



**Visualising and quantifying the RNA products of
influenza A virus polymerase for insights into
regulation and host restriction**

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Submitted for degree of Doctor of Philosophy

DECLARATION OF ORIGINALITY

I confirm that all work contained in this thesis, unless clearly stated otherwise, is my own. Any material from other sources has been acknowledged.

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ACKNOWLEDGEMENTS

Firstly, enormous thanks are due to my supervisor, Wendy. I am so grateful for the energy you have put into supervising me over the last four years and that you've always had time for a meeting - despite being incredibly busy saving the world from SARS-CoV-2. I feel very lucky to have worked for such a dedicated and enthusiastic scientist, and hopefully have learnt a lot in the process!

To the rest of the Barclay lab, past and present: Carol, Jay, JB, Daniel, Jason, Raul, Becky, Ruthiran, Jack, Ada, Bhakti, Ecco, Rebecca, Laury, Maya, Amalie, Dorothee, Ollie, Ksenia, Jess, Joelle - thank you for making the Barclay lab such a fun and supportive place! It is entirely unclear whether my PhD would have taken half or twice as long without you all – I certainly would have drunk a lot less tea. Particular thanks go to Tom for taking on the daily supervision of a PhD student with a project only tentatively related to his own. Thank you for tolerating my endless 'Can I ask a question – please?'s, and always checking in on me.

I would also like to thank my secondary supervisor, Prof Peter O'Hare, especially for advice with the imaging approaches during my first year, as well as our collaborators Dr Lucy Collinson and Dr Alana Burrell at the Crick. There would be no microscopy in this project without the Imperial Facility for Imaging by Light Microscopy – thank you Andreas, David and Steven!

I would have lost motivation many times over if it weren't for my family and friends especially Mike, Lex, Natasha, Andrew, Jantien and Niall and the original support team: Mum and Dad, Bec and Tab. Thank you for the love and encouragement! I am also very grateful to Anne and Ken for unquestioningly allowing a writing hermit to temporarily take up residence in their home – thank you both!

Finally, thank you to Imperial College for hosting me and the Wellcome Trust for funding this project.

ABSTRACT

Host restriction limits the emergence of pandemic strains from the influenza A virus avian reservoir. One important host range barrier is the RNA-dependent RNA polymerase protein (FluPol), which must adapt to undergo efficient replication within the mammalian nucleus. The restriction of avian FluPol in mammalian cells is believed to occur due to incompatibilities with mammalian orthologues of the host factor ANP32, which lack a 33 amino acid insertion relative to avian ANP32A. The quintessential mammalian-adapting mutation that overcomes this restriction is a single amino acid change from a glutamic acid to a lysine at position 627 on the PB2 protein of the FluPol (PB2 E627K).

In this project, we set out to establish an approach for visualising the RNA products of FluPol within the nucleus. We developed a strand-specific RNA fluorescence *in situ* hybridisation (RNA FISH) approach, using the commercial RNAscope assay. Subsequently, this protocol was adapted for correlative light and electron microscopy (CLEM) in collaboration with the electron microscopy platform at the Crick Institute. Simultaneously, we used RNAscope and RT-qPCR to investigate the mechanism by which ANP32 supports viral replication, and functions in host restriction. We discovered that FluPol requires ANP32 proteins to support both steps of genome replication: cRNA and vRNA synthesis. However avian FluPol (PB2 627E) is only restricted in the second step, vRNA synthesis, in mammalian cells. This suggests ANP32 may have an additional role in supporting vRNA synthesis, and it is this activity that is specifically blocked when avian influenza infects mammalian cells.

In summary, this study has established a strand-specific RNA FISH assay, suitable for dissecting the spatiotemporal details of IAV infection within the nucleus on a single-cell level. Moreover, this approach has been utilised, alongside RT-qPCR, to dissect the mechanism of the essential host factor ANP32. In combination, this has improved our understanding of the IAV polymerase, a major contributor to host restriction, and an important therapeutic target.

