Polymerase mutations underlie early adaptation of H5N1 influenza virus to dairy cattle and other mammals.

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Abstract

In early 2024, an unprecedented outbreak of H5N1 high pathogenicity avian influenza was detected in dairy cattle in the USA. The epidemic remains uncontrolled, with spillbacks into poultry, wild birds and other mammals including humans. Here, we present molecular and virological evidence that the cattle B3.13 genotype H5N1 viruses rapidly accumulated adaptations in polymerase genes that enabled better replication in bovine cells, as well as cells of other mammalian species including humans and pigs. We find evidence of several mammalian adaptations gained early in the evolution of these viruses in cattle including PB2 M631L, which is found in all cattle sequences, and PA K497R, which is found in the majority. Structurally, PB2 M631L maps to the polymerase-ANP32 interface, an essential host factor for viral genome replication. We show this mutation adapts the virus to co-opt bovine ANP32 proteins and thereby enhances virus replication in bovine and primary human airway cells. Importantly, we show that ongoing evolution during 2024 in the PB2 gene, including a convergently arising D740N substitution, further increases polymerase activity in a range of

mammalian cells. Thus, the continued circulation of H5N1 in dairy cattle allows virus adaption improving replicative ability in cattle and increasing zoonotic spillover risk.

Introduction

The natural host reservoirs of influenza A viruses (IAV) are wild waterfowl and shorebirds. However, IAV can spill over and adapt to infect mammalian species, such as pigs, humans, horses and dogs[1]. In 2020, a novel genotype of H5N1 clade 2.3.4.4b emerged in Europe and spread around the world causing a panzootic in birds[2]. This panzootic genotype and its progeny are notable for their frequent spillover into mammals, including confirmed or potential mammal-to-mammal spread[3] in farmed mink in Europe[4-6] (2022-2023), semi-aquatic marine mammals in South America[7-11] (2023-), and recently, dairy cattle in the USA[12,13] (2024-).

In early 2024, an unexplained drop in milk production in cattle in the USA was attributed to infection with H5N1 clade 2.3.4.4b, of the B3.13 genotype[12,13]. Infected cows were initially observed in Texas but as of December 2024, over 900 infected herds have been recorded in 16 US states[12,13]. This outbreak was most likely caused by a single spillover from wild birds[12-14], with continuous transmission between dairy cattle (hereafter referred to as ‘cattle’) mediated by human activities such as contamination of milking machinery within farms[15], and movement of infected animals or contaminated equipment between farms and states[13]. The virus does not cause high mortality in cattle, instead mostly causing mastitis and modest respiratory distress[12,16]. In addition to cattle, several other species have been infected following exposure directly or indirectly to infected cattle including chickens, turkeys, peridomestic birds, farmed alpaca, domestic cats[13], and humans[17,18]. Infections have been repeatedly fatal in cats that ingested infected milk, but in humans the B3.13 virus has been associated with conjunctivitis and/or mild respiratory disease[17,18].

Avian influenza viruses (AIVs) usually have restricted replication in mammalian hosts[1]. However, they can rapidly accumulate mammalian adaptations that overcome this block, particularly in the viral RNA-dependent RNA polymerase[3,19]. In particular, ANP32 proteins are essential host-encoded factors co-opted by the virus to support

genome replication[20,21]. All birds excluding palaeognaths (e.g. ostriches and related birds), have an ANP32A gene with an additional exon resulting in splice variants encoding proteins with an extra 33 amino acids relative to mammalian orthologues [22]. Avian-origin influenza viruses require polymerase mutations to utilise the shorter mammalian ANP32A/B proteins[23]. The most widespread, and best characterised adaptive mutations are those in the PB2 subunit of the polymerase including PB2 E627K[22], Q591R/K[24,25], and D701N[25,26]; all of which have been detected during other mammalian H5N1 outbreaks[5-11] but are absent in USA dairy cattle viruses. However, alternative adaptive mutations in the influenza polymerase that enable the virus to co-opt suboptimal ANP32 proteins are possible[27-29]. Here, we investigated potential mammalian adaptations in the polymerase of cattle-adapted H5N1 viruses and identify two key mutations that allow the B3.13 virus to replicate in cells from cattle and other mammalian species, including humans.

Results

Phylogenetic analysis of the cattle H5N1 polymerase identified potential adaptive mutations.

To understand how an avian influenza virus had adapted to efficiently replicate in cells of a novel mammalian species, we constructed a phylogeny using concatenated genomes of all influenza A viruses isolated from cattle, virus from the first human case and wild avian viruses from September 2023-March 2024 (Supplementary Figure S1). Viruses from cattle formed a monophyletic group suggesting a single spillover event from wild birds[14] (Figure 1A). Interestingly, the sequence of the first case of human infection with a B3.13 H5N1 virus[17] was basal to all subsequent cattle viruses (Figure 1A, Supplementary Figure S1) and contained the known mammalian adaptations PB2 E627K[30] as well as PA K142E[31] relative to the closest avian relatives (Supplementary Table S1). However, these adaptive mutations were not found in any cattle virus sequences, which instead all contained PB2 M631L as well as PB2 E362G and PA L219I. PB2 M631L has previously been reported to adapt avian H1N1, H5N1 and H10N7 viruses to mammals[30,32,33], as well as H9N2 viruses to gene-edited chickens with

a non-functional ANP32A[29]. Early cattle sequences fell into three clades with additional mutations in the polymerase segments including i) PB2 E677G[34,35], ii) PA I13V and E613K, and iii) PA K497R, which was found in the largest cattle clade (Figure 1A, Supplementary Figure S1), consisting of ~95% of current sequences (as of December 2024) and has previously been associated with influenza adaptation to mammals[36]. Minor clade I (PB2 E677G) was found solely in Texas and appeared only at the beginning of the outbreak whereas minor clade ii (PA I13V) was seen across multiple states, including, most recently, in Colorado.

Activity of bovine H5N1 polymerase in mammalian cells maps to PB2 M631L and PA K497R

To test whether the mutations identified from the phylogenetic analysis were adaptive in cattle, we first constructed a 'minireplicon' system suitable for testing AIV polymerase activity in bovine cells. This consisted of co-transfecting bovine cells with a custom-made minigenome reporter that uses a bovine RNA polymerase I (Pol I) promoter to express a viral-like RNA encoding firefly luciferase flanked with non-coding segments from influenza A segment 8/NS[37] along with plasmids expressing influenza virus ribonucleoprotein components, PB2, PB1, PA and nucleoprotein (NP). This species-specific minireplicon system had far higher activity in bovine cells than one using an equivalent human PolI reporter (Supplementary Figure S2). Using this bovine-specific minireplicon reporter, the reconstituted polymerase complex of A/dairy cattle/Texas/24-008749-001-original/2024 (cattle/Texas) was highly active in MAC-T bovine mammary gland cells (Figure 1B).

We next reconstructed the avian-like ancestor (avian -4) to represent the polymerase complex of the virus that mostly likely entered the cattle population by removing mutations from the PB2 and PA segments of cattle/Texas: PB2 M631L, PB2 E362G, PA K497R and PA L219I (Figure 1A, Supplementary Table S1). To identify the key changes, we then assessed the impact of adding back the mutations that arose between the reconstructed ancestor and the cattle shared common ancestor (PB2 M631L, PB2 E362G and PA L219I). Neither PB2 E362G nor PA L219I increased

polymerase activity in bovine cells, individually or in combination and PB2 E362G even reduced activity (Figure 1B). In contrast PB2 M631L increased activity of the avian -4 ancestor by more than 12-fold indicating this was the mutation that arose early in the adaptation of H5N1 to dairy cattle.

Next, we investigated later mutations that arose in the major clade iii of the cattle H5N1 virus (Figure 1A). We first tested the effect of the PA mutation K497R that is maintained across all sequences of the major bovine clade. Reverting both PB2 631L to M and PA 497R to K resulted in a large decrease (approximately 80-fold) in polymerase activity in bovine cells (Figure 1C). The reintroduction of PB2 M631L alone increased the signal by 50-fold. Reintroducing PA K497R had a more modest effect (~20-fold increase in signal). Neither mutation alone fully recapitulated the activity of the WT polymerase, indicating that the PA change was further adaptive. Importantly the same mutations had minimal impacts in avian chicken fibroblasts (DF-1; Figure 1D), consistent with an adaptive function in cattle cells rather than simply altering the intrinsic activity of the viral polymerase.

We next tested the impact of these two mutations on polymerase activity in cells from two other mammalian hosts relevant to influenza viruses - humans and swine, again using host species-specific minireplicon reporters (Figure 1E, F). As seen in the bovine cells, both adaptations individually significantly enhanced polymerase activity, with PB2 M631L having the greater effect, implying it is the major mammalian adaptation. Also, as in bovine cells, we note a cumulative effect of the two substitutions together.

