken/MD/MINHMA/2004 (IT-003-008,
489 Immune Tech Ltd), mouse monoclonal anti-HA for H5N1 (8D2, Ab82455, AbCam),
490 laboratory-made rabbit polyclonal anti-NP (2915) (68), anti-PA residues 16-213 (expressed
491 as a fusion protein with β -galactosidase (69), anti-puromycin mouse monoclonal antibody
492 (Millipore MABE343), rabbit anti-PR8 PA-X peptide (residues 211-225) antibody (30) and
493 anti-tubulin- α rat monoclonal antibody (Serotec MCA77G). Secondary antibodies used were:
494 for immunofluorescence, Alexa fluor donkey anti-rabbit IgG 488 or 594 conjugates
495 (Invitrogen), for immunohistochemistry, goat anti-mouse horseradish peroxidase (Biorad
496 172-1011) and goat anti-rabbit horseradish peroxidase (Biorad 172-1019), for western blot,
497 donkey anti-rabbit IgG Dylight800 or Alexa fluor 680-conjugated donkey anti-mouse IgG
498 (Licor Biosciences).

499

500 **Site-directed mutagenesis**

501 The QuikChange® Lightning site-directed mutagenesis kit (Stratagene) was used according
502 to the manufacturer's instructions. Primers used for site-directed mutagenesis of the segment
503 3 gene were designed using the primer design tool from Agilent technologies. The strategies
504 used to disrupt the frameshift site (FS) as well as generating C-terminally truncated versions
505 of PA-X via PTCs were as described (30); the cited study used the PTC1 construct.

506

507

508 **Protein analyses**

509 Coupled *in vitro* transcription–translation reactions were carried out in rabbit reticulocyte
510 lysate supplemented with ³⁵S-methionine using the Promega TNT system according to the
511 manufacturer's instructions. SDS–PAGE followed by autoradiography was performed
512 according to standard procedures. Immunoprecipitations were performed as previously
513 described (70). Transfection-based reporter assays to assess host cell shut-off by PA-X
514 (described previously (30)) were performed by co-transfecting QT-35 cells with a reporter
515 plasmid containing the *Renilla* luciferase gene along with pHW2000 plasmids expressing the
516 appropriate segment 3 genes with or without the desired PA-X mutations. 48 h post-
517 transfection, cells were lysed and luciferase activity measured on a Promega GloMax 96-well
518 Microplate luminometer using the Promega *Renilla* Luciferase system.

519

520 **Reverse genetics rescue of viruses**

521 All viruses used in this study were made by reverse genetics. 293T cells were transfected
522 with eight pHW2000 plasmids each encoding one of the influenza segments using
523 Lipofectamine™ 2000 (Invitrogen). Cells were incubated for 6 hours post-transfection before
524 medium was replaced with DMEM serum-free virus growth medium. At 2 days post-
525 transfection, 0.5 µg/ml TPCK trypsin (Sigma) was added to cells. Cell culture supernatants

526 were harvested at 3 days post-transfection. 293T cell culture supernatants were clarified and
527 used to infect 10-11 day-old embryonated hens' eggs. At 3 days p.i., eggs were chilled over-
528 night and virus stocks were partially sequenced to confirm identity.

529

530 **RNA extraction, RT-PCR and sequence analysis.**

531 Viral RNA extractions were performed using the QIAamp viral RNA mini kit with on-
532 column DNase digestion (QIAGEN). Reverse transcription used the influenza A Uni12
533 primer (AGCAAAAGCAGG) using a Verso cDNA kit (Thermo Scientific). PCR reactions
534 were performed using Pfu Ultra II fusion 145 HS polymerase (Stratagene) or Taq Polymerase
535 (Invitrogen) according to the manufacturers' protocols. PCR products were purified for
536 sequencing by Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare).
537 Primers and purified DNA were sent to GATC biotech for Sanger sequencing (Lightrun
538 method). Sequences were analysed using the DNASTar software.

539

540 **Virus titration**

541 Plaque assays, TCID₅₀ assays and haemagglutination assays were performed according to
542 standard methods (71). MDCK or MDCK-SIAT cells were used for infectious virus titration,
543 and infectious foci were visualised by either toluidine blue or immunostaining for influenza
544 NP and visualising using a tetra-methyl benzidine (TMB) substrate.

545

546 **Virus purification and analysis**

547 Allantoic fluid was clarified by centrifugation twice at 6,500 x g for 10 mins. Virus was then
548 partially purified by ultracentrifugation at 128,000 x g for 1.5 hours at 4°C through a 30%
549 sucrose cushion. For further purification, virus pellets were resuspended in PBS, loaded onto
550 15-60% sucrose/PBS density gradients and centrifuged at 210,000 x g for 40 mins at 4°C.