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ACRONYMS AND ABBREVIATIONS

aa.....	amino acid	IAV.....	influenza A virus
ActD.....	actinomycin D	IDD.....	intrinsically disordered domain
AIV.....	avian influenza virus	IIF.....	indirect immunofluorescence
ANP32.....	Acidic leucine-rich nuclear phosphoprotein 32 kDa	IBV.....	influenza B virus
ANP32A ₃₃	avian ANP32A containing the full 33 aa avian insertion	ICV.....	influenza C virus
ANP32A ₂₉	avian ANP32A splice variant lacking the first 4 aa of the 33 aa avian insertion	IDV.....	influenza D virus
ANP32A Δ	avian ANP32A splice variant lacking the full 33 aa avian insertion	KO.....	knockout
bp.....	base pairs	LRR.....	leucine-rich repeat
BXA.....	baloxavir acid	LCAR.....	low-complexity acidic region
chANP32A.....	chicken ANP32A	M.....	matrix
chANP32B.....	chicken ANP32B	m ⁷ G.....	7-methylguanylate cap
chANP32E.....	chicken ANP32E	MOI.....	multiplicity of infection
CHX.....	cycloheximide	NA.....	neuraminidase
CLEM.....	correlative light and electron microscopy	NLS.....	nuclear localisation signal
cRNA.....	complementary RNA	NP.....	nucleoprotein
cRNP.....	complementary ribonucleoprotein	NS.....	non-structural
Cryo-EM.....	cryo-electron microscopy	NSV.....	negative-sense single-stranded RNA virus
CTD.....	carboxy-terminal domain	NS2/NEP.....	non-structural 2/ nuclear export protein
Da.....	Dalton	nt.....	nucleotide
dKO.....	huANP32A/huANP32B double knockout cells	NTD.....	amino-terminal domain
5-EU.....	5-ethynyluridine	ORF.....	open reading frame
EM.....	electron microscopy	PA.....	polymerase acidic
FluPol.....	influenza RNA dependent RNA polymerase	PA-C.....	C-terminal domain of the PA protein
FluPol _E	encapsidating FluPol	PB.....	polymerase basic
FluPol _R	replicating FluPol	PB2-N.....	N-terminal domain of the PB2 protein
FluPol _T	<i>trans</i> -activating FluPol	Pol I.....	host RNA polymerase I
FluPol 627E.....	avian-like FluPol with a glutamic acid at position 627 on the PB2 protein	Pol II.....	host RNA polymerase II
FluPol 627K.....	FluPol with a mammalian-adapting lysine at position 627 on the PB2 protein	PolyA.....	polyadenylated
HA.....	haemagglutinin	RdRp.....	RNA dependent RNA polymerase
hpi.....	hours post infection	RT-qPCR.....	reverse transcription-quantitative PCR
huANP32A.....	human ANP32A	RNA FISH.....	RNA fluorescence <i>in situ</i> hybridisation
huANP32B.....	human ANP32B	RNP.....	ribonucleoprotein
huANP32E.....	human ANP32E	RT.....	reverse transcription
		Ser5P CTD.....	serine-5 phosphorylated host pol II carboxy-terminal domain
		tKO.....	huANP32A/huANP32B/huANP32E triple knockout cells
		vRNA.....	viral RNA
		vRNP.....	viral ribonucleoprotein
		WT.....	wild-type
		3-8/3-5-8 mutants.....	viral-like RNAs with mutations introduced to the 3' vRNA promoter at positions 3 and 8, or 3, 5 and 8

CHAPTER ONE: INTRODUCTION

1.1 INFLUENZA BACKGROUND

1.1.1 Seasonal Influenza

Influenza A virus (IAV) and Influenza B virus (IBV) are jointly responsible for annual epidemics of acute respiratory viral illness (Ghebrehewet et al., 2016; Paules & Subbarao, 2017). In temperate regions, influenza epidemics occur seasonally in the winter (Paules & Subbarao, 2017), whilst outbreaks in tropical regions can occur throughout the year and are more difficult to predict (Yang et al., 2015). Normally, two seasonal IAV subtypes co-circulate in humans, H1N1 and H3N2, and two IBV lineages, Victoria and Yamagata (Petrova & Russell, 2018). The recurrence of seasonal epidemics is attributed to the efficient transmission of influenza viruses, combined with a high level of evolution (Petrova & Russell, 2018). Influenza viruses replicate using a low fidelity RNA-dependent RNA polymerase (RdRp or FluPol) that lacks exonuclease proofreading capability, resulting in an inherently high error rate and the formation of quasispecies (Taubenberger & Kash, 2010). This genetic diversity allows influenza to escape immunity caused by previous infection or vaccination, in a phenomenon described as antigenic drift (Petrova & Russell, 2018).

