

1 A rare variant in *Anp32B* impairs influenza virus replication 2 in human cells

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7 SUMMARY

8 Viruses require host factors to support their replication, and genetic variation in such factors
9 can affect susceptibility to infectious disease. Influenza virus replication in human cells relies
10 on ANP32 proteins, which serve essential but redundant roles to support influenza virus
11 polymerase activity. Here, we investigate naturally occurring single nucleotide variants
12 (SNV) in the human *Anp32A* and *Anp32B* genes. We note that variant rs182096718 in
13 *Anp32B* is found at a higher frequency than other variants in either gene. This variant results
14 in a D130A substitution in ANP32B, which is less able to support influenza virus polymerase
15 (FluPol) activity than wildtype ANP32B. Using a split luciferase binding assay, we also show
16 reduced interaction between ANP32B-D130A and FluPol. We then use CRISPR/Cas9
17 genome editing to generate the mutant homozygous genotype in human eHAP cells, and
18 show that FluPol activity and virus replication are attenuated in cells expressing wildtype
19 ANP32A and mutant ANP32B-D130A. This is in contrast to cells that completely lack
20 expression of ANP32B where no attenuation is seen. We conclude that ANP32B-D130A
21 exerts a dominant negative effect on the pro-viral activity of ANP32A in cells, and suggest
22 that carriers of rs182096718 may have some genetic protection against influenza viruses.

23 KEYWORDS

24 *Influenza virus, ANP32B, mutation, single nucleotide variant, host factor*

25 INTRODUCTION

26 All viruses rely on host factors to support their replication. Genetic variation in such proteins
27 can affect susceptibility to infectious disease (Ciancanelli et al., 2016; Karlsson et al., 2014;
28 Pittman et al., 2016; Zhang, 2020). Perhaps the best-known mutation conferring innate
29 genetic resistance to viral infection is the $\Delta 32$ variant of the HIV-1 co-receptor C-C
30 chemokine receptor type 5 (*CCR5*) (Carrington et al., 1999). Single nucleotide variants that
31 reduce susceptibility to virus infection include a P424A substitution in the filovirus endosomal
32 fusion receptor Niemann-PickC1 (*NPC1*) (Carette et al., 2011; Kondoh et al., 2018), and a
33 G428A mutation in fucosyltransferase 2 (*FUT2*) that renders homozygous carriers resistant
34 to norovirus (Lindesmith et al., 2003; Thorven et al., 2005). A mutation in interferon-induced
35 transmembrane protein 3 (*IFITM3*) has been associated with increased severity of infection
36 with influenza virus (Ciancanelli et al., 2016; Everitt et al., 2012; Prabhu et al., 2018; Zhang
37 et al., 2013), as have monogenic lesions of *GATA2*, *IRF7*, *IRF9* and *TLR3* (Zhang, 2020).
38 Besides a pair of polymorphisms in the non-coding region (NCR) of *LGALS1*, which may
39 confer some protection from H7N9 influenza A virus (IAV) in Chinese poultry workers (Chen
40 et al., 2015), no single nucleotide variants protective against influenza virus have thus far
41 been described.

42 Influenza replication and transcription are carried out in the host cell nucleus by the virus-
43 encoded heterotrimeric RNA-dependent RNA polymerase (Te Velhuis and Fodor, 2016),
44 which relies on host factors to support RNA synthesis (Peacock et al., 2019). Influenza

45 viruses co-opt the host proteins acidic nuclear phosphoprotein 32 kilodaltons A (ANP32A)
46 and ANP32B to support their replication (Long et al., 2016; Staller et al., 2019; Zhang et al.,
47 2019). ANP32A and ANP32B are functionally redundant in their support for influenza virus:
48 in human cells lacking either but not both ANP32A (AKO) or ANP32B (BKO), virus
49 proliferation is not impaired. In cells lacking both proteins (dKO), however, FluPol activity
50 and virus growth are completely abrogated (Staller et al., 2019; Zhang et al., 2019). ANP32
51 proteins are expressed in many different human tissues, including fibroblasts and lung
52 (Lonsdale et al., 2013), and involved in a wide range of cellular processes, including
53 apoptosis and transcriptional regulation (Reilly et al., 2014). ANP32A and ANP32B are 249
54 and 251 amino acids long, respectively, with two major domains: a structured N-terminal
55 leucine-rich repeat region (LRR) and an unresolved low-complexity acidic region (LCAR)
56 (Huyton and Wolberger, 2007; Tochio et al., 2010). Mutational analysis of ANP32 proteins of
57 several species relevant to influenza has shown that the identity of amino acid residues 129
58 and 130 dictates pro-viral function (Long et al., 2019; Peacock et al., 2020; Staller et al.,
59 2019; Zhang et al., 2019). ANP32 proteins that cannot be co-opted by influenza virus include
60 human ANP32E (unpublished data), chicken ANP32B, and mouse ANP32A. What these
61 orthologues have in common is divergence from the pro-viral dyad 129N-130D: human
62 ANP32E has glutamate at position 129 (129E) rather than asparagine, chicken ANP32B has
63 isoleucine at position 129 (129I) and asparagine at position 130 (130N), and mouse ANP32A
64 has alanine at position 130 (130A), suggesting that these amino acids are important for pro-
65 viral activity of ANP32 proteins.

66 Here we describe natural variation in the human *Anp32A* and *Anp32B* genes, in particular
67 the relatively enriched SNV rs182096718 in *Anp32B*, which has a global minor allele
68 frequency (MAF) of 0.0044 and is prevalent in 3.41% of individuals of Hispanic / Latino
69 ethnicity. SNV rs182096718 translates to a mutant ANP32B-D130A protein product, and is
70 the only variant for which homozygous carriers exist. We generated the homozygous mutant
71 genotype (wildtype *Anp32A* / mutant *Anp32B*) in low-ploidy human eHAP cells
72 (Essletzbichler et al., 2014) by CRISPR/Cas9 genome editing. Using minigenome reporter
73 assays and virus challenge, we found that IAV FluPol activity and replication were severely
74 restricted in monoclonal cells expressing wildtype ANP32A and ANP32B-D130A. We
75 conclude that cells expressing ANP32B-D130A alongside wildtype ANP32A are less able to
76 support influenza polymerase activity, even when compared with cells that lack ANP32B
77 expression (BKO). This suggests a potential dominant negative effect of ANP32B-D130A on
78 ANP32A pro-viral activity. We speculate that carriers of this variant may have some
79 resistance to influenza viruses.

80 RESULTS

81 A relatively common variant encodes ANP32B-D130A

82 We searched public databases for rare missense SNVs in the human *Anp32A* and *Anp32B*
83 genes that were predicted, on the basis of our previous work, to have functional impact on
84 the pro-influenza virus activity of the proteins. The databases included the dbSNP from the
85 National Center for Biotechnology Information (NCBI) (Sherry et al., 2001), the Avon
86 Longitudinal Study of Parents and Children (ALSPAC) (Walter et al., 2015), Trans-Omics for
87 Precision Medicine (TOPMed) (Taliun et al., 2019), the 1000 Genomes Project (Genomes
88 Project et al., 2015), the Grand Opportunity Exome sequencing Project (GO-ESP) (Fu et al.,
89 2013), and the genome aggregation database (gnomAD) (Karczewski et al., 2019). The
90 latter lists a total 54 missense SNVs in *Anp32A* and 82 in *Anp32B*.