To investigate whether PB2 M631L and PA K497R are universal mammalian adaptations, we introduced them into a distantly related H5N1 IAV polymerase constellation (A/turkey/England/50-92/1991; 50-92), which has 98% and 97% amino acid identity in PB2 and PA, respectively. In this background, both PB2 M631L and PA K497R significantly enhanced polymerase activity by ~10- and ~5-fold in bovine cells and 30-fold and 25-fold in human cells (Supplementary Figure S3). Neither mutation alone was as potent as the well characterised mammalian adaptation PB2 E627K. This suggests both PB2 M631L and PA K497R are *bona fide* mammalian adaptations, relevant across diverse AIVs.

PB2 M631L, and to a lesser extent PA K497R, enable efficient replication of bovine H5N1 in mammalian cells.

To further test the importance of PB2 M631L and PA K497R in adapting virus to replicate in mammalian cells, we generated a reverse genetics system for cattle/Texas and made viruses bearing mutations at these sites. To work at BSL2, we rescued viruses as 2:6 reassortants containing the HA and NA of the attenuated[38] vaccine strain, A/Puerto Rico/8/1934 (PR8) and the remaining genes from cattle/Texas. We compared multicycle replication of the WT cattle/Texas virus with that of either the individual or double PB2/PA reversion in MAC-T cells (Figure 2A). Compared to the minimal avian-like precursor (PB2 M631L and PA K497R reverted), the viruses with PB2 M631L alone or in combination with PA K497R showed a significant, >10-fold increase in infectious virus titres at 24 and 48 hours. However, PA K497R alone resulted in a smaller, non-significant increase in titre. Similar results were also seen in bovine alveolar cells (BAT II; Supplementary Figure S4A) where PB2 M631L was necessary for growth at 48 hours and the addition of PA K497R increased titre at 48 hours above PB2 M631L alone. Overall, these results in the context of infectious virus are consistent with the minireplicon data that show PB2 M631L, and to a lesser degree the PA K497R, are responsible for this virus's ability to replicate in bovine cells.

We next tested the replication of these viruses in a pair of human cell lines (A549 cells representing human lungs and human normal dermal fibroblasts; Figure 2B, Supplementary Figure S4B). Consistent with the minireplicon assays in human cells (Figure 1D) and virus replication phenotypes in bovine cells (Figure 2A, Supplementary Figure S4A), the PB2 M631L mutation alone, or combined with PA K497R, significantly enhanced virus titres compared to the minimal avian-like precursor virus across multiple time points in the human cell lines. PA K497R alone showed somewhat higher titres at later time points compared to the precursor in both human cell lines, reaching significance at 48 hours post-infection in A549 cells. Overall, these data show that PB2 M631L is a major mammalian adaptation that enhances virus replication in both bovine and human cells.

Finally, we tested the growth of these viruses in chicken lung cells (CLEC213; Figure 2C). Compared to the minimal avian-like precursor, only PB2 M631L showed significantly higher titres, and only at a single time point, suggesting that these mutations have a minimal impact on virus fitness/replication in avian cells.

Whilst all available H5N1 sequences from cattle contain PB2 M631L, PA K497R was only found in the major clade of cattle sequences (clade iii, which also contains of all subsequent human B3.13 infections other than the first Texas case[17]), with alternative PB2 or PA mutations seen in two other minor clades (Figure 1A, supplementary Figure 1). To investigate whether these mutations represent alternative bovine adaptations, we recapitulated these genotypes by using the WT cattle/Texas polymerase background with PA R497 reverted to the avian consensus 497K and individually introduced PA I13V and E613K from Clade i or PB2 E677G from Clade ii. PA I13V and E613K together exerted a small but significant increase in polymerase activity over PB2 M631L alone showing a ~3-fold increased minireplicon signal in bovine cells (Supplementary Figure S5A) and ~2-fold increase in human cells (Supplementary Figure S5B). The introduction of PB2 E677G resulted in a modest non-significant increase in polymerase activity in bovine, but not human cells. None of the polymerase mutations found in the minor clade i and ii cattle viruses had a significant impact in polymerase activity in avian cells (Supplementary Figure S5C). This suggests that PB2 M631L represented an initial potent mammalian adaptation, and a range of other early polymerase mutations had smaller impacts, ‘fine tuning’ polymerase activity in bovine cells.

Emerging mutations further enhance B3.13 H5N1 polymerase activity in mammalian but not avian cells.

By continually monitoring sequences from the ongoing dairy cattle outbreak, we detected incidences of further, known mammalian adaptations arising within polymerase genes of the cattle viruses[25]. This included a single emergence of a cluster of sequences containing PB2 Q591R, and at least three independent emergences of PB2 D740N across the tree (Supplementary Figure S1). To test the impact of these changes

we introduced them, as well as PB2 E627K, which has also been reported to transiently arise within cattle sequences[39] into the WT cattle/Texas polymerase which already contains PB2 M631L. PB2 D740N significantly boosted polymerase activity in bovine (5-fold increased signal), human (3-fold increase signal) and swine cells (1.2-fold increase; Figure 3A-C). PB2 Q591R led to a smaller, but still statistically significant increase over the WT polymerase in both bovine and human cells (Figure 3A, B). The introduction of PB2 E627K on top of the M631L mutation already present in cattle/Texas led to the highest polymerase activity (Figure 3A-C). Neither PB2 E627K nor D740N increased activity in avian cells, relative to the wild-type cattle/Texas polymerase (Figure 3D). We conclude that these mutations indicate ongoing adaptation of H5N1 viruses to further optimise polymerase function in cattle.

To further investigate the impact of these ongoing mammalian adaptations, we rescued the full, WT cattle/Texas virus, the minimal avian-like precursor virus (PB2 L631M and PA R497K), and a wildtype virus with the additional mutation D740N. This work was performed under CL3, SAPO4 conditions. We initially tested the ability of the virus to replicate in MAC-T cells and found that the wildtype and D740N virus had a significant ~10-fold growth advantage over the avian precursor at 48 hours (Figure 4A). No significant differences in titres were seen between the WT and WT + D740N mutants. Furthermore, in a human lung cell line (Calu-3), both the WT and WT + D740N virus showed significantly enhanced titres over the avian precursor of ~2.5 and >10-fold at 48 and 72 h post-infection, respectively (Figure 4B). Finally, we performed multicycle replication curves in primary differentiated human nasal epithelial cells, maintained at air liquid interface (Figure 4C). WT and WT+D740N virus reached significantly higher titres of ~100-fold more than the avian precursor by 72 h post-infection. Again, this confirms that the cattle virus is much better able to replicate in human cells than the minimal avian precursor due to the PB2 M631L and PA K497R polymerase mutations. The PB2 D740N containing virus did not replicate to significantly higher titres than the WT virus in the bovine or human cells, suggesting that if this mutation does impact the virus, the effect is subtle and not captured by these assays.

Mutations in the polymerase of the cattle H5N1 viruses adapt the polymerase to use bovine ANP32 proteins.

PB2 residue 631 is structurally located close to the interface between influenza polymerase and the host factor ANP32 (Figure 5A, Supplementary Figures S6), nearby several other mammalian ANP32-adapting mutations such as PB2 E627K and Q591R[25,40]. We therefore hypothesised that the B3.13 polymerase adapted to bovine cells by increasing its ability to co-opt bovine ANP32 proteins. To test this, we performed minigenome assays in human cells which lack ANP32A, ANP32B and ANP32E (eHAP triple knockout cells[28]) complemented with ANP32 proteins from different relevant species including cattle, human, pigs, and chicken. Consistent with previous findings[41,42], only chicken ANP32A, and to a much lower degree swine ANP32A, supported viral polymerases from avian (AIV07, an early European clade 2.3.4.4b panzootic genotype virus; Figure 5B) or avian-like viruses (the minimal avian-like precursor; Figure 5C). Neither bovine ANP32A nor B supported any significant activity of avian virus polymerases. Both bovine ANP32A and ANP32B supported mammalian-adapted polymerases: cattle/Texas (Figure 5D) and a pandemic 2009 H1N1 virus (Figure 5E), England/195. However, bovine ANP32A was consistently and significantly better able than bovine ANP32B to support polymerase activity of all constellations tested. We assessed the effect of of the individual cattle/Texas mammalian adaptations, PB2 M631L and PA K497R complemented by the different ANP32 homologues (Figure 5F). Either mutation alone significantly enhanced the complementation of polymerase activity by bovine ANP32A or ANP32B, with PB2 M631L showing a stronger complementation by all ANP32 orthologues than PA K497R. Thus, we conclude that the major adaptive mutation PB2 M631L detected in the bovine influenza outbreak has adapted IAV for replication in cattle by increasing its capacity to co-opt bovine ANP32A.