551 Virus bands were extracted from gradients and virus was pelleted by ultracentrifugation at
552 128,000 x g for 1.5 hours at 4°C. Pellets were resuspended in PBS and aliquots treated with
553 N-glycosidase F (New England Biolabs), according to the manufacturer's protocol. Virus
554 pellets were lysed in Laemmli sample buffer and separated by SDS-PAGE on 10% or 12%
555 polyacrylamide gels under reducing conditions. Protein bands were visualised by Coomassie
556 blue staining (ImperialTM protein stain, Thermo Scientific) or detected by immunostaining in
557 western blot. Coomassie stained gels were scanned and bands quantified using ImageJ
558 software. Western blots were scanned on a Li-Cor Odyssey Infrared Imaging system v1.2
559 after staining with the appropriate antibodies and bands were quantified using ImageStudio
560 Lite software (Odyssey).

561

562 **Chick embryo pathogenesis model**

563 Ten-day old embryonated hens' eggs were inoculated via the allantoic cavity route with 1000
564 PFU in 100 µl per egg or mock (serum-free medium only) infected. Embryo viability was
565 subsequently determined by examination of veins lining the shell (which collapse on death)
566 and embryo movement (for a few minutes). At 2 - 3 days p.i. (depending on experiment),
567 embryos were killed by chilling, washed several times in PBS and then scored blind for overt
568 pathology by two observers in each experiment. Scores were 0 = normal, 1 = intact but with
569 dispersed haemorrhages, 2 = small, fragile embryo with dispersed haemorrhages. For
570 histology, embryos were decapitated, washed several times in PBS, imaged and fixed for
571 several days in 4% formalin in PBS. Two embryos per virus condition were sectioned
572 longitudinally and mounted onto paraffin wax. Tissue sections were cut and mounted onto
573 slides and stained with haematoxylin and eosin (H&E) by the Easter Bush Pathology Service.
574 Further sections were examined by immunohistofluorescence performed for influenza NP
575 (62). Sections were deparaffinised and rehydrated and heat-induced antigen retrieval was

576 performed using sodium citrate buffer (10 mM sodium citrate, 0.05% Tween20, pH 6.0).
577 Sections were stained with anti-NP antibody followed by an Alexa fluor-conjugated
578 secondary antibody. Pre-immune bleed serum was also used to confirm specificity of staining
579 by anti-NP antibody. Sections were mounted using ProLong Gold anti-fade reagent
580 containing DAPI (Invitrogen). Stained tissue sections were scanned using a Nanozoomer XR
581 (Hamamatsu) using brightfield or fluorescence settings. Images were analysed using the NDP
582 view 2.3 software (Hamamatsu).

583

584 **Graphs and statistical analyses**

585 All graphs were plotted and statistical analyses (Mantel-Cox test, t-tests and Dunnett's and
586 Tukey's tests as part of one-way Anova) performed using Graphpad Prism software.