91 Four SNVs in *Anp32A* and five in *Anp32B* were selected for investigation, taking into
92 account changes in charge, bulk, polarity and putative solvent exposure of the encoded

93 amino acid substitutions (Huyton and Wolberger, 2007; Tochio et al., 2010), as well as the
 94 previously established importance of positions 129 and 130 for pro-influenza viral activity
 95 (Figure 1A). All the variants in *Anp32A* are exceedingly rare (MAF<0.001), and no
 96 homozygous carriers have been identified. The serine mutants at position 158 were selected
 97 as this is a putative phosphorylation site (Hong et al., 2004). The SNV responsible for a
 98 mutant ANP32A-D130N protein (rs771143708) is found in a single heterozygous individual
 99 out of 3,854 in the ALSPAC cohort, rs751239680 (ANP32A-R132Q) is found in a single
 100 heterozygous individual out of 10,052 in the African cohort of the gnomAD database,
 101 rs772530468 (ANP32A-S158T) is present in a single South Asian male in 23,070 in the
 102 gnomAD database, as well as two separate homozygous individuals in the TOPMed
 103 database. Finally, a single African male in 5,032 in the gnomAD database is a heterozygous
 104 carrier of rs772530468 (ANP32A-S158A) (Figure 1B).

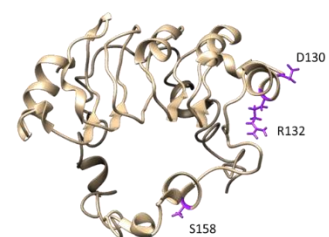
105 Most variants in *Anp32B* were also low-frequency (MAF<0.0001): rs377406514 (ANP32B-
 106 L128V) is found in a single African female out of 10,066 in the gnomAD database;
 107 rs771977254 (ANP32B-E133Q) occurs in two out of 4,900 Ashkenazi Jewish females in the
 108 gnomAD database; rs761932651 (ANP32B-L138H) is found in one South Asian male in
 109 23,070 in the gnomAD database; rs770020996 (ANP32B-L142F) occurs in one female in
 110 7,544 and one male in 23,072 from the South Asian cohort of the gnomAD database (Figure
 111 1C). There is, however, an exception. SNV rs182096718, encoding ANP32B-D130A, is
 112 relatively common in the Hispanic / Latino cohort of the gnomAD database, where in a total
 113 pool of 35,420 alleles 1,209 minority alleles were identified, as well as 25 homozygous
 114 carriers. The D130A substitution in ANP32B is thus present in 3.41% of the Latino cohort,
 115 which, compared with all other SNVs in either *Anp32A* or *Anp32B* is a relatively high
 116 frequency. We and others have previously shown that murine ANP32A, which naturally
 117 harbours 130A, does not support influenza virus polymerase. Substituting 130A in human
 118 ANP32A greatly reduced FluPol activity, while introducing 130D in mouse ANP32A rescued
 119 its capacity to support FluPol activity (Staller et al., 2019; Zhang et al., 2019). Indeed,
 120 ANP32B KO mice, but not ANP32A KO mice, showed reduced viral loads and mortality
 121 when infected with H3N2 or H5N1 influenza A virus (Beck et al., 2020).

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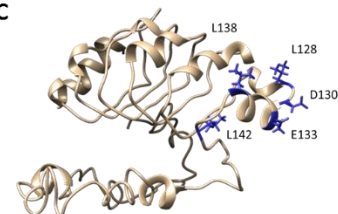
A

Gene	ID	Variant	Amino acid substitution	Minor allele frequency	Source	Heterozygous carriers	Homozygous carriers
<i>Anp32A</i>	rs771143708	C>T	D130N	T=0.0003	ALSPAC	1	0
	rs751239680	C>T	R132Q	T<0.0001	ExAC	1	0
	rs772530468	A>C	S158T	C<0.0001	gnomAD TOPMed	3	0
		A>T	S158A	T<0.0001	ExAC gnomAD	1	0
<i>Anp32B</i>	rs377406514	C>G	L128V	G<0.0001	gnomAD	1	0
	rs182096718	A>C	D130A	C=0.0044	gnomAD 1000G GO-ESP TOPMed	1,233	25
	rs771977254	G>C	E133Q	C<0.0001	gnomAD	2	0
	rs761932651	T>A	L138H	A<0.0001	gnomAD	1	0
	rs770020996	C>T	L142F	T<0.0001	gnomAD	2	0

B



C



123

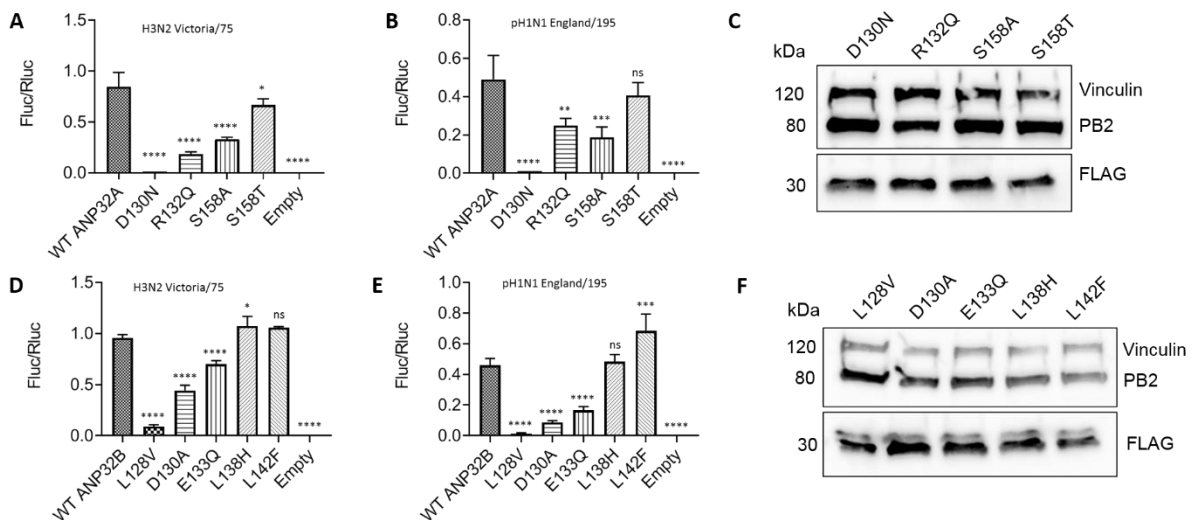
124 **Figure 1. Selected naturally occurring missense single nucleotide variants in *Anp32A* and**
 125 ***Anp32B*** (A) NCBI dbSNP Reference IDs, variant, amino acid substitution, global minor allele
 126 frequency (MAF), source database, and number of heterozygous and homozygous carriers for each

127 variant (B) Structural model of ANP32A showing amino acids affected by selected SNVs (C)
 128 Structural model of ANP32B showing amino acids affected by selected SNVs