The clinical outcome of seasonal influenza infection ranges from asymptomatic to death. Symptomatic infection is characterised by sudden onset fever, severe malaise and respiratory symptoms (Ghebrehewet et al., 2016; Paules & Subbarao, 2017). Severe disease is associated with complications including pneumonia, respiratory failure, sepsis and multiorgan failure, which may be driven directly by influenza or indirectly by secondary bacterial infections (Uyeki et al., 2022). Up to 5 million cases of IAV and IBV occur annually, resulting in an estimated 290,000-650,000 deaths (The World Health Organisation, 2018). The main risk factors for severe disease are pregnancy, age, an immunocompromised state, or underlying comorbidities (Ghebrehewet et al., 2016; Paules & Subbarao, 2017).

1.1.2 Pandemic Influenza

Influenza A virus is also responsible for occasional zoonoses and sporadic pandemics (Taubenberger & Kash, 2010). The natural reservoirs of IAV are wild aquatic birds, in which the infection is mostly asymptomatic (Webster et al., 1992). Within this reservoir, IAV has diverged into a vast pool of unique subtypes, which are defined based on the properties of the two external glycoproteins: neuraminidase (NA) and haemagglutinin (HA). There are 16 different HA subtypes (H1-H16) and 9 different NA subtypes (N1-N9) known to circulate in birds (Long, Mistry, et al., 2019). Furthermore, IAV subtypes have repetitively crossed species barriers to establish new lineages in diverse avian and mammalian species (Long, Mistry, et al., 2019; Webster et al., 1992). In combination, this has resulted in a huge zoonotic reservoir of antigenically distinct IAVs. Finally, two unique HA and NA subtypes (H17N10,

H18N11) have been isolated from bats. This may imply a previously unappreciated reservoir for IAV (Tong et al., 2012, 2013), although the bat subtypes have not been isolated from any other species (Ciminski et al., 2017). The complex ecology of IAV is illustrated in Figure 1.

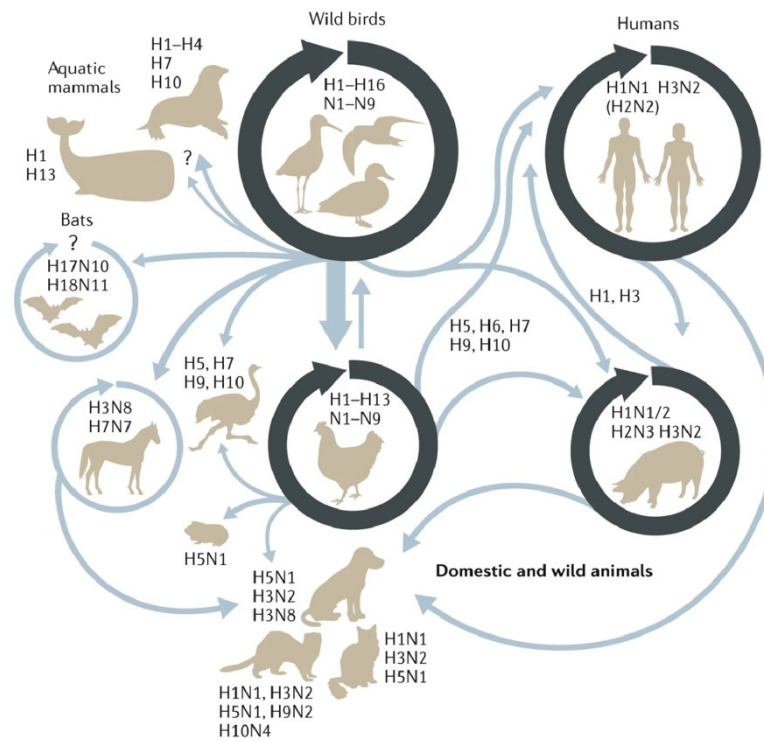


Figure 1: The complex ecology of influenza A virus (IAV).

Figure taken from Long, Mistry, et al., 2019. Reproduced with permission from Springer Nature. The primary reservoir of IAV haemagglutinin (HA) subtypes H1-16 and neuraminidase (NA) subtypes N1-9 are wild birds. Various subtypes from this viral reservoir can spill-over to infect other avian and mammalian species (light blue arrows), sometimes directly, and sometimes via an intermediate host (e.g., wild birds to chickens to humans). Adaptive mutations are typically required for such zoonoses. Dark circles illustrate where subtypes are established within the new host. HA subtypes H17-18 and NA subtypes N10-11 have only been detected in bats.