Discussion

Our data using reconstructed viral polymerases in host-specific minireplicon and infection assays in cells from different relevant influenza host species show that PB2

M631L is the key adaptive mutation that allowed B3.13 H5N1 high pathogenicity avian influenza virus to efficiently replicate in cattle. PB2 M631L enables the polymerase complex to co-opt bovine ANP32 proteins to support its activity, with a strong preference for bovine ANP32A over ANP32B. Structurally, in the complex between the influenza polymerase and host ANP32, PB2 residue 631 is located near amino acids 627 and 591 which are well characterised sites that mutate to allow adaptation of AIV polymerase to mammalian ANP32 proteins[25,40] but the exact mechanism of how PB2 M631L allows the use of mammalian ANP32s remains unresolved. Structural studies show that ANP32 bridges the asymmetric dimer of influenza polymerase comprising a replicating subunit and a recruited subunit that will be used to encapsidate the nascent RNA strand. PB2 E627K results in a switch from a negative to a positive charge enhancing the interaction between the encapsidating subunit of the polymerase replication dimer and ANP32 residue E151, while also avoiding the clash between the negatively charged LCAR region of mammalian ANP32s and the replicating polymerase subunit[40,43]. This mechanism also likely applies to adaptive charge switch mutations at residue 591 from Q to R/K[40]. PB2 M631L does not result in an alteration of charge but presumably changes the polymerase structure in this region to better accommodate the interaction between either or both polymerase subunits and ANP32. Recent work from Gu et al. using the cattle virus polymerase confirmed that PB2 M631L increases activity in human cells at both 33°C and 37°C[30]. Previous work has shown that PB2 M631L adapts avian H10N7 viruses to mice[32] and enables an avian H9N2 virus to use otherwise suboptimal ANP32 proteins[29]. These reports, combined with our results in the context of an older non-goose/Guangdong H5N1 suggest that M631L is a general mammalian adaptive mutation for influenza polymerase that is not specific to a particular virus genetic background. However, why this mutation emerged in cattle rather than the more potent PB2 E627K remains unclear. Indeed E627K could well have emerged in cattle prior to the dead-end branch containing the first human infection[14,17], and E627K arose experimentally in cattle infected with a contemporary European H5N1 2.3.4.4b virus[16]. This suggests a high degree of stochasticity in the emergence of mammalian adaptations.

Following fixation of PB2 M631L in cattle, additional mutations in the polymerase arose in three separate clades. Most cattle sequences contained PA K497R which increased polymerase activity in the absence of M631L and increased replication in bovine mammary cells. Structurally, PA K497R sits in a loop close to the PB2 M631L and ANP32 interface, though may not be directly interacting with them (Figure 5A). Of the mutations defining the other two early cattle virus clades, PA I13V + E613K showed a significant increase in the minigenome above PB2 M631L whereas PB2 E677G showed no significant increase in activity and did not spread beyond Texas. Neither PA I13V or E613K have previously been associated with mammalian adaptation but E613K does fall on the polymerase symmetric dimer interface (Supplementary Figure S6B) and could be altering polymerase regulation[40].

Cattle/Texas virus has extremely robust replication in the bovine mammary gland, with very high titres of infectious virus being reported from milk[12,13,16]. However, further mutations that increase polymerase activity in bovine cells are still being reported as the outbreak continues. There have been three separate emergences of PB2 D740N which we show here boosted polymerase activity above the wild-type virus. PB2 E627K has been detected as a minor variant in conjunction with M631L in one cow but has not spread despite it giving the highest polymerase activity in combination with M631L in our minireplicon assay. It will be vital to continue to monitor evolution of the polymerase, and how mutations may impact infectivity and transmissibility of these viruses in cattle and other mammals.

What is the current risk to humans posed by the cattle virus? Our results show that the cattle virus containing mammalian adaptations can replicate better in mammalian cells than the minimal avian-like precursor virus, and is therefore a clear risk to mammals which have consumed infected milk such as cats and raccoons[13]. Thus far, these cattle viruses show inefficient airborne transmissibility in ferret experiments[30,44] and have not adapted to transmit via respiratory route or to efficiently use human receptors (α-2,6-linked sialic acids)[45-48]. Nonetheless, frequent exposure of an antigenically novel, highly pathogenic influenza virus that can replicate well in human cells is concerning. Our data suggest that the emerged cattle influenza virus is also capable of efficiently infecting avian and swine cells, further increasing the risk of spill

over from cattle to other species. In fact, the mammalian-adapted virus appeared to have no fitness cost in avian cells, explaining the high propensity of this virus to spillover into poultry, and suggesting such a virus could maintain its mammalian adaptations if it spilt back into wild birds. With each human infection and increased polymerase activity leading to higher levels of replication, there is a danger of further evolution changing viral receptor properties. Additionally, a reassortment event with human seasonal influenza viruses could lead to a novel virus, particularly during the Northern hemisphere winter influenza season.

Recent experimental infections in cows suggest that the ability of the North American B3.13 cattle influenza virus to spread via milk is not unique and that other mammalian viruses are capable of transmitting via this route[16]. Phylogenetic data suggests that this originated from a single spillover event from wild birds to cattle and such spillovers must be rare as IAV has rarely been recorded in cattle. Nonetheless, in the absence of an effective control strategy, the high pathogenicity H5N1 virus may now become endemic in US dairy cattle, requiring continuous monitoring even in the absence of overt disease. Moreover, many other clades and strains of H5N1 virus continue to emerge through reassortment, causing zoonotic infections. For example, the emergence of the D1 genotype of H5N1 virus in North America has caused a recent uptick in spillover from birds to mammals including humans and swine[49]. Urgent development and testing of broadly reactive H5 influenza vaccines for both animals and humans is a priority.

Methods

Ethics and biosafety

Virus work was undertaken at either containment level 3 (CL3), SAPO4 (for whole reverse genetics-derived viruses) or CL2 (for reverse genetics-derived viruses with HA and NA from the attenuated vaccine strain A/Puerto Rico/8/1934 (PR8) and the remaining internal genes from H5N1 viruses). Viruses carrying H5 HA with a multibasic cleavage site are categorised as specified animal pathogens order (SAPO) 4 and

Advisory Committee on Dangerous Pathogens (ACDP) hazard group 3 by United Kingdom regulations. Work with these viruses was undertaken in a licensed CL3/SAPO4 facility of The Pirbright Institute under GMRA (BAG-RA-226). CL2 work with ACDP Hazard Group 2 recombinant influenza viruses was performed at the Roslin Institute under biological risk assessment BARA 1011 approved by a University of Edinburgh biosafety committee and GMRA 1811. All virus and GM risk assessments were approved by the appropriate internal committees, as well as the UK Health and Safety Executive (HSE) and, where necessary, the UK scientific advisory committee for genetic modification (SACGM).

Ethics for the use of the primary human airway epithelial cells were as described previously[50]. Briefly, donors provided written consent and Ethics approval was given through the Living Airway Biobank, administered through the UCL Great Ormond Street Institute of Child Health (REC reference: 19/NW/0171, IRAS project ID: 261511, Northwest Liverpool East Research Ethics Committee). Nasal brushings were obtained by trained clinicians from adult (30–50 years) donors who reported no respiratory symptoms in the preceding 7 weeks. Brushings were taken from the inferior nasal concha zone using cytological brushes (Scientific Laboratory Supplies, CYT1050). All methods were performed following the relevant guidelines and regulations.

Cells

Human Embryonic Kidney 293T (293T), Madin-Darby Canine Kidney (MDCK), chicken immortalised fibroblast cells (DF-1), human adenocarcinoma cells (A549), normal human dermal fibroblasts (NHDF), and bovine mammary epithelial cells (MAC-T) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% Pen-Strep. Human lung adenocarcinoma (Calu-3) cells were maintained in DMEM with 10% FCS, 1% Pen-Strep, 2 mM L-glutamine. Swine fetal testes (ST) cells were maintained in Advanced DMEM, 5% FCS, 1% Pen-Strep, 2 mM L-glutamine. Chicken lung epithelial cell (CLEC213[51], kindly gifted by Dr Sascha Trapp) were cultured in DMEM F12 supplemented with 8% FBS, 2 mM glutamine, 1% Pen-Strep. Triple knockout cells (tKO- Engineered human haploid

(eHAP) cells with ANP32A, ANP32B and ANP32E knocked out) were generated as previously described[52] and maintained in Iscove's modified Dulbecco's medium (IMDM), 10% FBS, 1% NEAA, 1% Pen-Strep. All cells were maintained at 37°C and 5% CO_2.

Primary human airway cells were generated and differentiated as described previously[50]. Briefly, human nasal brushings were expanded on 3T3-J2 fibroblasts and frozen down as Passage 1. Basal cells were then revived as needed, expanded in co-culture with 3T3-J2 fibroblasts until passage 2 and differentiated at an air liquid interface as previously described [50].

Plasmids

Reverse genetics plasmids of the viruses used in this study were produced as previously described (Table 1)[45,53,54]. Mutants were generated by site directed mutagenesis. pCAGGS expression plasmids of the polymerase subunits were subcloned from reverse genetics plasmids.