587

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608

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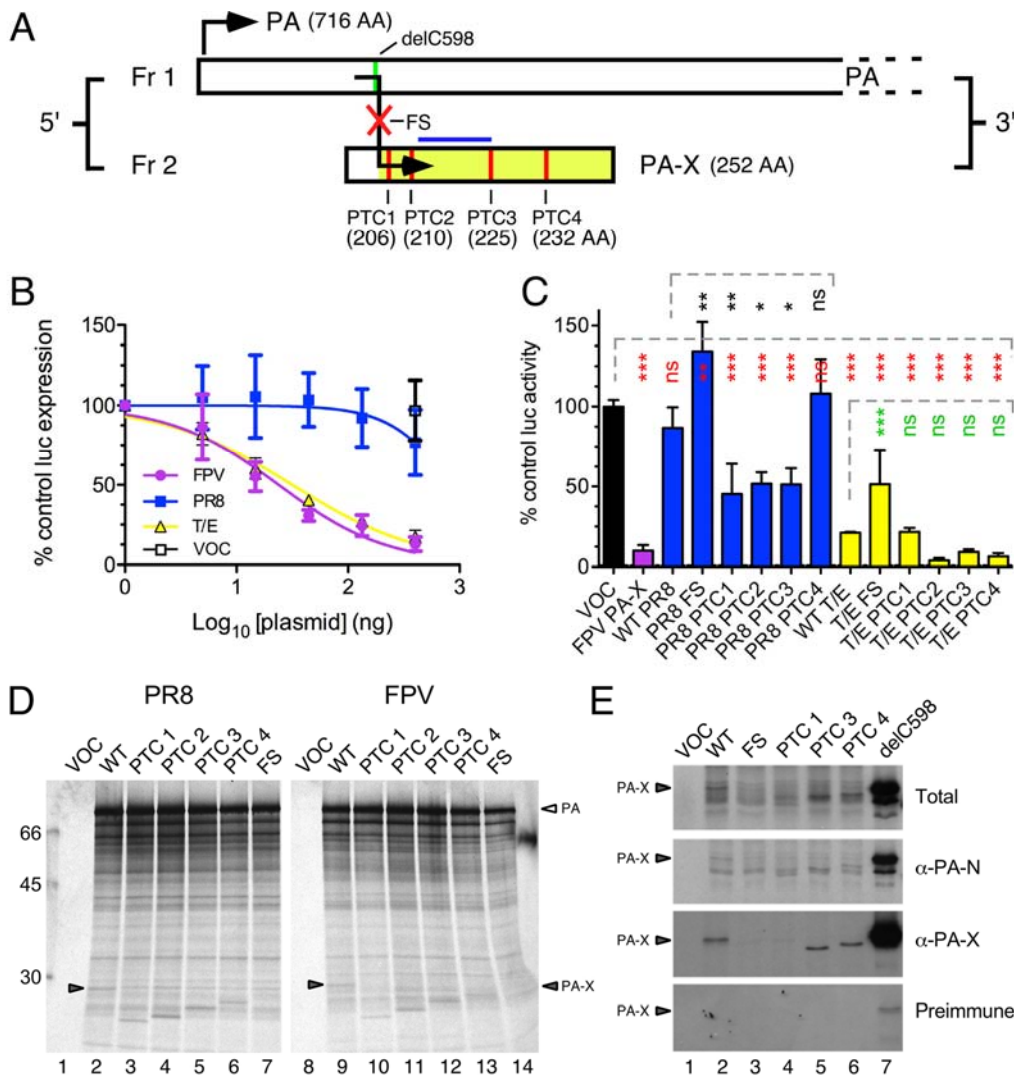
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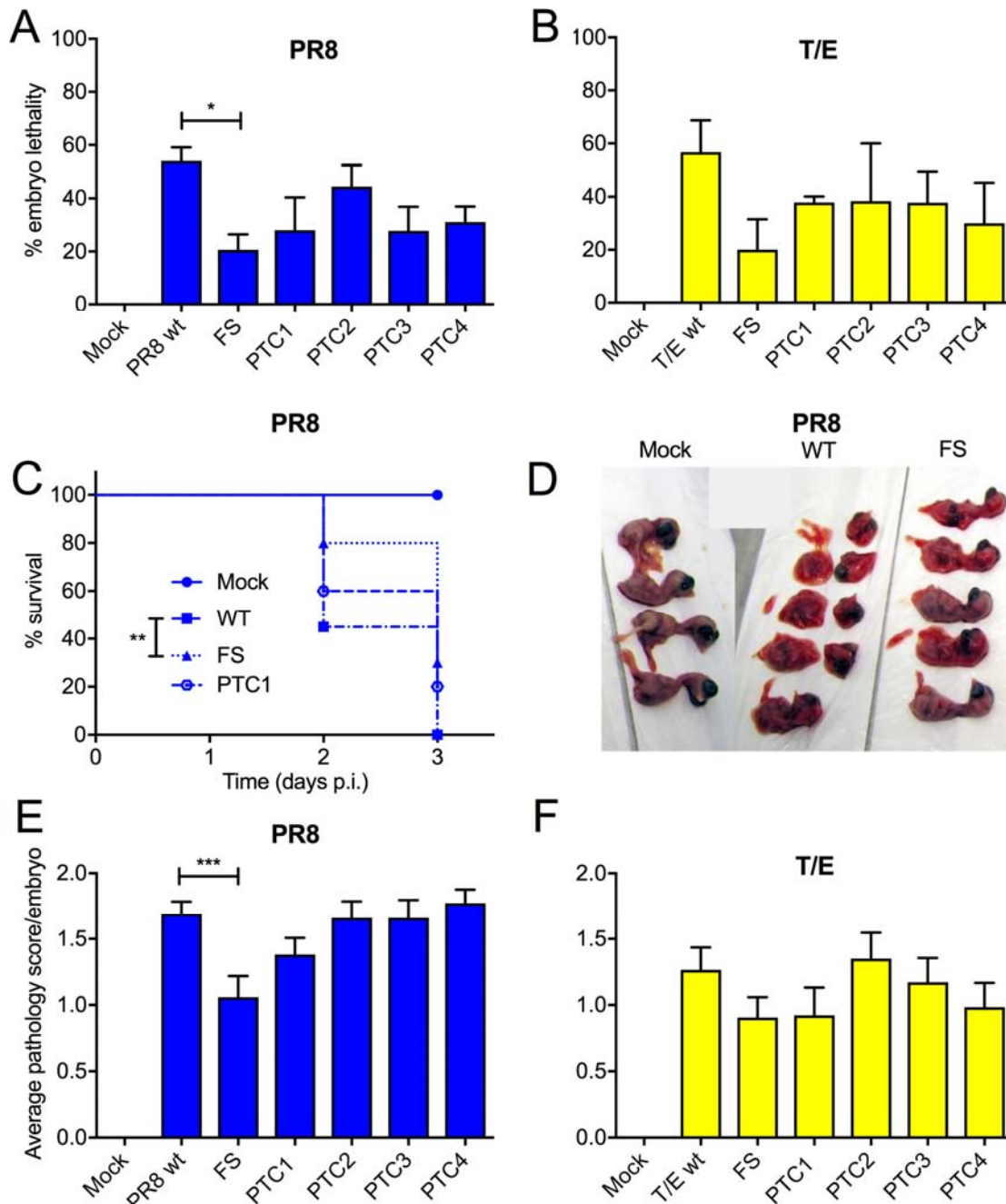
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853 **Figures**