Occasionally, influenza viruses in the animal reservoir infect humans. Such zoonoses are typically associated with exposure to infected poultry or swine and can result in severe disease, especially in the case of H5N1 and H7N9 subtypes, which have an estimated case fatality rate of 56% and 39%, respectively (Richard et al., 2014; World Health Organisation Western Pacific Region, 2022). Nevertheless, these infections are mostly unable to transmit between individuals, and outbreaks are limited (World Health Organisation Western Pacific Region, 2022). Very rarely, a zoonotic virus adapts to efficiently infect and transmit between humans. When this occurs, the combination of a poorly adapted virus with an antigenically naïve population can result in widespread severe disease (Long, Mistry, et al., 2019). The consequences of such zoonotic events are illustrated by the historical 1918 ‘Spanish flu’ pandemic, as well as the coronavirus pandemic caused by SARS-CoV-2. Over time, pandemic IAV becomes better adapted to human infection, causes milder disease, and eventually replaces or co-circulates with existing seasonal strains (Long, Mistry, et al., 2019).

The host range of IAV is limited by the ability of the virus to efficiently interact with species-specific host factors and conditions. Multiple adapting mutations must be accumulated prior to productive infection of a new species. Key barriers include receptor binding preference (Rogers & Paulson, 1983), efficient polymerase activity (Almond, 1977; Gabriel et al., 2005; Mänz et al., 2012) and evasion of host specific immune defences or restriction factors (Dittmann et al., 2008; Mänz et al., 2013; M. Weber et al., 2015) (reviewed in detail in Long, Mistry, et al., 2019). Nevertheless, the risk of new pandemic strains is significantly increased by the phenomenon of reassortment. Due to the segmented nature of the influenza genome (Section 1.2), if two or more different IAV co-infect the same cell, then new genetic combinations can arise through genome segments from different parental strains combining in progeny virus. When this phenomenon results in viruses with novel antigenicity it is described as antigenic shift (Taubenberger & Kash, 2010). Infection of intermediate hosts that are amenable to both human and avian strains, such as (debatably) pigs (Moncorgé et al., 2013; Nelli et al., 2010; Peacock, Swann, et al., 2020; Vincent et al., 2014; H. Zhang et al., 2020), may be important in driving reassortment (Kida et al., 1994; Webster et al., 1992).

Since the start of the 20th century, there have been five global influenza pandemics with varied origins: 1918, 1957, 1968, 1977 and 2009 (summarised in Figure 2) (Long, Mistry, et al., 2019; Taubenberger & Kash, 2010). It is clear from this historic record that new pandemic IAVs emerge with unpredictable frequency and in disparate and unexpected ways, making it difficult to allocate resources for surveillance and predict the next event (Vincent et al., 2014; Webster & Govorkova, 2014). This links to the complicated ecology of the virus (Webster et al., 1992). In direct contrast, IBV is almost exclusively isolated from humans, and has not been recorded as causing pandemics (R. Chen & Holmes, 2008).

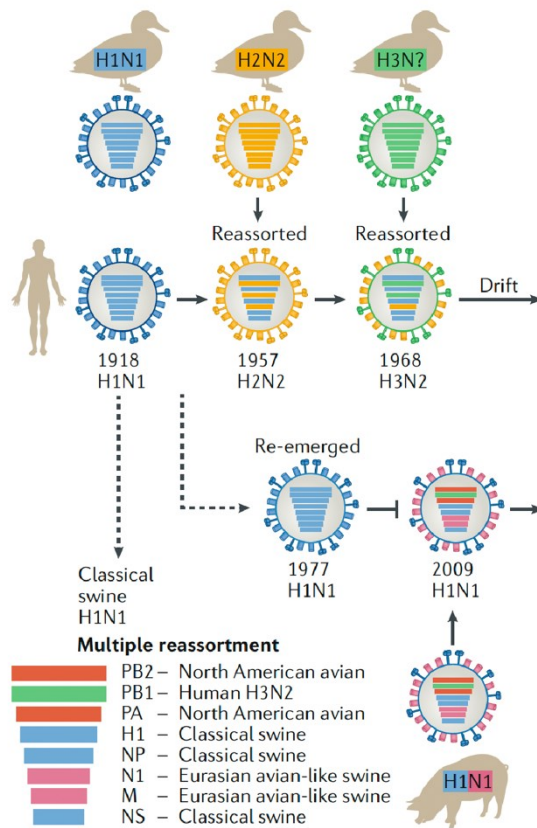


Figure 2: The unpredictable origins of IAV pandemics.