Construction of the minireplicon luciferase reporter plasmid for use in bovine cells was performed using a previous strategy used for an equivalent swine reporter, pSPOM2-Firefly[37] with swine polI promoter replaced with 428bp bovine polI promoter from the bovine genome, bosTau8 chrUn_GJ059828v1 (1165-1592)[55]. Plasmid map available at github.com/Flu1/bovinePolI. *Bos taurus Anp32A* (NCBI reference sequence NM_001195019.1) and *Anp32B (NM_001035074.1)* were ordered as synthetic GeneArt constructs from ThermoFisher Scientific and subcloned into pCAGGS as described previously[41].

Virus strain name	Alias	GISAID ID	Whole virus, 2:6 or minireplicon
A/dairy cattle/Texas/24-008749-001-original/2024 (H5N1)	cattle/Texas	EPI_ISL_19094762	All
A/chicken/England/053052/2021(H5N1)	AIV07	EPI_ISL_9012457	minireplicon only

A/turkey/England/50-92/1991(H5N1)	50-92	EPI_ISL_5888	minireplicon only
A/England/195/2009	Eng195	EPI_ISL_29994	Whole virus and minireplicon only

Table 1. Virus strains, aliases, accessions and uses in this study.

Viruses

Viruses were rescued using 8 plasmid PHW2000 sets containing each genome segment from the named virus. HEK 293T cells were transfected with a mix containing 250 ng of each plasmid using lipofectamine 2000 according to the manufacturer's instructions. Media was replaced 24 h post-transfection with serum-free DMEM with 0.14% bovine serum albumin and 1 µg/ml of L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin. Virus-containing supernatants were collected after 48 h, clarified by centrifugation and used to inoculate 10-day-old embryonated hen's eggs. Eggs were incubated for 48 h before humane euthanization at 4°C, after which virus-containing allantoic fluid was collected, clarified and stored at -80°C before titration by plaque assay.

Phylogenetics

Dairy Cattle sequences submitted prior to 1st December 2024 , avian H5N1 sequences from the USA submitted between 1st September 2023-31st March 2024 and A/Texas/37/2024 were downloaded from GISAID. Sequences from isolates which contained PB2, PB1, PA, NP, HA, NA, NS1, NS2/NEP, M1 and M2 segments were concatenated. Sequences were aligned using MAFFT v7.490 trimmed using pytrimal with a gap threshold of 0.85 to ensure correct residue numbering (total length = 4466 amino acids). Duplicate sequences and sequences with > 30 ambiguous residues or gaps were removed. A phylogenetic tree from the cleaned alignment (n=902) was made in IQTree2 running automatic ModelFinder and the FLAVI+F+I+R2 model[56,57]. Data manipulation and visualisation was carried out using Python 3.12.2 and R 4.4.1. Code

and list of GISAID accessions can be found at https://github.com/fernandocapelastegui/Cattle-H5N1-polymerase-adaptation.

Minireplicon assay

293T, MAC-T, or eHAP tKO cells were transfected in 24-well plates with Lipofectamine 3000 transfection reagent (Thermo Fisher) with the following amounts of pCAGGS expression plasmids: 40 ng PB2, 40 ng PB1, 20 ng PA, 80 ng NP, 40 ng Renilla luciferase, 80 ng species-specific (human, bovine, swine or chicken) polI vRNA Firefly luciferase and 40 ng ANP32 or empty pCAGGS (where appropriate). DF-1 and ST cells were transfected in 12 well plates using quadruple the plasmid/transfection reagent mixes to those used for the other cells in 24-well plates. Cells were lysed 24h post-transfection using passive lysis buffer (Promega) and polymerase activity measured using the Dual-luciferase Reporter assay system (Promega) and a FLUOstar Omega plate reader (BMG Labtech). Assays in MAC-T cells were read using Dual-Glo® Luciferase Assay System (Promega) on an Infinite MPlex plate reader (Tecan). Firefly luciferase signal has been normalised to Renilla luciferase signal to give relative luminescence units (RLU).

Virus replication kinetics

Confluent monolayers of CLEC213, A549, NHDF, MAC-T, BAT II, and Calu-3 cells were washed once with PBS and infected at multiplicity of infection (MOI) 0.01 with virus diluted in serum-free medium for 1 h at 37 °C. Inoculum was replaced with serum-free medium supplemented with 0.14% BSA and 1 µg/ml TPCK-treated bovine pancreas trypsin. Calu-3 and MAC-T experiments performed with whole H5N1 virus excluded TPCK trypsin. Supernatants were collected at various times post infection and stored at -80°C until infectious titres were determined by plaque assay.

Infection of primary human airway cells was performed as follows. Before infection cells were washed with Dulbecco's phosphate-buffered saline supplemented with Calcium and Megnesium (DPBS++) to remove mucus and debris. Cells were

infected with 200 µL of virus in DPBS++ at an MOI of 0.01 and incubated at 37°C for 1 h. Inoculum was removed, and cells were washed twice with DPBS++. Time points were taken by adding 200 µL of DPBS++ and incubating for 10 mins and 37°C before removal and titration. The time course was performed at 37°C, 5% CO_2. Time points were taken at 24-, 48-, 72- and 96-hours post-infection. Infectious titres were determined by plaque assay on MDCKs.

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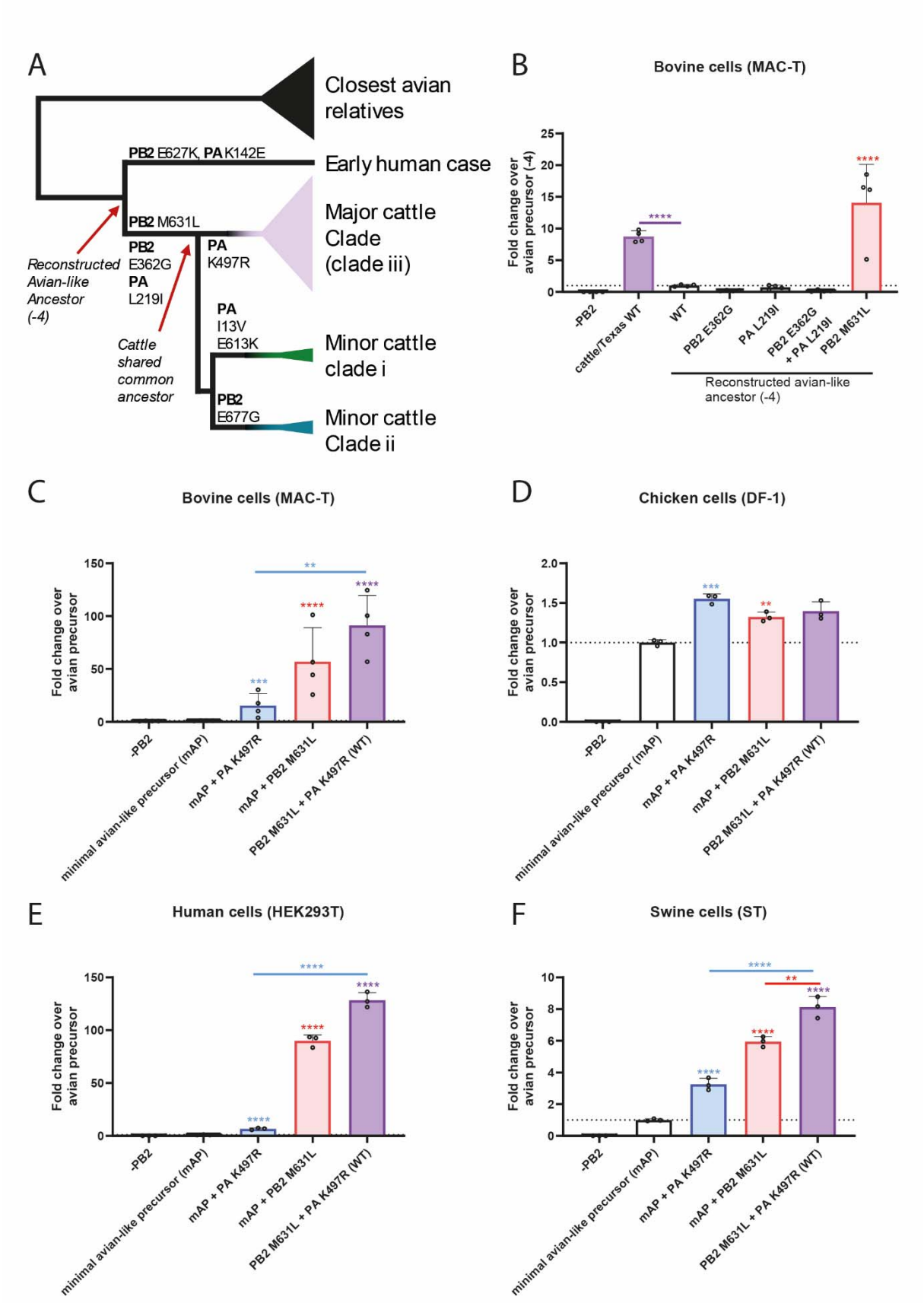
Credit Contributions:
VD – investigation, resources, visualisation
JLQ – investigation, resources
SR – investigation, Writing – review & editing
NP – investigation, Writing – review & editing
MDB – investigation, resources, Writing – review & editing
JY – investigation, resources, Writing – review & editing
FC – data curation, investigation, software, Writing – review & editing
TM - resources
K-MC - resources
JA - resources
ES – resources, Writing – review & editing
CMSheppard – investigation, supervision, Writing – review & editing
IHB – funding acquisition, supervision, Writing – review & editing
CMSmith - funding acquisition, resources, supervision
MI - funding acquisition, resources, supervision
PD - funding acquisition, methodology, supervision, Writing – review & editing
WSB – conceptualisation, funding acquisition, methodology, supervision, Writing – review & editing
RMP – investigation, methodology, project administration, supervision, validation
TPP – conceptualisation, formal analysis, investigation, methodology, project administration, supervision, validation, visualisation, Writing – original draft
DHG – conceptualisation, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, software, supervision, visualisation, Writing – original draft