129

130 **Selected mutant ANP32 proteins affect FluPol activity**

131 The capacity of each mutant protein, when expressed exogenously in human eHAP cells
 132 lacking ANP32A and ANP32B (dKO), to support reconstituted polymerases from a seasonal
 133 H3N2 virus (A/Victoria/3/75) and a 2009 pandemic H1N1 isolate (A/England/195/2009) was
 134 tested in minigenome reporter assays (Figure 2). ANP32A-D130N did not support IAV
 135 polymerase, while R132Q and S158A substitutions were significantly reduced in their
 136 capacity to support FluPol activity. ANP32A-S158T, in contrast, supported FluPol to an
 137 extent similar to wildtype ANP32A (Figure 2A and B). ANP32B-D130A had a large
 138 deleterious effect on the support for FluPol activity, as did the leucine to valine substitution at
 139 position 128 (ANP32B-L128V). ANP32B-E133Q was also significantly less able to support
 140 FluPol activity. Substitutions of the leucines at positions 138 and 142 to histidine (L138H)
 141 and phenylalanine (L142F), respectively, did not reduce the ability of the mutant ANP32B
 142 proteins to support FluPol activity (Figure 2D and E). None of the differences were explained
 143 by changes in expression (Figure 2C and F) or nuclear localisation of the mutant proteins
 144 (Supplementary Figure 1).



145

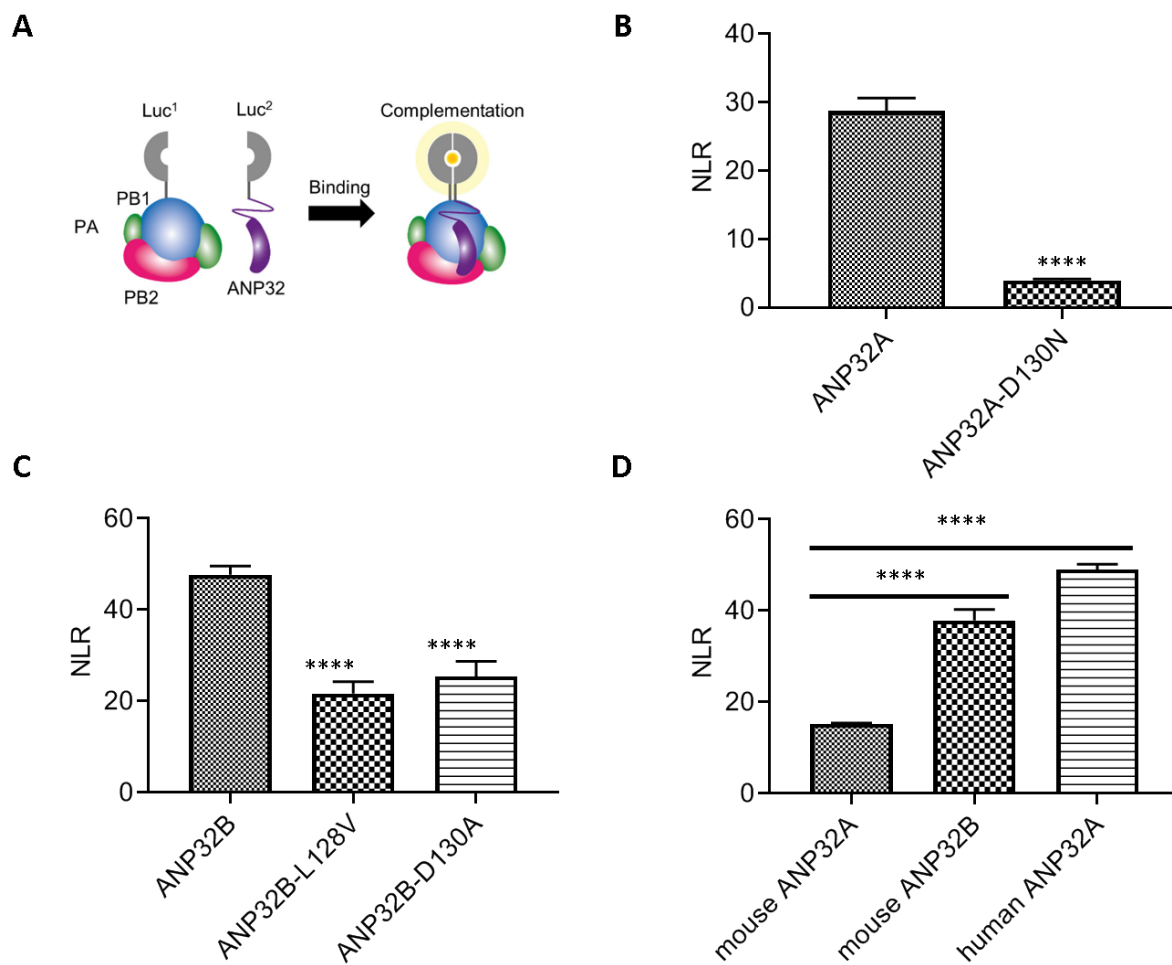
146 **Figure 2 ANP32A and B mutant proteins show variable capacity to rescue IAV FluPol activity**
 147 (A) and (B) Minigenome reporter assays in eHAP dKO cells with co-transfected FLAG-tagged
 148 ANP32A variants with H3N2 Victoria (A) or pH1N1 Eng/195 (B) RNP components (PB1, PB2, PA and
 149 NP), pPoll-firefly luciferase minigenome reporter, and Renilla luciferase control. Data show mean (SD)
 150 of firefly activity normalized to Renilla and analysed by one-way ANOVA from one representative
 151 repeat (n = 2 independent triplicate experiments). Panels (D) and (E) show identical experiments with
 152 ANP32B constructs. ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. (C)
 153 and (F) Western blotting analysis showing expression of H3N2 Victoria PB2 and FLAG tagged mutant
 154 ANP32A (C) or ANP32B (F) constructs compared with Vinculin loading control. Blots are
 155 representative of minigenome assays shown in (A) and (D), respectively.

156

157

158 Using a split luciferase complementation assay previously developed in our laboratory
 159 (Cassonnet et al., 2011; Mistry et al., 2020) (Figure 3A) we next assessed whether the

160 ANP32 mutants whose pro-viral function was most impaired (ANP32A-D130N, ANP32B-
161 L128V, and ANP32B-D130A) showed reduced binding to FluPol. The luciferase signal
162 indicating binding of ANP32A-D130N to FluPol was reduced >7-fold, relative to wildtype
163 ANP32A (Figure 3B). FluPol interactions of ANP32B-L128V and ANP32B-D130A were also
164 reduced, resulting in luciferase signals of around half those measured with the wildtype
165 ANP32B (Figure 3C). The poor pro-viral function of these mutants is thus, at least in part,
166 explained by reduced interaction with trimeric influenza polymerase. Interestingly, binding of
167 mouse ANP32A to Vic/75 H3N2 FluPol was reduced 2.5-fold compared with mouse
168 ANP32B, and 3.2-fold compared with human ANP32A (Figure 3D)
169