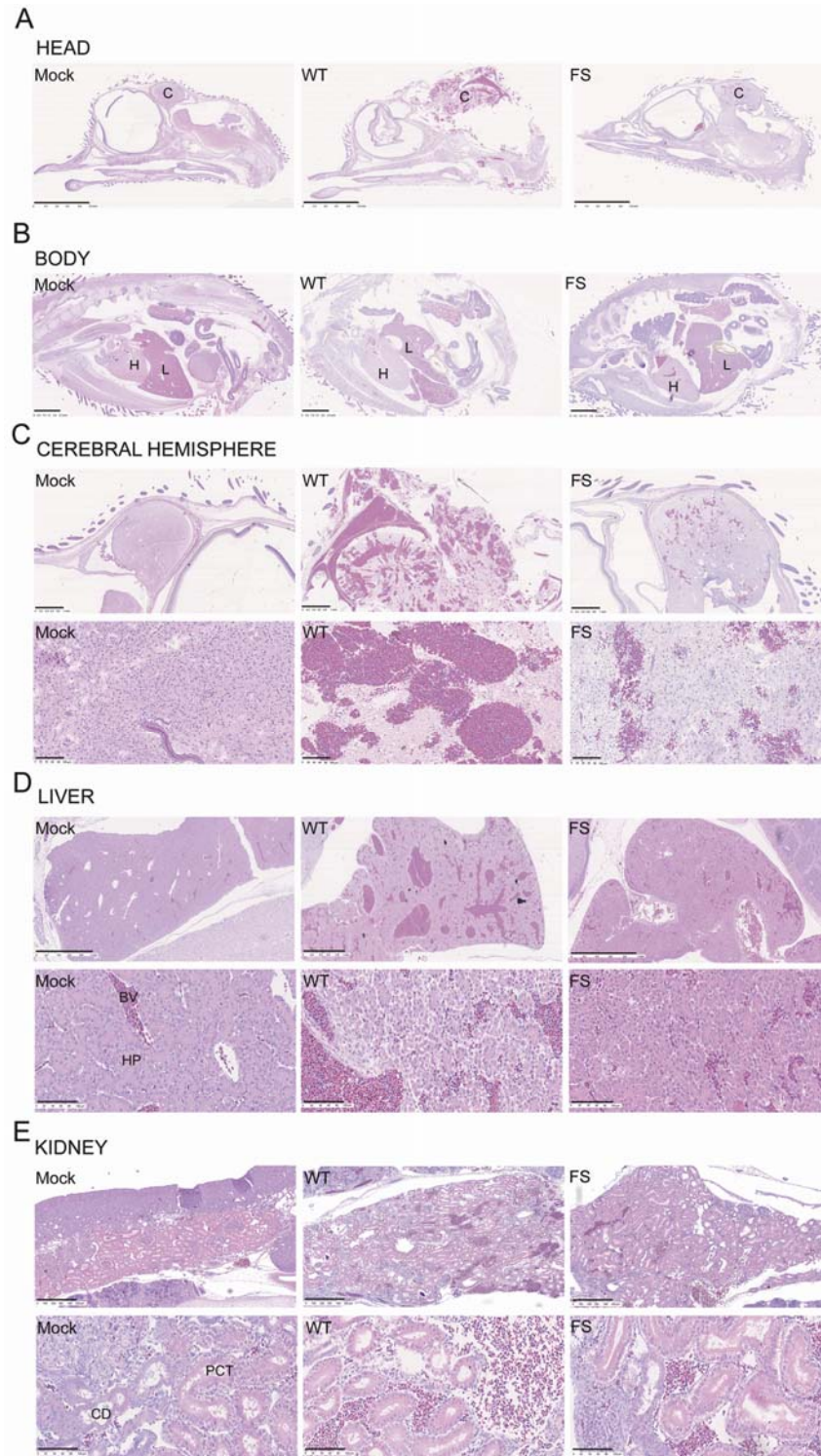


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 855 **FIGURE 1. Virus strain dependent variation in PA-X-mediated host cell shut-off**
 856 **activity.** **A)** Schematic showing mutations in segment 3: at the frameshift (FS) site to generate a PA-X null
 857 virus, or in the X-ORF so that segment 3 expresses C-terminally truncated versions of PA-X (PTCs 1-4, size of
 858 products indicated), or removing cytosine 598 (delC598) to place the X ORF in frame with PA such that only
 859 PA-X is expressed. **B, C)** PA-X-mediated inhibition of cellular RNA polymerase II-driven gene expression in
 860 QT-35 cells. **B)** Cells were co-transfected with 100 ng of pRL plasmid constitutively expressing *Renilla*
 861 luciferase and a dilution series of the indicated segment 3 pHW2000 plasmids or with a fixed amount of the
 862 empty pHW2000 vector (VOC). Luciferase activity was measured 48 h later and plotted as % of a pRL only
 863 sample. Dose-inhibition curves were fitted using GraphPad Prism software. Data are mean \pm SD of two
 864 independent experiments each performed in triplicate. **C)** Cells were co-transfected with 100 ng of pRL plasmid
 865 and 400 ng of effector pHW2000 plasmids expressing segment 3 products. Luciferase activity was measured 48
 866 h later and plotted as the % of a pHW2000 vector only control. Data are the mean \pm SD from 2 independent
 867 experiments performed in duplicate. Dashed lines indicate groups of statistical tests (against the left hand bar in
 868 each case; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) as assessed by Dunnett's test. **D, E)** *In vitro* translation of PA-X