Figure taken from Long, Mistry, et al., 2019. Reproduced with permission from Springer Nature. Since the start of the 20th century there have been five separate IAV pandemics. The origin of the 1918 H1N1 pandemic is unknown but suggested to be avian for all eight segments (Reid et al., 2004). In contrast, both the 1957 H2N2 and 1968 H3N2 pandemics arose through reassortment of a contemporary human-adapted strain with avian influenza viruses. The 1957 H2N2 derived its HA, NA and polymerase basic 1 (PB1) from a Eurasian H2N2 avian strain, and remaining segments from a seasonal H1N1 descended from the 1918 pandemic strain (Kawaoka et al., 1989; Reid et al., 2004; Schafer et al., 1993). Subsequently the 1968 H3N2 derived its HA and PB1 from a further Eurasian avian strain, and remaining segments from a contemporary seasonal H2N2 (Bean et al., 1992; Kawaoka et al., 1989; Reid et al., 2004). The 1977 H1N1 pandemic strain was caused by the re-emergence of a human-adapted strain from the 1950s, to which pre-existing immunity was absent in younger age groups. The source remains unknown (Rozo & Gronvall, 2015; Webster et al., 1992). Finally, the 2009 H1N1 pandemic emerged from pigs and has a very complex lineage including segments with origins from a Eurasian-avian-like lineage of swine influenza, a classical swine virus, an H3N2 human seasonal IAV and a North American avian IAV (Dawood et al., 2009; Vincent et al., 2014).

1.1.3 Therapeutic Options

Vaccination provides the most effective protection against influenza infection (Paules & Subbarao, 2017). However, high antigenic diversity between strains, combined with antigenic drift and shift requires a multi-valent vaccination with annual reformulation. In the event of a pandemic, it is unlikely the existing vaccine would give cross-reactive protection (Kim et al., 2018; Kumar et al., 2018). Furthermore, strain selection is made 6-8 months prior to distribution, thus there is an opportunity for dominant strains to be incorrectly predicted (Webster & Govorkova, 2014). To overcome this issue, there is much interest in the development of a universal influenza vaccine, which would provide cross-reactive protection regardless of subtype and strain (Kim et al., 2018; Kumar et al., 2018).

In the case of a new pandemic, antiviral drugs are likely to be the first line of defence (Hurt, 2014). There are four classes of antiviral drug approved for treatment of influenza: the adamantanes (IAV Matrix 2 (M2) ion channel inhibitors), NA inhibitors (NAIs), membrane fusion inhibitors and RdRp inhibitors (Paules & Subbarao, 2017). Nevertheless, the effectiveness of approved antivirals in treating seasonal influenza is contentious (Paules & Subbarao, 2017), and antiviral resistance emerges readily, limiting long-term efficacy (Hurt, 2014). Thus, there is a need for ongoing research and development into novel broad-acting and effective antivirals (Kotey et al., 2019). Current candidates in clinical trials include monoclonal antibodies for passive immunotherapy and novel viral polymerase inhibitors (Behzadi & Leyva-Grado, 2019; Kotey et al., 2019). There is also interest in targeting host factors essential for viral activity. This approach expands the number of therapeutic targets and may make it more difficult for the virus to evolve resistance (König et al., 2010). Finally, as much of the pathogenesis of severe influenza is triggered by an excessive inflammatory reaction, combining immunomodulatory therapies with antivirals may improve outcomes in severe/at risk cases (Ramos & Fernandez-Sesma, 2015).

Interestingly, non-pharmaceutical interventions enacted during the coronavirus pandemic, such as school closures and social distancing, appeared to significantly suppress seasonal influenza epidemics in several countries (Davis et al., 2022; Fricke et al., 2021; Q. S. Huang et al., 2021; Karlsson et al., 2021), and it has been suggested that the influenza B strain IBV/Yamagata may have been eradicated (Koutsakos et al., 2021). Nevertheless, whether such measures would be effective in response to a novel pandemic strain with different transmission dynamics is unknown (Karlsson et al., 2021; Rashid et al., 2015). Consequently, influenza virus continues to represent a significant threat to global public health and remains an important area of both fundamental and translational research.