170

171 **Figure 3 Poor pro-viral function of ANP32 mutants correlates with poor binding to FluPol (A)**
172 *Schematic of split luciferase complementation assay (adapted from Mistry et al. 2019) (B-D) Equal*
173 *amounts (15 ng) of pCAGGS expression plasmids encoding H5N1 50-92 polymerase or H3N2*
174 *components (PB1-luc1, PB2-627K and PA), as well as ANP32-luc2, were transfected into 293T cells.*
175 *24 hours post-transfection cells were lysed and luminescence was measured. Two separate sets of*
176 *control wells were transfected with either untagged PB1, PB2-627K, PA, ANP32-luc2 and unbound*
177 *luc1, or untagged ANP32, PB1-luc1, PB2-627K, PA and unbound luc2. Total amount of plasmid (in*
178 *ng) was kept constant by using empty pCAGGS. Normalised luminescence ratio (NLR) was obtained*
179 *by dividing luminescence in the experimental condition (tagged PB1 + tagged ANP32) by the sum of*
180 *the luminescence measured in the control conditions (i.e. background interaction of unbound luc1 with*
181 *ANP32-luc2, and unbound luc2 with PB1-luc1, respectively). (B) Interaction between ANP32A-D130N*
182 *mutant and H5N1 50-92 FluPol, compared with wildtype ANP32A (C) Interactions between ANP32B-*
183 *L128V and ANP32B-D130A mutants and H5N1 50-92 FluPol, compared with wildtype ANP32B. (D)*

184 *Interaction between mouse ANP32A and Vic/75 FluPol compared with mouse ANP32B and human*
185 *ANP32A. Data shown are mean (SD) representative of two independent triplicate experiments;*
186 *statistical analysis in (B) by Student t-test, in (C and D²) by one-way ANOVA. ****, P < 0.0001*

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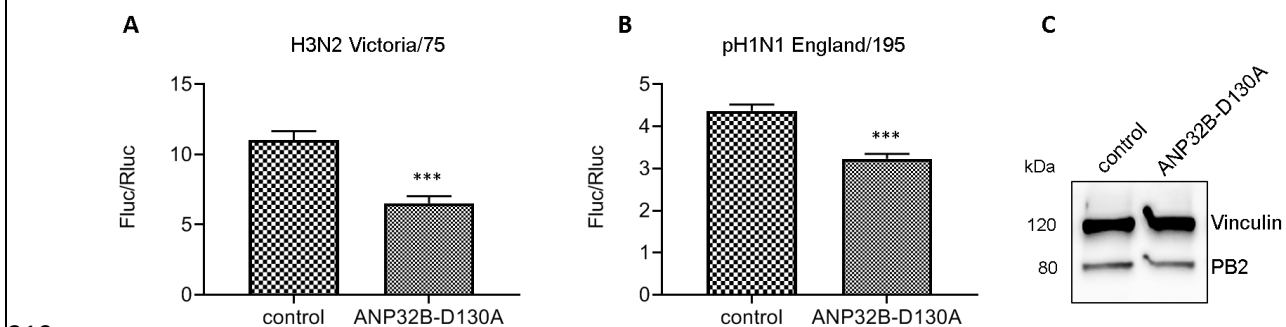
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189 As ANP32B-D130A is by far the most common SNV, and homozygous carriers of this variant
190 exist, we focused our further efforts on this mutant. Although ANP32A and ANP32B are
191 functionally redundant in their pro-viral activity, we reasoned the SNV might confer a
192 phenotype if it exerted a dominant negative effect over wildtype ANP32A. To test this we
193 titrated increasing concentrations of either wildtype ANP32B or ANP32B-D130A in dKO
194 cells, alongside a constant amount of wildtype ANP32A (Supplementary Figure 2).
195 Overexpressing ANP32B-D130A in the presence of wildtype ANP32A in dKO cells resulted
196 in decreased FluPol activity. This effect was not seen when co-expressing wildtype ANP32B.

197

198 **Influenza A virus replication is attenuated in a monoclonal mutant cell line**

199 To further probe the potential significance of the ANP32B-D130A variant, we generated the
200 homozygous wildtype *Anp32A* / mutant *Anp32B* genotype by CRISPR/Cas9 genome editing
201 in eHAP cells. Using a human codon-optimized DNA endonuclease SpCas9 and a single-
202 stranded DNA homology-directed repair (HDR) template to introduce the point mutation, we
203 obtained a monoclonal cell line carrying the desired genotype (Supplementary Figure 3A-C).
204 We selected an unsuccessfully edited clone to serve as a negative control, and carried out
205 Western blotting analysis to ensure wildtype ANP32A and mutant ANP32B were expressed
206 in control and mutant cells (Supplementary Figure 3D). Using minigenome reporter assays,
207 we found that reconstituted polymerases from H3N2 Vic/75 and pH1N1 Eng/195 were less
208 active in edited cells expressing wildtype ANP32A and ANP32B-D130A (Figure 4A and B).
209 This was not due to differential expression of transfected RNP components (Figure 4C).



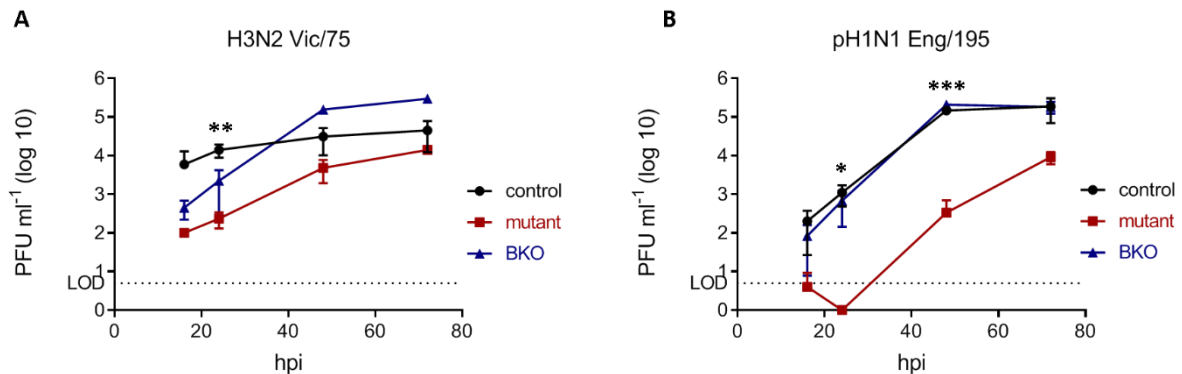
210

211 **Figure 4 FluPol activity is attenuated in ANP32B-D130A mutant cell line** Minigenome reporter
212 assays in monoclonal CRISPR/Cas9-edited cells with H3N2 Victoria (A) and pH1N1 Eng/195 (B) RNP
213 components (PB1, PB2, PA and NP), pPoll-firefly luciferase minigenome reporter, and *Renilla*
214 luciferase control. Data show mean (SD) of firefly activity normalized to *Renilla* and analysed by
215 Student t-test from one representative repeat ($n = 2$ independent triplicate experiments). ****, $P <$
216 0.001 (C) Western blot analysis showing Vic/75 FluPol component PB2 expression in control and
217 mutant cells.