Figures


A
Closest avian relatives
PB2 E627K, PA K142E
Early human case
PB2 M631L
Major cattle Clade (clade iii)
PB2 E362G
PA L219I
PA K497R
Reconstructed Avian-like Ancestor (-4)
Cattle shared common ancestor
PA I13V E613K
Minor cattle clade i
PB2 E677G
Minor cattle Clade ii
B
Bovine cells (MAC-T)
Fold change over avian precursor (-4)
-PB2
cattle/Texas WT
WT
PB2 E362G
PA L219I
PB2 E362G + PA L219I
PB2 M631L
Reconstructed avian-like ancestor (-4)
C
Bovine cells (MAC-T)
Fold change over avian precursor
-PB2
minimal avian-like precursor (mAP)
mAP + PA K497R
mAP + PB2 M631L
PB2 M631L + PA K497R (WT)
D
Chicken cells (DF-1)
E
Human cells (HEK293T)
F
Swine cells (ST)

550

Figure 1. Identification of key mammalian polymerase adaptations in the major clade of bovine H5N1 that enhance polymerase activity in bovine cells. A) Simplified schematic of cattle polymerase phylogeny and polymerase mutations that arose in early branches. B-F) Minigenome in (B, C) bovine MAC-T cells (D) chicken DF-1 (E) human HEK293T, (F) swine ST cells with cattle Texas (WT). B) Reconstructed avian-like ancestor (-4) has a reversion of PB2 E362G, M631L and PA L219, K497R. C-F) Minimal avian-like precursor has the reversions of PB2 M631L and PA K497R. Data normalised to minimal avian-like precursor. Data throughout plotted as triplicate (D,E,F) or quadruplicate (B, C) technical repeats of a representative repeat of N = 3 independent repeats. Data throughout plotted as mean + SD. Statistics throughout performed by one-way ANOVA with multiple comparisons using log-transformed data. Log-normality determined by Shapiro-Wilk test and QQ plot. Significance shown by asterisks indicating: **, 0.01 ≥ P > 0.001; ***, 0.001 ≥ P > 0.0001; ****, P ≤ 0.0001.

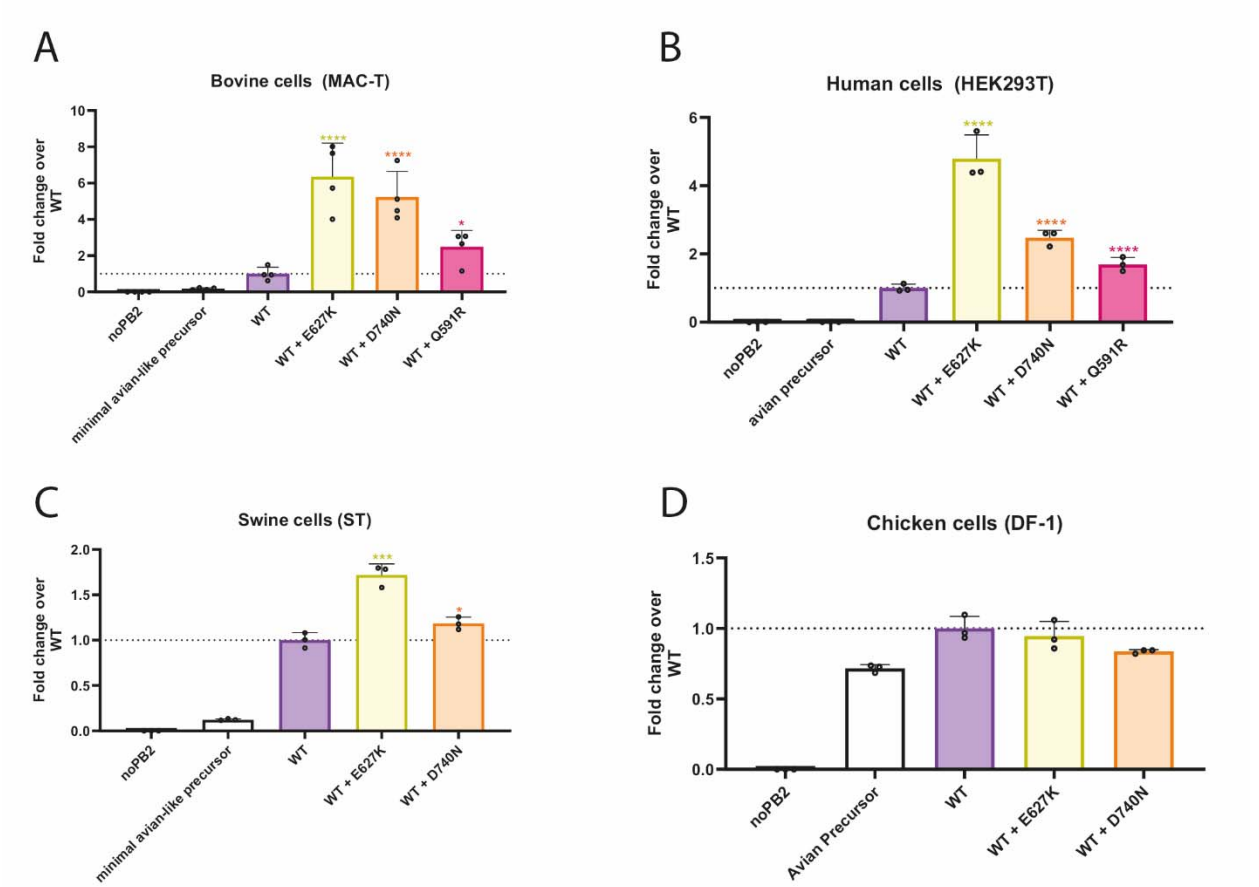


Figure 2. PB2 M631L, and to a lesser extent PA K497R, enhance virus replication in mammalian, but not avian cell lines. Reverse-genetics derived viruses, containing the HA and NA genes of the attenuated laboratory strain A/Puerto Rico/1/34, and the remaining genes from Cattle Texas (2:6 viruses) were used to infect A) bovine mammary cells (MAC-T) B) human lung cells (A549), or C) chicken lung cells (CLEC213). Cells were infected with an MOI of 0.01. Infectious virus titres were determined by plaque assay on MDCKs. Data throughout plotted as N = 3 technical repeats from an assay representing N = 3 independent biological repeats. Data throughout plotted as mean ± SD. Statistics throughout performed by two-way ANOVA on log-transformed data with multiple comparisons to the minimal avian-like precursor. Significance shown by asterisks indicating: **, 0.01□≥□P□>□0.001; ***, 0.001□≥□P□>□0.0001; ****, P□≤□0.0001.