 869 from PR8 segment 3 constructs. Aliquots of rabbit reticulocyte lysate supplemented with ³⁵S-methionine were
 870 programmed with the indicated plasmids and radiolabelled polypeptides visualised by SDS-PAGE and
 871 autoradiography before **(D)** or after **(E)** immunoprecipitation with the indicated antisera. Arrowheads in **(D)**
 872 indicate full length PA-X while molecular mass (kDa) markers are shown on the left.
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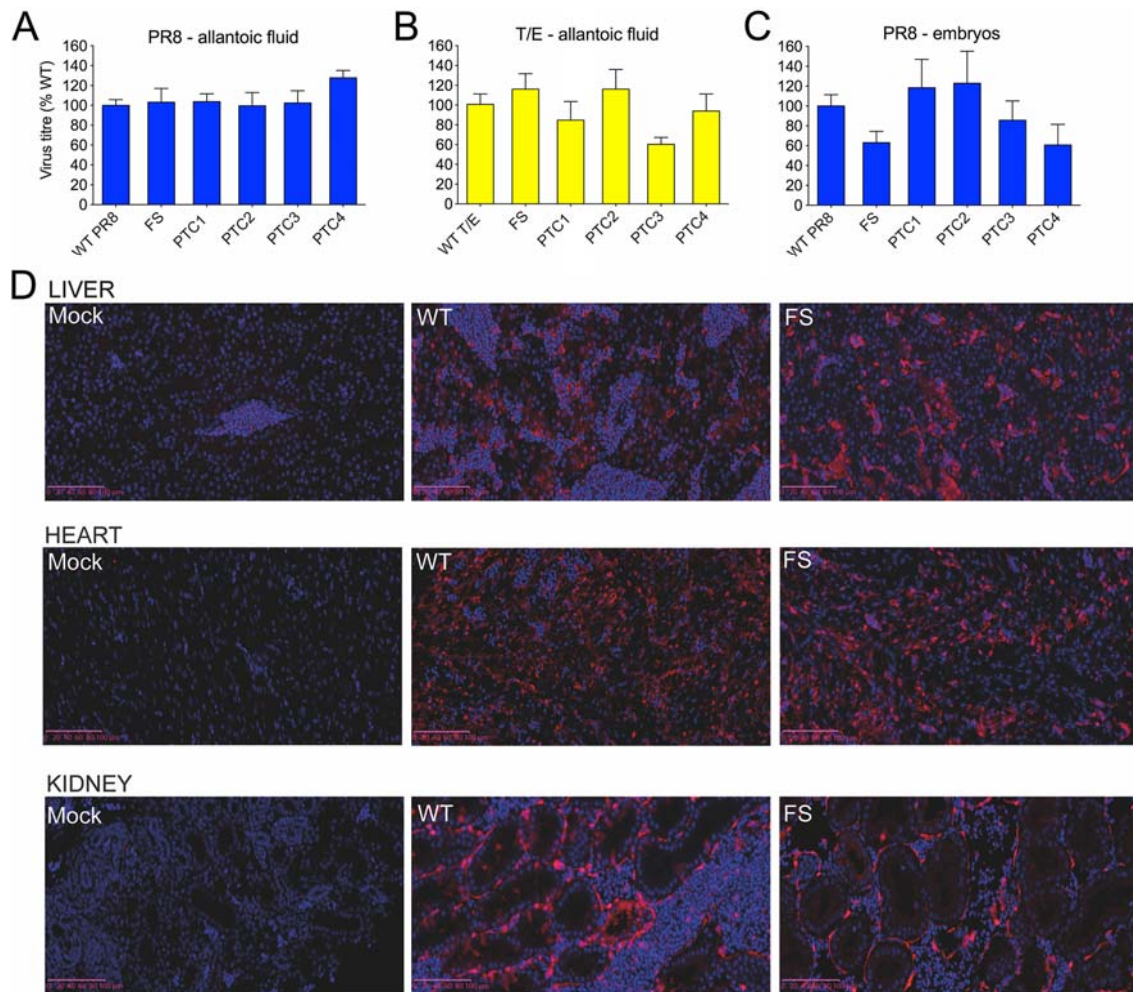
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FIGURE 2. Effect of PA-X mutations in a chick embryo pathogenicity model. Groups of 5-6 embryonated hens' eggs were infected with 1000 PFU of the indicated viruses and **A, B**) embryo viability was determined by candling at 2 days p.i. Data are plotted as mean \pm SEM % embryo lethality from 3-4 independent experiments. Horizontal bars indicate statistical significance (* $p < 0.05$) as assessed by Dunnett's test. **C**) Infected eggs were monitored daily for embryo viability and survival was plotted versus time. Data are from 3 independent experiments with 5 - 10 eggs per experiment. Statistical significance between WT and FS viruses (** $p < 0.01$) was assessed by log-rank (Mantel-Cox) test. **D-F**) From the experiments described in **A**) and **B**), embryos were imaged **D**) and **E, F**) scored blind by two observers as 0 = normal, 1 = intact but bloody, 2 = small, damaged and with severe haemorrhages. Data are the average \pm SEM pathology scores from 3-4 independent experiments. Horizontal bar indicates statistical significance (***) $p < 0.001$) as assessed by Dunnett's test.