1.2 VIROLOGY AND STRUCTURE

1.2.1 The Orthomyxoviridae

IAV and IBV are both single-stranded, negative-sense, segmented RNA viruses belonging to the family Orthomyxoviridae (Bouvier & Palese, 2008). The International Committee on Taxonomy of Viruses (ICTV) currently suggests 9 members of the family (Alphainfluenzavirus (IAV), Betainfluenzavirus (IBV), Gammainfluenzavirus, Deltainfluenzavirus, Isavirus, Mykissvirus, Quaranjavirus, Sardinivirus and Thogotovirus) and 21 individual species (International Committee on Taxonomy of Viruses (ICTV), 2021). IAV, IBV and Influenza C Virus (ICV, Gammainfluenzavirus) infect humans, however only IAV and IBV are associated with severe disease (Paules & Subbarao, 2017). The recently identified Influenza D Virus (IDV, Deltainfluenzavirus) infects cattle, goats and pigs, whilst Isavirus, Sardinivirus and Mykissvirus are pathogens of fish (Batts et al., 2017; Godwin et al., 2020; Plarre et al., 2012; Zhai et al., 2017). Finally, Thogotovirus and Quaranjavirus are arboviruses. Thogotoviruses mainly infect domestic mammals, and Quaranjavirus mainly infect avian species, however both occasionally cause severe disease in humans (R. Peng et al., 2017; Sameroff et al., 2021). Thus, the Orthomyxoviridae represent a family of significant animal and human pathogens.

1.2.2 Structure

All influenza virus form enveloped particles, of pleiomorphic shape. Lab-adapted strains are primarily spherical, with a diameter of ~120nm, but also produce elongated 'baciliform' particles (120-250nm). In addition, clinical isolates form extended particles, known as filaments, which have a narrowed width (~80nm) and can reach several microns in length (Figure 3A) (Dadonaite et al., 2016). It has been suggested that much like genetic diversity, morphological heterogeneity may contribute to viral survival in a fluctuating environment (Vahey & Fletcher, 2019). A schematic illustrating the architecture of a spherical influenza A virus is illustrated in Figure 3B. The viral glycoproteins HA and NA, and the ion channel M2, are embedded in the host-derived lipid membrane, which envelopes a capsid core of viral M1 protein (Bouvier & Palese, 2008). Both IAV and IBV have eight genome segments, which are packaged within the capsid and are proposed to adopt a 7+1 configuration with one segment in the centre, and the others arranged around the outside, as visualised in figure 3C (Noda et al., 2006). The genome segments are named after the main proteins they encode. In order of size, these are: polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase acidic (PA), HA, nucleoprotein (NP), NA, matrix (M) and non-structural (NS) (Bouvier & Palese, 2008). The six largest segments encode a single core protein, whilst the two smallest genome segments (M, NS) encode two core proteins each. The unspliced M and NS segments express M1 and NS1 proteins, respectively, whilst M2 and NS2/Nuclear Export Protein (NEP) are expressed following splicing (Dubois et al., 2014; Mor et al., 2016). In addition, several accessory proteins have been described which are produced via alternative translation initiation sites, ribosomal frameshifts and alternative splicing (Dubois et al., 2014).

Each of the eight genome segments (known as viral RNAs (vRNAs)) are packaged into replicative structures known as viral ribonucleoproteins (vRNPs) (Figure 3D) (te Velthuis & Fodor, 2016). The conserved 13 5' terminal and 12 3' terminal nucleotides of the vRNA constitute the promoter and interact to form a partially double stranded 'hook' structure to which a single molecule of the heterotrimeric RdRp protein (FluPol), consisting of PB2, PB1 and PA, binds (Ferhadian et al., 2018; Pflug et al., 2017; te Velthuis & Fodor, 2016). The remainder of the resulting vRNA loop is coated by multiple copies of NP, and twists into a flexible double-stranded anti-parallel helical structure (10-15 nm wide and 30-120 nm long), with the bound polymerase on one end, and a terminal loop at the other (Arranz et al., 2012; Compans et al., 1972; Moeller et al., 2012; Noda et al., 2006). NP binds RNA without sequence-specificity via the phosphate backbone, leaving the base pairs exposed (Eisfeld et al., 2015). NP binding is non-uniform, with sections of exposed RNA folding into secondary structures (Dadonaite et al., 2019; Eisfeld et al., 2015; le Sage et al., 2018; N. Lee et al., 2017; Williams et al., 2018). These structures may be important for interactions between vRNP segments, and incorporation of the correct complement of genome segments into nascent virions (Dadonaite et al., 2019).