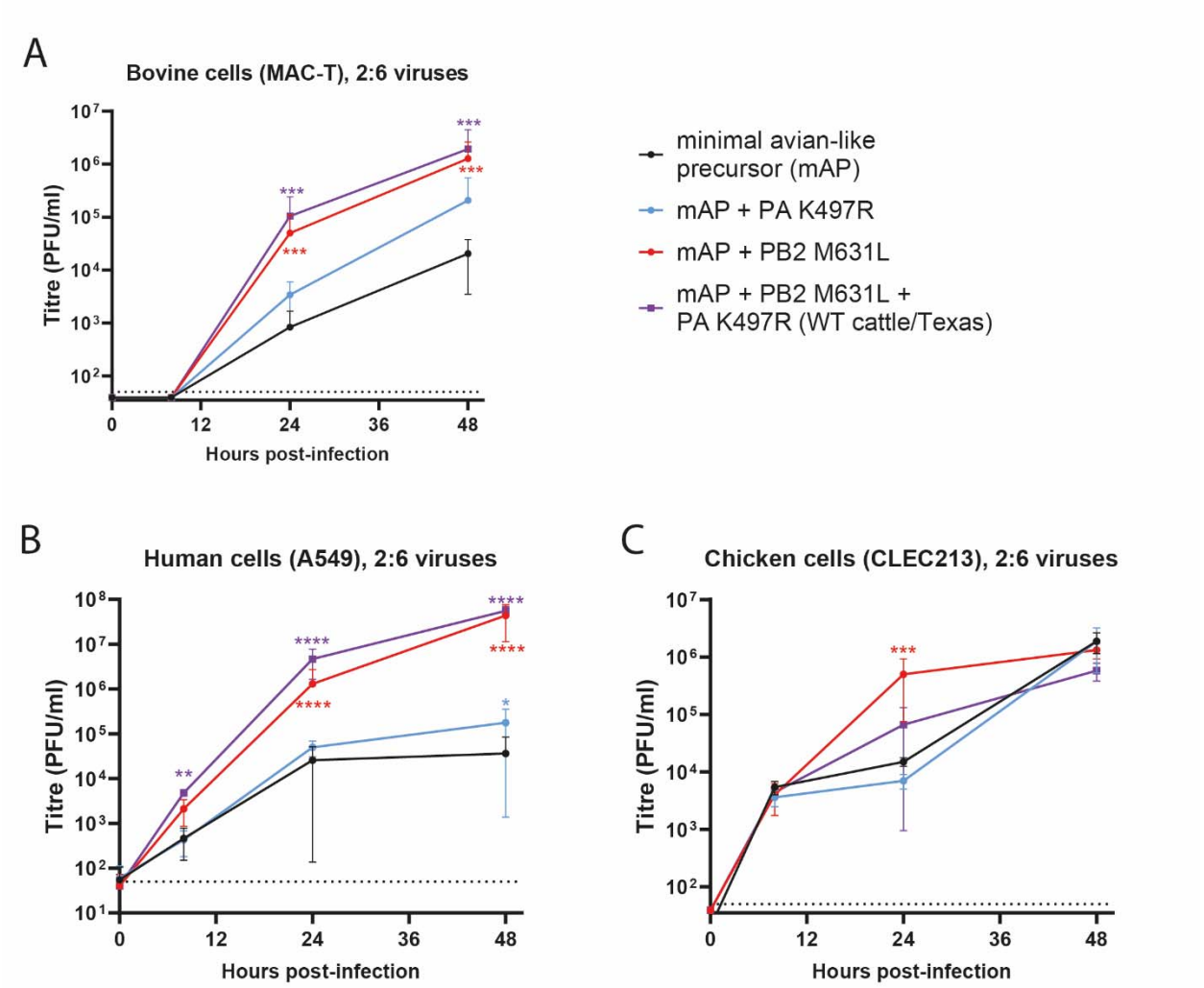


Figure 3. Ongoing adaptations found in cattle H5N1 viruses further enhance polymerase activity in mammalian cells. Minigenome in (A) bovine MAC-T cells (B) human HEK293T, (C) swine ST cells or (D) chicken DF-1 with mutant versions of cattle Texas representing potential ongoing adaptation. Data normalised to WT cattle Texas. Data throughout plotted as triplicate (B, C, and D) or quadruplicate (A) technical repeats of a representative repeat of N = 3 independent repeats. Data throughout plotted as mean + SD Statistics throughout performed by one-way ANOVA with multiple comparisons against WT polymerase using log-transformed data. Log-normality determined by Shapiro-Wilk test and QQ plot. Significance shown by asterisks indicating: *, 0.05□≥□P□>□0.01; **, 0.01□≥□P□>□0.001; ***, 0.001□≥□P□>□0.0001; ****, P□≤□0.0001.

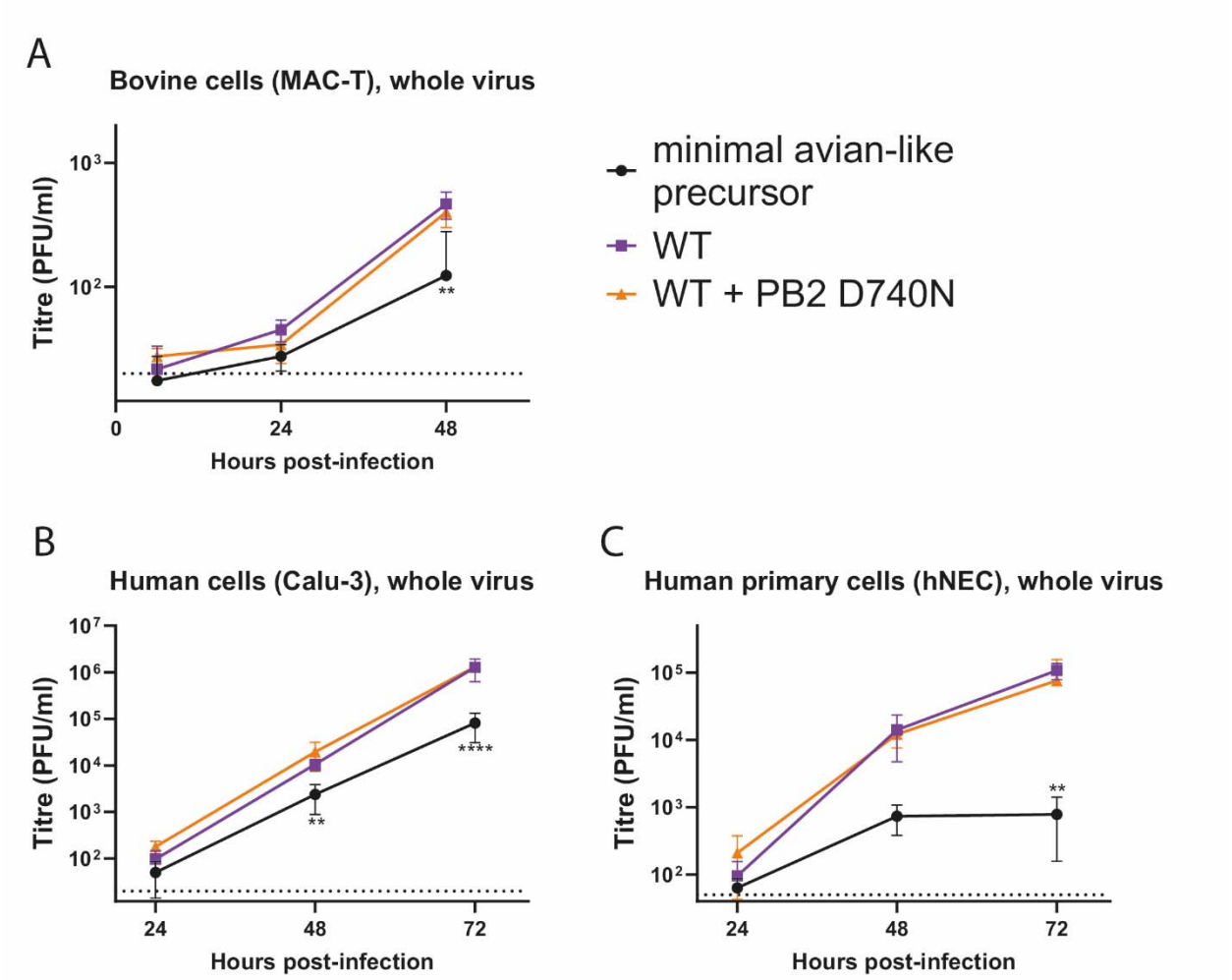


Figure 4. Mammalian adaptations in the cattle H5N1 polymerase enhance its replication in human and bovine cell lines and human primary airway cultures. Reverse-genetics derived viruses, containing all eight gene segments from Cattle Texas, and handled at ACDP3/SAPO4 conditions were used to infect **A)** bovine mammary cells (MAC-T) **B)** human airway cells (Calu-3) (C) primary differentiated human nasal epithelial cells airway maintained at air liquid interface (hNECs). Cells were infected with an MOI of 0.01 for all experiments. Infectious virus titres were determined by plaque assay on MDCKs. Data throughout plotted as N = 3 technical repeats. Data throughout plotted as mean ± SD. Statistics throughout performed by two-way ANOVA on log-transformed data with multiple comparisons against WT cattle/texas. Significance shown by asterisks indicating: **, 0.01 ≥ P > 0.001; ****, P ≤ 0.0001.