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FIGURE 3. Histopathology of chick embryos following infection with PR8 viruses. Embryonated hens' eggs were infected with segment 3 WT or mutant viruses or mock infected. 2 days p.i. embryos were fixed, sectioned and stained with H&E before imaging with a Nanozoomer XR (Hamamatsu) using brightfield settings; representative pictures are shown: **A)** head, scale bar = 5 mm, and **B)** body, scale bar = 2.5 mm, and **C)** cerebral hemisphere, **D)** liver and **E)** kidney, scale bars for low and high magnification images = 1mm and 100 μ m or 500 μ m, respectively. C = cerebral hemisphere, H = heart, L = liver, PCT = proximal convoluted tubule, CD = collecting duct, HP=hepatocytes, BV= blood vessel).



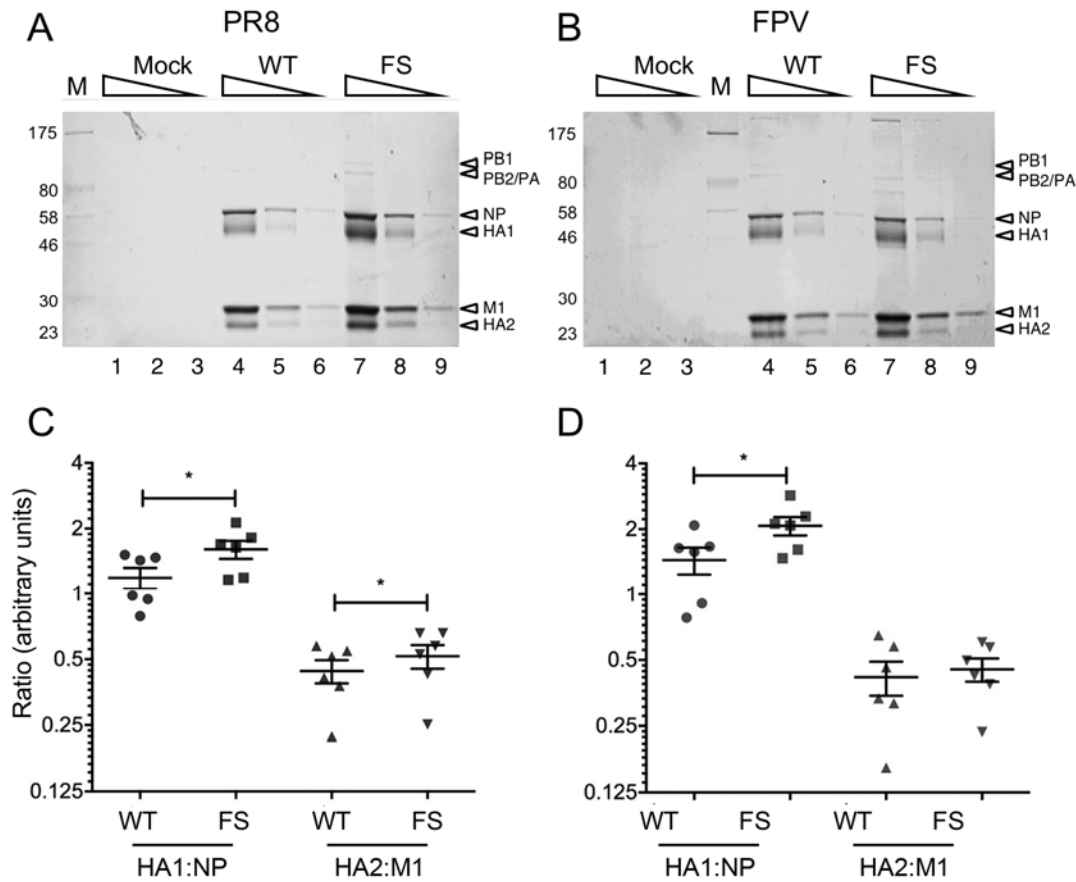
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897 **FIGURE 4. Effects of mutating PA-X expression on virus replication in chick embryos.**