218

219 Next we infected mutant and control cells with low-MOI (0.005) recombinant influenza A
220 viruses containing internal genes from H3N2 Vic/75 and pH1N1 Eng/195 viruses, with the

221 neuraminidase (NA) and haemagglutinin (HA) external genes of the laboratory-adapted
222 H1N1 PR/8 virus. In addition, we infected eHAP cells lacking ANP32B (BKO). Influenza A
223 virus replication was severely attenuated in mutant cells, in comparison with either control or
224 BKO cells, in particular at early time points. H3N2 Vic/75 infectious virus production in
225 mutant cells was almost 100-fold lower than in control cells at 16h and 24h post-infection
226 (Figure 5A). This difference was even more pronounced in cells infected with pH1N1
227 Eng/195 virus, which at 24h and 48h post-infection is approximately 1000-fold less
228 productive in mutant cells (Figure 5B). Both viruses replicated to higher titres in cells that
229 completely lack ANP32B (BKO) than in cells expressing the ANP32B-D130A mutant.



230

231 **Figure 5 IAV replication is attenuated in ANP32B-D130A mutant cells** Control (black), mutant
232 (red), and BKO (blue) monoclonal cells were infected with H3N2 Victoria/75 (A) or pH1N1
233 England/195 (B) 6:2 reassortant viruses with PR8 hemagglutinin (HA) and neuraminidase (NA) genes
234 at MOI = 0.005 and incubated at 37°C in the presence of 1 µg/ml trypsin to allow multicycle
235 replication. Supernatants were harvested at the indicated times post-infection and pfu/ml established
236 by plaque assay on MDCK cells. LOD (dotted line) denotes the limit of detection in plaque assays.
237 Shown are representative data from one of two independent infection experiments carried out in
238 triplicate. P values were calculated per time point by student's t test and represent differences
239 between control and mutant cell lines. *P<0.05; **P<0.01; ***P<0.01

240

241 DISCUSSION

242 Here, we used publicly available databases to perform a biased screen for naturally
243 occurring single nucleotide variants in human *Anp32A* and *Anp32B*, under the hypothesis
244 that some mutations may affect susceptibility to influenza virus infection. One of the
245 mutations, which translates to an aberrant ANP32B-D130A protein, is highly enriched in
246 carriers of Latino descent. Although most carriers of the SNV will be heterozygous for the
247 mutation, 25 homozygotes are reported in the gnomAD database. We generated this
248 naturally occurring homozygous genotype by CRISPR/Cas9 genome editing in low-ploidy
249 human eHAP cells, and found that IAV FluPol activity and virus replication were significantly
250 attenuated in the mutant cell line, compared to wildtype control cells or cells lacking
251 ANP32B. Thus we have provided the first example of a single nucleotide variant in the
252 coding region of a human gene that might confer some protection against influenza virus
253 infection. The nucleotide substitution, an A>C transversion in exon 4 of the *Anp32B* gene,
254 gives rise to a protein product bearing an alanine residue at position 130, in lieu of the
255 canonical aspartate (ANP32B-D130A). This substitution entails a loss of a negatively
256 charged amino acid with the potential to form a salt bridge or hydrogen bond with a potential
257 binding partner. Indeed, using a split luciferase assay, we show reduced interaction of
258 ANP32B-D130A with IAV polymerase.

259 Although we and others have previously reported redundancy in reconstituted systems for
260 ANP32A and B in their support of FluPol activity (Staller et al., 2019; Zhang et al., 2019), the
261 data presented here implies that deleterious mutations in one ANP32 protein might exert
262 dominant negative effects on the pro-viral activity of the other. The mechanism behind the
263 dominant negative effect cannot be readily explained by the split luciferase assay we used
264 here that indicates that the D130A mutation weakens the interaction between ANP32B and
265 FluPol. Further studies to elucidate the binding interfaces and valency of this virus-host
266 interaction will be required to account for our findings.

267 In addition, it is important to bear in mind that the outcome of genetic variation will also
268 depend on gene dosing in relevant tissues. The genotype-tissue expression (GTEx) portal
269 uses whole genome sequencing and RNA-Seq data to estimate gene expression in 54 non-
270 diseased human tissue types from nearly 1,000 individuals (Lonsdale et al., 2013). ANP32A
271 and ANP32B are both clearly detectable in healthy lung tissue, at a median transcripts per
272 kilobase million (TPM) of 42 and 201, respectively. There are thus 4.8-fold more ANP32B
273 transcripts than ANP32A transcripts in the lung, suggesting that mutations that impair that
274 pro-viral function of ANP32B such as ANP32B-D130A might have significant effects on virus
275 replication in the major target tissue.

276 A caveat of the work here presented is the use of human eHAP cells where (primary)
277 respiratory epithelium would have been preferable. We selected eHAP cells for their low
278 ploidy, which renders CRISPR/Cas9 genome editing more straightforward. More commonly
279 used laboratory cell lines like A549 or HEK-293 derivatives are polyploid (Giard et al., 1973;
280 Graham et al., 1977; Lin et al., 2014). We have shown previously that eHAP cells are a
281 suitable model for influenza virus infection (Long et al., 2019; Mistry et al., 2020; Peacock et
282 al., 2020; Staller et al., 2019). A drawback of using a haploid cell line is that heterozygous
283 genotypes cannot be generated. Thus far we have been unable to trace carriers of the
284 variants but future work using diploid epithelial cells that reflect the heterozygous genotype
285 could indicate whether heterozygotes are also likely to display reduced influenza
286 susceptibility.

287 In conclusion, we have provided the first example of a single nucleotide variant in the coding
288 region of a human gene that may offer carriers some protection against influenza virus. This
289 work has the potential to help elucidate the mechanism through which ANP32 proteins
290 support influenza virus replication, and can inform future intervention.

291

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302 **AUTHOR CONTRIBUTIONS**

303 Conceptualization, E.S. and W.S.B.; Methodology, E.S., C.M.S., V.S.S., and W.S.B.;
304 Investigation, E.S., L.B., and R.F.; Resources, T.P.P.; Writing – Original Draft, E.S.; Writing
305 – Review and Editing, all authors; Visualization, E.S. and C.M.S.; Supervision, C.M.S,
306 V.S.S., and W.S.B.; Funding Acquisition, W.S.B.

307 **DECLARATION OF INTERESTS**

308 The authors declare no competing interests.

309

310 **EXPERIMENTAL PROCEDURES**

311 **Cell culture**

312 Human eHAP cells (Horizon Discovery) were cultured in Iscove's modified Dulbecco's
313 medium (IMDM; Thermo Fisher) supplemented with 10% fetal bovine serum (FBS; Labtech),
314 1% nonessential amino acids (NEAA; Gibco), and 1% penicillin/streptomycin (Invitrogen).
315 Human embryonic kidney (293T) cells (ATCC) and Madin-Darby canine kidney (MDCK) cells
316 (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen)
317 supplemented with 10% FBS, 1% NEAAs, and 1% penicillin/streptomycin. All cells were
318 maintained at 37°C in a 5% CO² atmosphere.