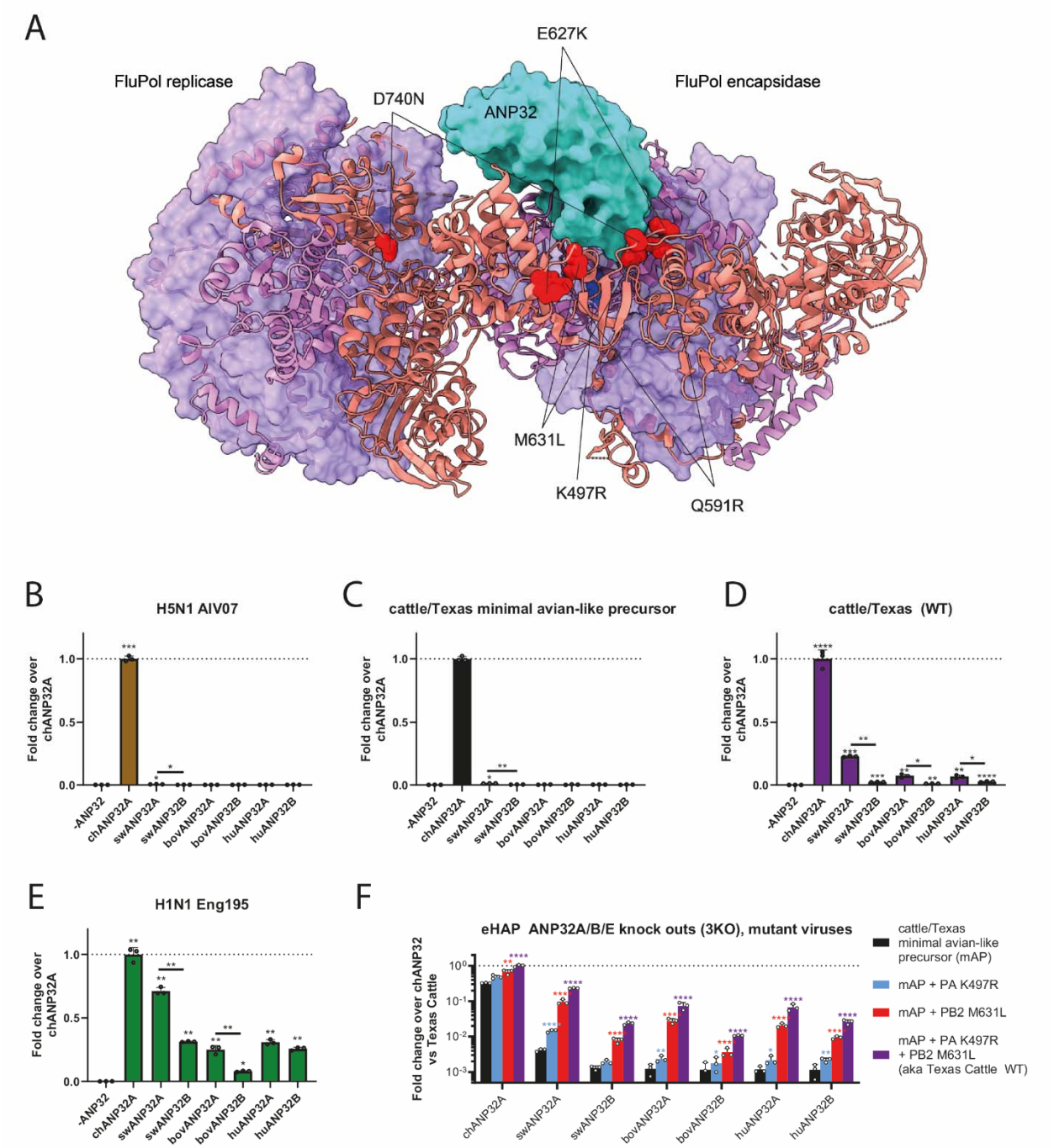


Figure 5. Bovine ANP32A and B proteins are able to support mammalian-adapted polymerases, although bovine ANP32A is dominant. A) Key mammalian adaptations identified in this study mapped to the structure of H5N1 influenza A replicase dimer complex with human ANP32B (PDB: 8R1J). ANP32B is shown in cyan, PB1 in purple, PA in violet and PB2 as a salmon ribbon representation. H5N1 cattle PB2 substitutions (Q591, E627, M631 and D740) and PA (K497R) shown in red. B-F) Minigenomes in human engineered haploid cells (eHAP) with endogenous ANP32A, ANP32B and ANP32E knocked out were supplemented with ANP32 proteins from different species

(chANP32A = chicken ANP32A, swANP32A = swine ANP32A, swANP32B = swine ANP32B, bovANP32A = bovine ANP32A, bovANP32B = bovine ANP32B, huANP32A = human ANP32A, huANP32B = human ANP32B, -ANP = no ANP32 control). Avianised cattle = PB2 M631L and PA K497R reversed. Data normalised throughout to chANP32A. Data throughout plotted as triplicate technical repeats of a representative repeat of N = 3 independent repeats. Data throughout plotted as mean + SD. Statistics throughout performed by one-way ANOVA with multiple comparisons - against -ANP32 control, or between species-matched ANP32A and ANP32B proteins, using log-transformed data. Log-normality determined by Shapiro-Wilk test and QQ plot. Significance shown by asterisks indicating: *, 0.05□≥□P□>□0.01; **, 0.01□≥□P□>□0.001; ***, 0.001□≥□P□>□0.0001; ****, P□≤□0.0001.

References

1 Long, J. S., Mistry, B., Haslam, S. M. & Barclay, W. S. Host and viral determinants of influenza A virus species specificity. *Nature Reviews Microbiology* **17**, 67-81 (2019). https://doi.org/10.1038/s41579-018-0115-z

2 Xie, R. *et al.* The episodic resurgence of highly pathogenic avian influenza H5 virus. *bioRxiv*, 2022.2012.2018.520670 (2022). https://doi.org/10.1101/2022.12.18.520670

3 Peacock, T. *et al.* The global H5N1 influenza panzootic in mammals. *Nature* (2024). https://doi.org/10.1038/s41586-024-08054-z

4 Agüero, M. *et al.* Highly pathogenic avian influenza A(H5N1) virus infection in farmed minks, Spain, October 2022. *Eurosurveillance* **28**, 2300001 (2023). https://doi.org/doi:https://doi.org/10.2807/1560-7917.ES.2023.28.3.2300001

5 Kareinen, L. *et al.* Highly pathogenic avian influenza A(H5N1) virus infections on fur farms connected to mass mortalities of black-headed gulls, Finland, July to October 2023. *Eurosurveillance* **29**, 2400063 (2024). https://doi.org/doi:https://doi.org/10.2807/1560-7917.ES.2024.29.25.2400063

6 Lindh, E. *et al.* Highly pathogenic avian influenza A(H5N1) virus infection on multiple fur farms in the South and Central Ostrobothnia regions of Finland, July 2023. *Eurosurveillance* **28**, 2300400 (2023). https://doi.org/doi:https://doi.org/10.2807/1560-7917.ES.2023.28.31.2300400

7 Uhart, M. M. *et al.* Epidemiological data of an influenza A/H5N1 outbreak in elephant seals in Argentina indicates mammal-to-mammal transmission. *Nature Communications* **15**, 9516 (2024). https://doi.org/10.1038/s41467-024-53766-5

8 de Carvalho Araujo, A. *et al.* Mortality in sea lions is associated with the introduction of the H5N1 clade 2.3.4.4b virus in Brazil October 2023: whole genome sequencing and phylogenetic analysis. *BMC Veterinary Research* **20**, 285 (2024). https://doi.org/10.1186/s12917-024-04137-1

9 Gamarra-Toledo, V. *et al.* First Mass Mortality of Marine Mammals Caused by Highly Pathogenic Influenza Virus (H5N1) in South America. *bioRxiv*, 2023.2002.2008.527769 (2023). https://doi.org/10.1101/2023.02.08.527769

10 Leguia, M. *et al.* Highly pathogenic avian influenza A (H5N1) in marine mammals and seabirds in Peru. *Nat Commun* **14**, 5489 (2023). https://doi.org/10.1038/s41467-023-41182-0

11 Tomás, G. *et al.* Highly pathogenic avian influenza H5N1 virus infections in pinnipeds and seabirds in Uruguay: Implications for bird-mammal transmission in South America. *Virus Evol* **10**, veae031 (2024). https://doi.org/10.1093/ve/veae031

12 Caserta, L. C. *et al.* Spillover of highly pathogenic avian influenza H5N1 virus to dairy cattle. *Nature* (2024). https://doi.org/10.1038/s41586-024-07849-4

13 Nguyen, T.-Q. *et al.* Emergence and interstate spread of highly pathogenic avian influenza A(H5N1) in dairy cattle. *bioRxiv*, 2024.2005.2001.591751 (2024). https://doi.org/10.1101/2024.05.01.591751

14 Worobey, M. *et al.* Preliminary Report on Genomic Epidemiology of the 2024 H5N1 Influenza A Virus Outbreak in US Cattle. *Virological.org* (2024).

15 Le Sage, V., Campbell, A. J., Reed, D. S., Duprex, W. P. & Lakdawala, S. S. Persistence of Influenza H5N1 and H1N1 Viruses in Unpasteurized Milk on Milking Unit Surfaces. *Emerg Infect Dis* **30**, 1721-1723 (2024). https://doi.org/10.3201/eid3008.240775

16 Halwe, N. J. *et al.* H5N1 clade 2.3.4.4b dynamics in experimentally infected calves and cows. *Nature* (2024). https://doi.org/10.1038/s41586-024-08063-y

17 Uyeki, T. M. *et al.* Highly Pathogenic Avian Influenza A(H5N1) Virus Infection in a Dairy Farm Worker. *N Engl J Med* **390**, 2028-2029 (2024). https://doi.org/10.1056/NEJMc2405371

18 Garg, S. *et al.* Highly Pathogenic Avian Influenza A(H5N1) Virus Infections in Humans. *N Engl J Med* (2024). https://doi.org/10.1056/NEJMoa2414610

19 Peacock, T. P., Sheppard, C. M., Staller, E. & Barclay, W. S. Host determinants of influenza RNA synthesis. *Annual review of virology* **6**, 215-233 (2019).