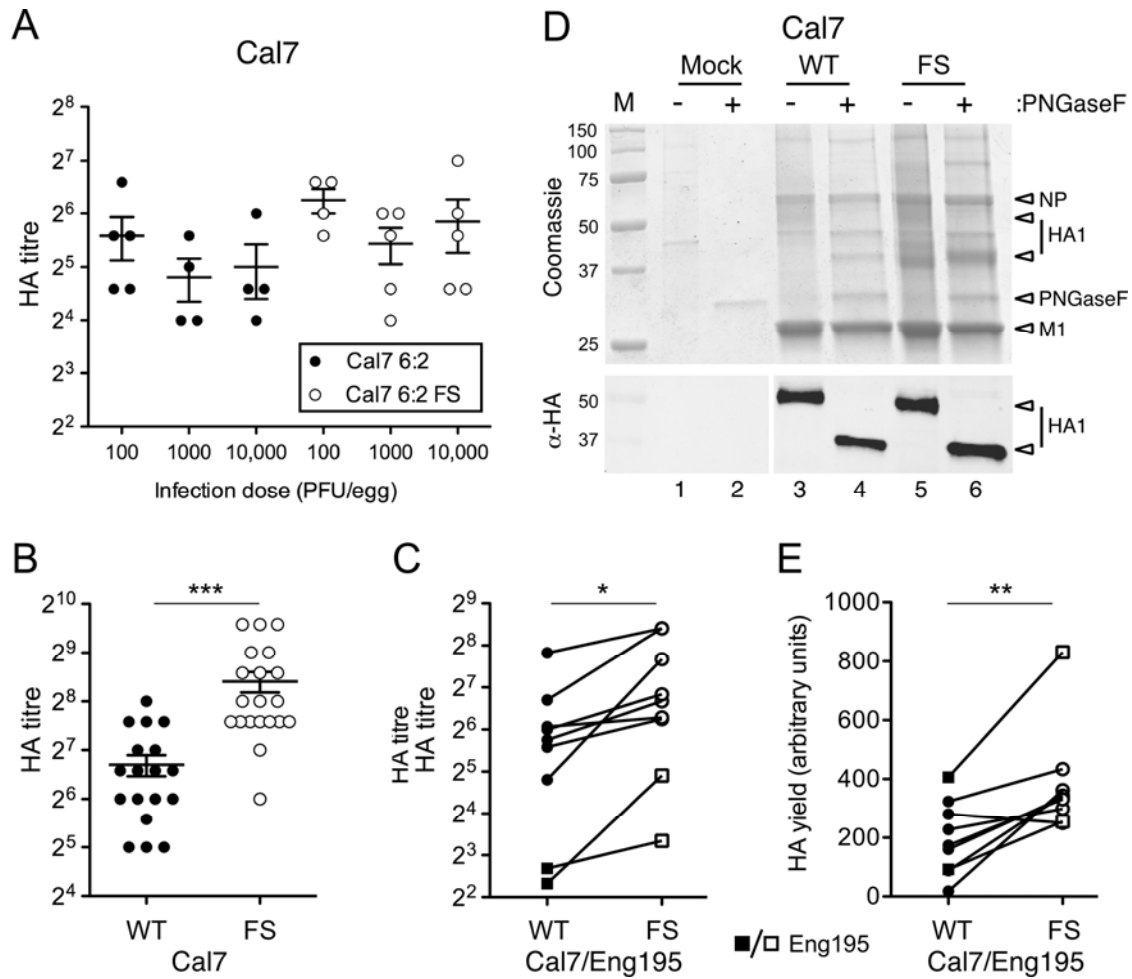
898 Groups of 5-6 embryonated hens' eggs were infected with the indicated viruses and at 2 days p.i., virus titres
899 determined by plaque assay from (A, B) allantoic fluid or (C) washed and macerated chick embryos. Graphs
900 represent the mean \pm SEM from 3 (A, B) or 2-4 independent experiments (C). Titres of mutant viruses were not
901 significantly different compared to WT virus (Dunnett's test). (D) Embryos were fixed at 2 days p.i., sectioned
902 and stained for IAV NP and DNA before imaging using a Nanozoomer XR (Hamamatsu) on fluorescence
903 settings. Representative images of liver, heart and kidney are shown. Scale bars = 100 μ m. NP = red, DAPI =
904 blue.