319 **Plasmids and cloning**

320 Human FLAG-tagged pCAGGS-ANP32A and ANP32B expression plasmids have been
321 described (Staller et al., 2019). Mutant proteins were cloned from these plasmids by
322 overlapping touchdown PCR, using primers CCAACCTGAATAACTACCGCGAGAAC and
323 GTTCTCGCGGTAGTTATTCAGGTTGG (ANP32A-D130N),
324 GAATGACTACCAAGAGAACGTGTTC and GAACACGTTCTCTTGGTAGTCATTC
325 (ANP32A-R132Q), GAGGCCCTGATGCTGACGCCGAGG and
326 CCTCGGCGTCAGCATCAGGGGCCTC (ANP32A-S158A),
327 GAGGCCCTGATACTGACGCCGAGGGC and GCCCTCGGCGTCAGTATCAGGGGCCTC
328 (ANP32A-S158T), GTGACAAACGTGAATGACTATCGG and
329 CCGATAGTCATTCACGTTTGTAC (ANP32B-L128V),
330 CAAACCTGAATGCCTATCGGGAGAGC and GCTCTCCCGATAGGCATTCAGGTTTG
331 (ANP32B-D130A), GACTATCGGCAGAGCGTGTTTAAG and
332 CTAAACACGCTCTGCCGATAGTC (ANP32B-E133Q),
333 GAGAGCGTGTTTAAGCACCTGCCACAGCTG and
334 CAGCTGTGGCAGGTGCTTAAACACGCTCTC (ANP32B-L138H),
335 GTTGCTGCCACAGTTTACTTATCTCGA and TCGAGATAAGTAACTGTGGCAGCAAC
336 (ANP32B-L142F). Expression plasmids encoding H3N2 Vic/75 and pH1N1 RNP
337 components PB1, PB2, PA and NP have been described (Staller et al., 2019), as have
338 pCAGGS-ANP32A_{luc2C}, pCAGGS-luc1, pCAGGS-luc2, and H5N1 50-92 PB1_{luc1C} (Long et al.,
339 2019; Mistry et al., 2020). pPoll reporter plasmid containing firefly luciferase flanked by IAV-
340 specific promoters, and pCAGGS-*Renilla* luciferase transfection / cellular transcription
341 control have been previously described (Staller et al., 2019). Expression plasmids pCAGGS-
342 ANP32B_{luc2C}, ANP32A-D130N_{luc2C}, ANP32B-L128V_{luc2C}, and ANP32B-D130A_{luc2C} were
343 cloned by overlapping touchdown PCR. All plasmid constructs were verified by Sanger
344 sequencing and analyzed in Geneious prime 2019.

345

346 **CRISPR/Cas9 genome editing**

347 Guide RNA GCACTCTCTCGGTAGTCATTC was designed manually against the
348 protospacer sequence in exon 4 of *Anp32B* to target DNA endonuclease SpCas9, expressed
349 from Addgene plasmid # 62988 (PX459), to the target nucleotide (Supplementary Figure
350 3A). The guide RNA itself was cloned into Addgene plasmid # 80457 (pmCherry_gRNA). A
351 custom-designed 88 base ssODN (single strand DNA) homology-directed repair template,
352 harbouring the point mutation (cytosine in bold) and a silent PAM mutation (thymidine in italic
353 script) –
354 GAAAAGCCTGGACCTCTTTAACTGTGAGGTTACCAA TCTGAATGCCTACCGAGAGAGTG
355 TCTTCAAGCTCCTGCCCCAGCTTACCTAC – was obtained from Integrated DNA
356 Technologies (IDT). Equal amounts of PX459 and pmCherry_gRNA (total 1 µg), and 1.0 µl
357 10 µM ssODN template, were transfected by electroporation into approximately 400,000
358 eHAP cells using the Neon™ transfection system (Invitrogen). Cells and DNA were mixed
359 into a 10 µl volume of suspension buffer, which was subjected to a single 1,200 Volt pulse
360 for a duration of 40 ms. Cells were incubated at 37°C for 24 hours in IMDM growth medium
361 without antibiotics. A fluorescence-activated cell sorter (FACS) Aria IIIU (BD Biosciences)
362 with an 85-µm nozzle was used to sort cells expressing mCherry (550-650 nm emission) into
363 96-well plates containing growth medium. Single cells were grown out into monoclonal
364 populations over a period of 10 to 14 days. Total genomic DNA was extracted using the
365 Purelink™ Genomic DNA Mini Kit (Invitrogen) and amplified by touchdown PCR to generate
366 a 1,561-base pair fragment of the edited locus (primers TACCTCTGCCCTCTCAATCTCT
367 and ACGCACACAAACACACACTATT). PCR products were then incubated at 65°C for 30
368 minutes in the presence of BsmI restriction enzyme (NEB). The resulting DNA fragments
369 were separated by 1.5% agarose gel electrophoresis. Potentially successfully edited clones
370 were verified by Sanger sequencing (primers TAAAGACCGCTTGATACCCAGG and
371 TGAGGCTGAGTGGGTAGTGG), and analysed in Geneious prime 2019.

372

373 **Minigenome reporter assays**

374 In order to measure influenza virus polymerase activity, pCAGGS expression plasmids
375 encoding H3N2 Vic/75 or pH1N1 Eng/195 PB1 (0.02 µg), PB2 (0.02 µg), PA (0.01 µg), and
376 NP (0.04 µg) were transfected into 100,000 eHAP cells using Lipofectamine 3000 (Thermo
377 Fisher) at ratios of 2 µl P3000 reagent and 3 µl Lipofectamine 3000 reagent per µg plasmid
378 DNA. As a reporter construct, we transfected 0.02 µg pPoll-luc, which encodes a
379 minigenome containing a firefly luciferase reporter flanked by influenza A virus promoter
380 sequences. pCAGGS-*Renilla* luciferase (0.02 µg) was co-transfected as a transfection and
381 toxicity control. 0.04 micrograms of the indicated ANP32-FLAG constructs was co-expressed
382 to determine their effect on polymerase activity. Twenty-four hours after transfection, cells
383 were lysed in 50 µl passive lysis buffer (Promega) for 30 minutes at room temperature with
384 gentle shaking. Bioluminescence generated by firefly and *Renilla* luciferases was measured
385 using the dual-luciferase system (Promega) on a FLUOstar Omega plate reader (BMG
386 Labtech).

387

388 **Split luciferase complementation assay**

389 pCAGGS expression plasmids encoding H5N1 50-92 or H3N2 Vic/75 PB1_{luc1C}, PB2-627K,
390 PA, and the relevant ANP32_{luc2C} construct were transfected into 100,000 293T cells at a ratio
391 of 1:1:1:1 (15 ng per well). Control conditions contained pCAGGS-luc1 and untagged PB1,
392 or pCAGGS-luc2 and untagged ANP32A, respectively, with all other components remaining
393 constant. Empty pCAGGS plasmid was used to ensure total transfected DNA was equal

394 across conditions. Twenty-four hours after transfection, cells were lysed in 50 μ l *Renilla* lysis
395 buffer (Promega) for 1 h at room temperature with gentle shaking (*Gaussia* and *Renilla*
396 luciferase share the same substrate). Bioluminescence generated by *Gaussia* luciferase was
397 measured using the Renilla luciferase kit (Promega) on a FLUOstar Omega plate reader
398 (BMG Labtech). Normalized luminescence ratios (NLR) were calculated by dividing the
399 signal from the potential interacting partners by the sum of the two controls, as described
400 (Mistry et al., 2020).