20 Staller, E. *et al.* ANP32 Proteins Are Essential for Influenza Virus Replication in Human Cells. *J Virol* **93** (2019). https://doi.org/10.1128/jvi.00217-19

21 Zhang, H. *et al.* Fundamental Contribution and Host Range Determination of ANP32A and ANP32B in Influenza A Virus Polymerase Activity. *J Virol* **93** (2019). https://doi.org/10.1128/jvi.00174-19

22 Long, J. S. *et al.* Species difference in ANP32A underlies influenza A virus polymerase host restriction. *Nature* **529**, 101-104 (2016). https://doi.org/10.1038/nature16474

23 Long, J. S. *et al.* Species specific differences in use of ANP32 proteins by influenza A virus. *eLife* **8**, e45066 (2019). https://doi.org/10.7554/eLife.45066

24 Yamada, S. *et al.* Biological and Structural Characterization of a Host-Adapting Amino Acid in Influenza Virus. *PLoS Pathog* **6**, e1001034 (2010). https://doi.org/10.1371/journal.ppat.1001034

25 Peacock, T. P. *et al.* Mammalian ANP32A and ANP32B Proteins Drive Differential Polymerase Adaptations in Avian Influenza Virus. *Journal of Virology*, e00213-00223 (2023).

26 Mehle, A. & Doudna, J. A. An Inhibitory Activity in Human Cells Restricts the Function of an Avian-like Influenza Virus Polymerase. *Cell Host & Microbe* **4**, 111-122 (2008). https://doi.org/https://doi.org/10.1016/j.chom.2008.06.007

27 Gilbertson, B., Duncan, M. & Subbarao, K. Role of the viral polymerase during adaptation of influenza A viruses to new hosts. *Curr Opin Virol* **62**, 101363 (2023). https://doi.org/10.1016/j.coviro.2023.101363

28 Sheppard, C. M. *et al.* An Influenza A virus can evolve to use human ANP32E through altering polymerase dimerization. *Nat Commun* **14**, 6135 (2023). https://doi.org/10.1038/s41467-023-41308-4

29 Idoko-Akoh, A. *et al.* Creating resistance to avian influenza infection through genome editing of the ANP32 gene family. *Nature Communications* **14**, 6136 (2023). https://doi.org/10.1038/s41467-023-41476-3

30 Gu, C. *et al.* A human isolate of bovine H5N1 is transmissible and lethal in animal models. *Nature* (2024). https://doi.org/10.1038/s41586-024-08254-7

31 Mänz, B., Brunotte, L., Reuther, P. & Schwemmle, M. Adaptive mutations in NEP compensate for defective H5N1 RNA replication in cultured human cells. *Nature Communications* **3**, 802 (2012). https://doi.org/10.1038/ncomms1804

32 Zhang, X. *et al.* Enhanced pathogenicity and neurotropism of mouse-adapted H10N7 influenza virus are mediated by novel PB2 and NA mutations. *J Gen Virol* **98**, 1185-1195 (2017). https://doi.org/10.1099/jgv.0.000770

33 Patrono, L. V. *et al.* Archival influenza virus genomes from Europe reveal genomic variability during the 1918 pandemic. *Nature Communications* **13**, 2314 (2022). https://doi.org/10.1038/s41467-022-29614-9

34 Mänz, B. *et al.* Multiple Natural Substitutions in Avian Influenza A Virus PB2 Facilitate Efficient Replication in Human Cells. *J Virol* **90**, 5928-5938 (2016). https://doi.org/10.1128/jvi.00130-16

35 Welkers, M. R. A. *et al.* Genetic diversity and host adaptation of avian H5N1 influenza viruses during human infection. *Emerg Microbes Infect* **8**, 262-271 (2019). https://doi.org/10.1080/22221751.2019.1575700

36 Yamayoshi, S. *et al.* Enhanced Replication of Highly Pathogenic Influenza A(H7N9) Virus in Humans. *Emerg Infect Dis* **24**, 746-750 (2018). https://doi.org/10.3201/eid2404.171509

37 Moncorgé, O. *et al.* Investigation of influenza virus polymerase activity in pig cells. *J Virol* **87**, 384-394 (2013). https://doi.org/10.1128/jvi.01633-12

38 Beare, A. S., Schild, G. C. & Craig, J. W. TRIALS IN MAN WITH LIVE RECOMBINANTS MADE FROM A/PR/8/34 (H0 N1) AND WILD H3 N2 INFLUENZA VIRUSES. *The Lancet* **306**, 729-732 (1975). https://doi.org/10.1016/S0140-6736(75)90720-5

39 Singh, G. *et al.* Detection and characterization of H5N1 HPAIV in environmental samples from a dairy farm. *Virus Genes* **60**, 517-527 (2024). https://doi.org/10.1007/s11262-024-02085-4

40 Staller, E. *et al.* Structures of H5N1 influenza polymerase with ANP32B reveal mechanisms of genome replication and host adaptation. *Nature Communications* **15**, 4123 (2024). https://doi.org/10.1038/s41467-024-48470-3

41 Peacock, T. P. *et al.* Swine ANP32A supports avian influenza virus polymerase. *Journal of virology* **94**, e00132-00120 (2020).

42 Zhang, H. *et al.* A unique feature of swine ANP32A provides susceptibility to avian influenza virus infection in pigs. *PLoS Pathog* **16**, e1008330 (2020). https://doi.org/10.1371/journal.ppat.1008330

43 Carrique, L. *et al.* Host ANP32A mediates the assembly of the influenza virus replicase. *Nature* **587**, 638-643 (2020). https://doi.org/10.1038/s41586-020-2927-z

44 Eisfeld, A. J. *et al.* Pathogenicity and transmissibility of bovine H5N1 influenza virus. *Nature* **633**, 426-432 (2024). https://doi.org/10.1038/s41586-024-07766-6

45 Yang, J. *et al.* The Haemagglutinin Gene of Bovine Origin H5N1 Influenza Viruses Currently Retains an Avian Influenza Virus phenotype. *bioRxiv*, 2024.2009.2027.615407 (2024). https://doi.org/10.1101/2024.09.27.615407

46 Chopra, P. *et al.* Receptor Binding Specificity of a Bovine A(H5N1) Influenza Virus. *bioRxiv*, 2024.2007.2030.605893 (2024). https://doi.org/10.1101/2024.07.30.605893

47 Santos, J. J. S. *et al.* Bovine H5N1 influenza virus binds poorly to human-type sialic acid receptors. *bioRxiv*, 2024.2008.2001.606177 (2024). https://doi.org/10.1101/2024.08.01.606177

48 Good, M. R., Ji, W., Fernández-Quintero, M. L., Ward, A. B. & Guthmiller, J. J. A single mutation in dairy cow-associated H5N1 viruses increases receptor binding breadth. *bioRxiv*, 2024.2006.2022.600211 (2024). https://doi.org/10.1101/2024.06.22.600211

49 US Centre for Disease Control. Genetic Sequences of Highly Pathogenic Avian Influenza A(H5N1) Viruses Identified in a Person in Louisiana. (2024).

50 Woodall, M. N. J. *et al.* Age-specific nasal epithelial responses to SARS-CoV-2 infection. *Nature Microbiology* **9**, 1293-1311 (2024). https://doi.org/10.1038/s41564-024-01658-1

51 Esnault, E. *et al.* A novel chicken lung epithelial cell line: characterization and response to low pathogenicity avian influenza virus. *Virus Res* **159**, 32-42 (2011). https://doi.org/10.1016/j.virusres.2011.04.022

52 Sheppard, C. M. *et al.* An Influenza A virus can evolve to use human ANP32E through altering polymerase dimerization. *Nature Communications* **14**, 6135 (2023).

53 Schafers, J. *et al.* Pasteurisation temperatures effectively inactivate influenza A viruses in milk. *medRxiv*, 2024.2005.2030.24308212 (2024). https://doi.org/10.1101/2024.05.30.24308212

54 Yang, J. *et al.* The Haemagglutinin Genes of the UK Clade 2.3.4.4b H5N1 Avian Influenza Viruses from 2020 to 2022 Retain Strong Avian Phenotype. *bioRxiv*, 2024.2007.2009.602706 (2024). https://doi.org/10.1101/2024.07.09.602706

55 Perez, G. *et al.* The UCSC Genome Browser database: 2025 update. *Nucleic Acids Res* (2024). https://doi.org/10.1093/nar/gkae974

56 Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A. & Jermiin, L. S. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature Methods* **14**, 587-589 (2017). https://doi.org/10.1038/nmeth.4285

57 Minh, B. Q. *et al.* IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Mol Biol Evol* **37**, 1530-1534 (2020). https://doi.org/10.1093/molbev/msaa015