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FIGURE 5. Virion composition of WT or FS mutant viruses. Embryonated hens' eggs were infected with WT or segment 3 mutant viruses or mock infected. At 2 days p.i., virus was purified from allantoic fluid by sucrose density gradient ultracentrifugation and 3-fold serial dilutions **A, B**) analysed by SDS-PAGE on 10% polyacrylamide gels and staining with Coomassie blue. **C, D**) For PR8 and FPV, respectively, the ratios of NP:HA1 and M1:HA2 were determined by densitometry of SDS-PAGE gels. Scatter plots with the mean and SEM of 6 measurements from 3 independent experiments using 2 independently rescued virus stocks are shown. Horizontal bars indicate statistical significance (* $p < 0.05$) as assessed by paired t-test.



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917 **FIGURE 6. Effect of the PA-X FS mutation on HA yield of A(H1N1) pdm09 CVV**
 918 **mimics.** Embryonated hens' eggs were infected as indicated and **A-C)** HA titres in allantoic fluid measured at
 919 3 days post-infection for (A) groups of 5 or (B) 20 eggs per condition. (A, B) Scatter plots of titres from
 920 individual eggs with mean and SEM are shown. (***) $p < 0.001$ assessed by unpaired t-test. C) Average HA
 921 titres from groups of eggs inoculated at the infection dose which gave maximum yield are shown as paired
 922 observations. Statistical significance ($* p < 0.05$, $n=9$) assessed by paired t-test. **D, E)** Allantoic fluid was
 923 clarified and virus pelleted by ultracentrifugation through 30% sucrose pads. Equal volumes of resuspended
 924 virus pellets were separated by SDS-PAGE on a 12% polyacrylamide gel and visualized by (D) staining with
 925 Coomassie blue (upper panel) or western blot for HA1 (lower panel) with (+) or without (-) prior treatment with
 926 PNGase F. Molecular mass (kDa) markers and specific polypeptides are labelled. **E)** De-glycosylated HA1 yield
 927 was quantified by densitometry of the western blots. Data points represent 8 independent experiments using 3
 928 independently rescued RG virus stocks shown as paired observations. (** $p < 0.01$, $n=8$) as assessed by paired t-
 929 test. Circles represent Cal7 and squares represent Eng195 CVV mimics.

930 **TABLE 1. Effects of the Δ PA-X FS mutation on HA yield of CVVs grown in eggs.**

Lineage	Subtype	Strain	No. of independent rescues	HA titre 6:2 virus ^a	HA titre 6:2FS virus	Relative ^b HA titre	Relative ^b HA1 yield	No. of expts.	Small scale	Large scale
Human pdm2009	H1N1	A/California/07/2009	2	106 ± 86.6	249 ± 109	2.65 ± 2.16	1.9 ± 1.07 ^c	7	4	3
Human pdm2009	H1N1	A/California/07/2009 chimeric HA (NIBRG-119)	1	-	-	-	1.54 ± 0.43	2	1	1
Human pdm2009	H1N1	A/England/195/2009	1	5.71 ± 0.71	20.1 ± 9.92	3.79 ± 2.21	2.4 ± 0.37	2	1	1
Human pdm1968	H3N2	A/Udorn/307/72	2	2200 ± 929	2100 ± 720	1.26 ± 0.47	1.35 ± 0.36	5	3	2
Human pdm1968	H3N2	A/Hong Kong/1/68	1	801 ± 117	843 ± 140	1.05 ± 0.06	1.22 ± 0.39	3	2	1
Avian	H7N3	A/mallard/Netherlands/12/2000 (NIBRG-60)	2	33.5 ± 29.1	45.0 ± 36.9	1.34 ± 0.18	1.55 ± 0.14	5	3	2
Avian	H5N1	A/turkey/Turkey/1/2005/1/2005 (NIBRG-23)	2	47.1 ± 27.3	48.8 ± 23.2	1.13 ± 0.23	1.10 ± 0.30	5	3	2
Avian	H1N1	A/mallard/Netherlands/10/99	2	123 ± 59	128 ± 37	1.22 ± 0.37	1.13 ± 0.36	5	4	1
Avian	H9N2	A/chicken/Pakistan/UDL-01/2008	2	302 ± 364	312 ± 264	0.92 ± 0.30	1.01 ± 0.18	4	2	2

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^aValues are mean ± SD.

^bRelative means the ratio of the average HA titres (of eggs incubated at 35°C) or HA1 yield of FS (Δ PA-X) viruses to their WT counterparts

^cAn outlier from one experiment was ignored when taking the average.