401 **Western blotting**

402 At least 250,000 cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.8; Sigma-
403 Aldrich), 100 mM NaCl, 50 mM KCl, and 0.5% Triton X-100 (Sigma-Aldrich), supplemented
404 with a cOMplete EDTA-free protease inhibitor cocktail tablet (Roche) and prepared in
405 Laemmli 4x buffer (Bio-Rad) after protein concentration had been established by
406 spectrophotometry (DeNovix DS-11 FX+ spectrophotometer). Equal amounts of total protein
407 (20-50 μ g per lane) was resolved by SDS-PAGE using Mini Protean TGX precast gels 4% to
408 20% (Bio-Rad). Immunoblotting by semi-dry transfer (Bio-Rad Trans-Blot SD semidry
409 transfer cell) onto nitrocellulose membranes (Amersham Protran Premium 0.2 μ m NC; GE
410 Healthcare) was carried out using the following primary antibodies: rabbit α -vinculin (catalog
411 number ab129002, 1/1,000; Abcam), rabbit α -ANP32A (catalog number ab51013, 1/500;
412 Abcam), rabbit α -ANP32B (10843-1-AP, 1/1,000; Proteintech), mouse α -FLAG (catalog
413 number F1804, 1/500; Sigma-Aldrich) and rabbit α -IAV PB2 (catalog number GTX125926,
414 1/2,000; GeneTex). The following secondary antibodies were used: sheep α -rabbit
415 horseradish peroxidase (HRP) (catalog number AP510P, 1/10,000; Merck) and goat α -
416 mouse HRP (STAR117P, 1/5,000; AbD Serotec). Protein bands were visualized by
417 chemiluminescence using SuperSignal™ West Femto substrate (ThermoFisher Scientific) on a
418 Fusion-FX imaging system (Vilber Lourmat).

419 **Immunofluorescence microscopy**

420 Approximately 100,000 eHAP cells were cultured on sterilised glass coverslips and
421 transfected as per minigenome reporter assay protocol. Twenty-four hours after transfection,
422 cells were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100. FLAG-
423 tagged ANP32 constructs were visualised with primary antibody mouse α -FLAG (F1804;
424 1/200; Sigma) for 2 hours at 37°C in a humidified chamber. Cells were incubated with
425 secondary antibody goat α -mouse Alexa Fluor-568 (1/200; Life Technologies) for 1 hour at
426 37°C in a humidified chamber, and counterstained with DAPI. Coverslips were mounted on
427 glass slides using Vectashield mounting medium (H-1000-10; Vector Laboratories). Cells
428 were imaged with a Zeiss Cell Observer widefield microscope with ZEN Blue software, using
429 a Plan-Apochromat x100 1.40-numerical aperture oil objective (Zeiss), an Orca-Flash 4.0
430 complementary metal-oxide semiconductor (CMOS) camera (frame, 2,048 x 2,048 pixels;
431 Hamamatsu), giving a pixel size of 65 nm, and a Colibri 7 light source (Zeiss). Channels
432 acquired and filters for excitation and emission were 4',6-diamidino-2- phenylindole (DAPI)
433 (excitation [ex], 365/12 nm, emission [em] 447/60 nm), and TexasRed (ex 562/40 nm, em
434 624/40 nm). All images were analyzed and prepared with Fiji software.

435 **Influenza virus infection**

436 500,000 CRISPR/Cas9-modified monoclonal eHAP cells were infected with ~2,500 plaque-
437 forming units (PFU) H3N2 Vic/75 6:2 or pH1N1 Eng/195 6:2 virus diluted in 200 μ l serum-
438 free IMDM for 1 hour at 37°C (MOI 0.005) to allow virus to adsorb and enter the cells. The
439 inoculum was removed and cells were incubated in room-temperature phosphate-buffered
440 saline / HCl at pH 3.0 for 3 minutes to inactivate residual virus. Cells were incubated at 37°C

441 in serum-free cell culture medium (IMDM) supplemented with 1 µg/ml L-1-tosylamide-2-
442 phenylethyl chloromethyl ketone (TPCK) trypsin (Worthington-Biochemical). Cell
443 supernatants were harvested at indicated time points post-infection. Infectious titres were
444 determined by plaque assay on MDCK cells. Virus infection assays were performed in
445 triplicate on two separate occasions.

446 **Safety/biosecurity**

447 All work with infectious agents was conducted in biosafety level 2 facilities, approved by the
448 Health and Safety Executive of the United Kingdom and in accordance with local rules, at
449 Imperial College London, United Kingdom.

450 **Structural modelling**

451 Structural models of ANP32A and B were created using iTASSER structural prediction
452 software (based primarily on huANP32B [GenBank accession number 2RR6A] and
453 huANP32A [accession number 2JQDA], and 2JEOA). The three-dimensional structural
454 models were visualized and created in UCSF Chimera. Amino acid residues affected by
455 selected SNVs are highlighted in purple (ANP32A) or blue (ANP32B) stick format.

456 **Bioinformatics**

457 Human genomic information was obtained using the following publicly available databases:
458 gnomAD (<https://gnomad.broadinstitute.org/>); NCBI dbSNP
459 (<https://www.ncbi.nlm.nih.gov/snp/>); ALSPAC (<http://www.bristol.ac.uk/alspac/>); TOPMed
460 (<https://www.nhlbiwgs.org/>); 1000G (<https://www.internationalgenome.org/>); GO-ESP
461 (<https://esp.gs.washington.edu/drupal/>)

462

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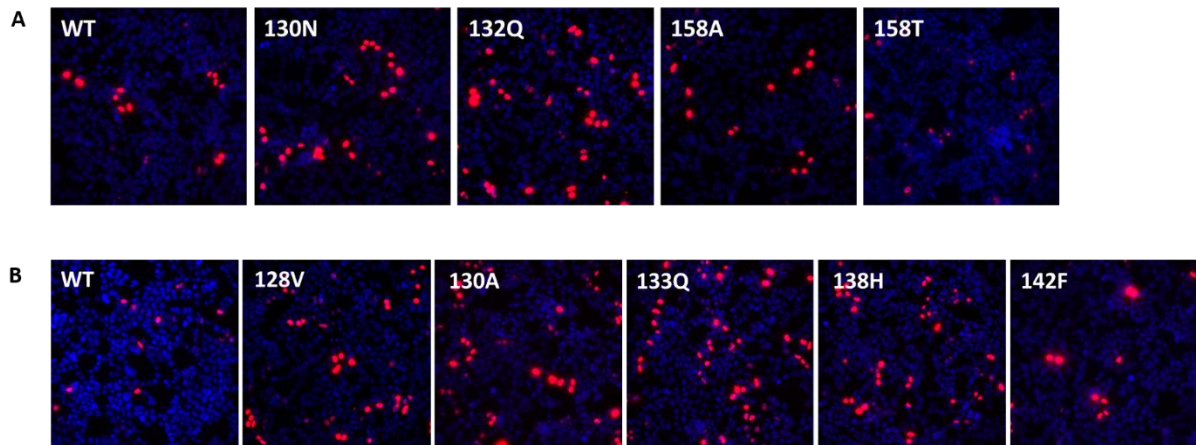
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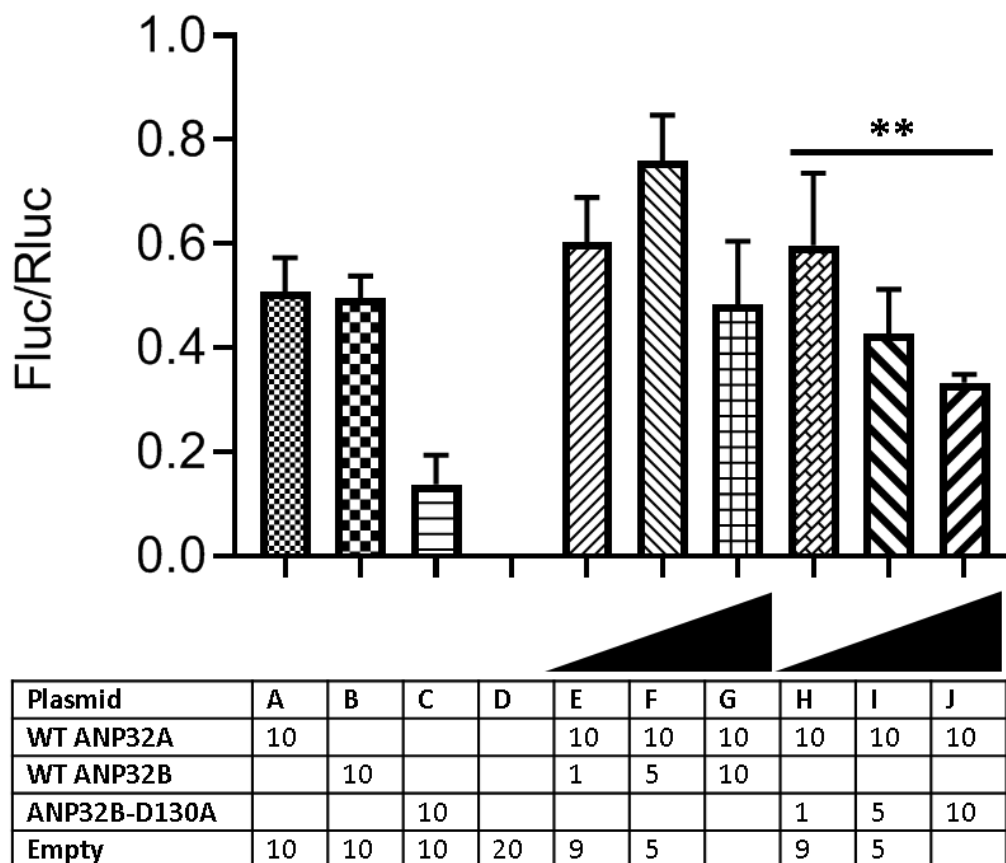
574 **SUPPLEMENTARY FIGURES**



575

576 **Supplementary Figure 1. Mutant ANP32 proteins localise to the cell nucleus**
 577 Immunofluorescence analysis showing nuclear localisation of mutant FLAG-tagged ANP32A (A) or
 578 ANP32B (B) proteins, detected with anti-FLAG primary antibody and Alexa Fluor-568 anti-mouse
 579 conjugate and counterstained with DAPI

580

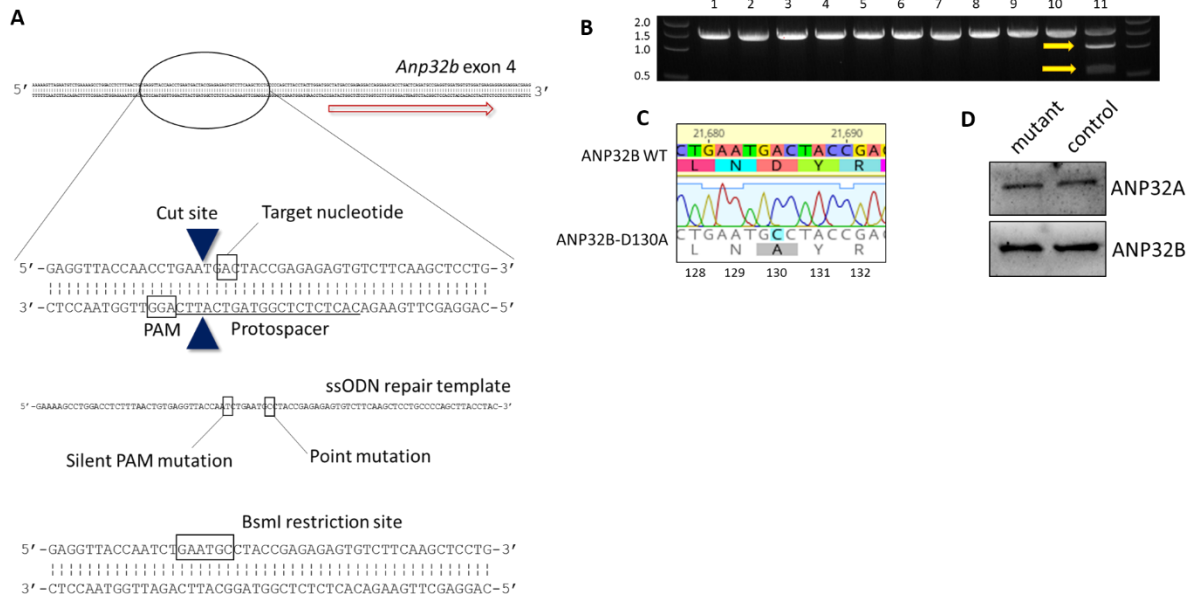


581

582 **Supplementary Figure 2 ANP32B-D130A exerts a dominant negative effect over ANP32A**
 583 Minigenome reporter assay in approximately 100,000 dKO cells with reconstituted pH1N1 Eng/195
 584 RNP components (PB1, PB2, PA and NP), pPoll-firefly luciferase minigenome reporter, and Renilla
 585 control. Conditions contain indicated amounts (in ng) of co-transfected FLAG-tagged pCAGGS-

586 *ANP32A* (WT *ANP32A*), *ANP32B* (WT *ANP32B*) or *ANP32B-D130A*. Empty *pCAGGS* was used to
 587 make sure equal amounts of total DNA were transfected across conditions. Data show mean (SD) of
 588 firefly activity normalized to *Renilla* and analysed by one-way ANOVA. **, $P < 0.01$.

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596 **Supplementary Figure 3 Generation of naturally occurring mutant genotype by genome editing**
 597 (A) *Anp32B* is located on Chromosome 9, with the codon encoding 130D in exon 4 of the gene. The
 598 red arrow indicates the reading direction. A protospacer-adjacent motif (PAM) was identified near the
 599 target adenine on the opposite DNA strand, and a guide RNA was designed against the protospacer
 600 (underlined). SpCas9 cut two base pairs upstream of the target nucleotide (dark blue arrowheads). An
 601 88-base single-stranded DNA (ssODN) homology-directed repair (HDR) template was designed
 602 bearing the missense A>C substitution as well as a silent PAM mutation between homology arms. A
 603 successful edit introduced a BsmI restriction site (GAATGC) not present in the wildtype allele,
 604 enabling screening by PCR amplification of a 1,561 base pair genome fragment followed by restriction
 605 digestion of the PCR product (B). A successfully edited allele is cleaved into 1,008 and 553 base pair
 606 DNA fragments. Potentially positive clones were verified by Sanger sequencing (C). (D) Western
 607 blotting analysis with specific antibodies against ANP32A and ANP32B in a mutant and control
 608 monoclonal cell line shows equal expression of both proteins.

609