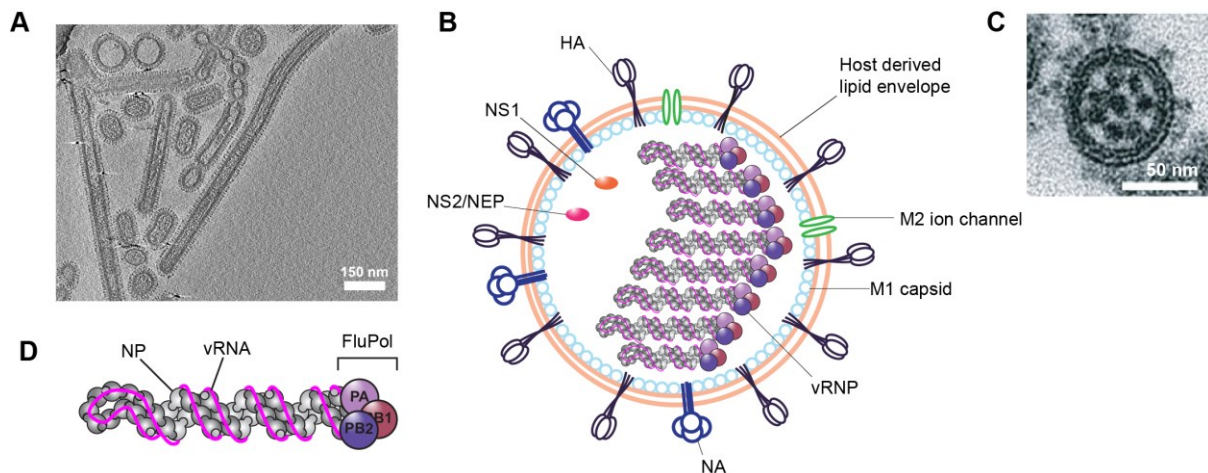


Figure 3: Structure of IAV.

(A) Tomogram demonstrating the diverse morphologies of IAV particles. Image reproduced from Vijayakrishnan et al., 2013. (B) Cartoon depiction of the structure of a spherical IAV particle. The viral surface glycoproteins HA and NA and the ion channel M2 are embedded in the host derived lipid viral envelope, which envelops a capsid core of the M1 protein. The genome consists of eight segments, each packaged as individual replicative complexes known as vRNPs. In addition, the virus packages copies of the NS1 and NS2/NEP proteins. (C) Transmission electron micrograph of a transverse sectioned viral particle, depicting the 7+1 orientation of the vRNPs. Image reproduced from Noda et al., 2006, with permission from Springer Nature. (D) Enlarged cartoon of the structure of a vRNP. The 5' and 3' termini of the vRNA form a partially double stranded 'hook' promoter structure to which a single copy of the heterotrimeric RNA-dependent RNA polymerase (FluPol), consisting of PB1, PB2 and PA, binds. The rest of the vRNA is coated with multiple copies of NP, and twists into a double-stranded helix.

In addition to the genome segments, viral particles have been shown to package NS1, NS2/NEP and an abundance of host proteins (Hutchinson et al., 2014). The majority of these are present at low levels. A minority however, such as ubiquitin, annexins, cytoskeletal proteins, and the influenza restriction factor cyclophilin A, are highly abundant, suggesting they are consistent components of the virion (Hutchinson et al., 2014).

1.2.3 The IAV life cycle

In humans, the primary cellular target of influenza virus are the airway epithelial cells. Immune cells including airway macrophage and dendritic cells are also permissive, however these routes of infection are not thought to be productive (Villalón-Letelier et al., 2017). A brief overview of the influenza A virus life cycle is outlined in Figure 4. Significantly, as an obligate intracellular parasite encoding a minimal number of proteins, influenza is highly dependent on co-opted host proteins and pathways to facilitate its infectious cycle (Peacock et al., 2019). Equally however, many host factors are inhibitory to viral replication (Villalón-Letelier et al., 2017). Adapting to correctly mediate interactions with host-specific factors is a major barrier limiting the emergence of novel zoonoses (Long, Mistry, et al., 2019).

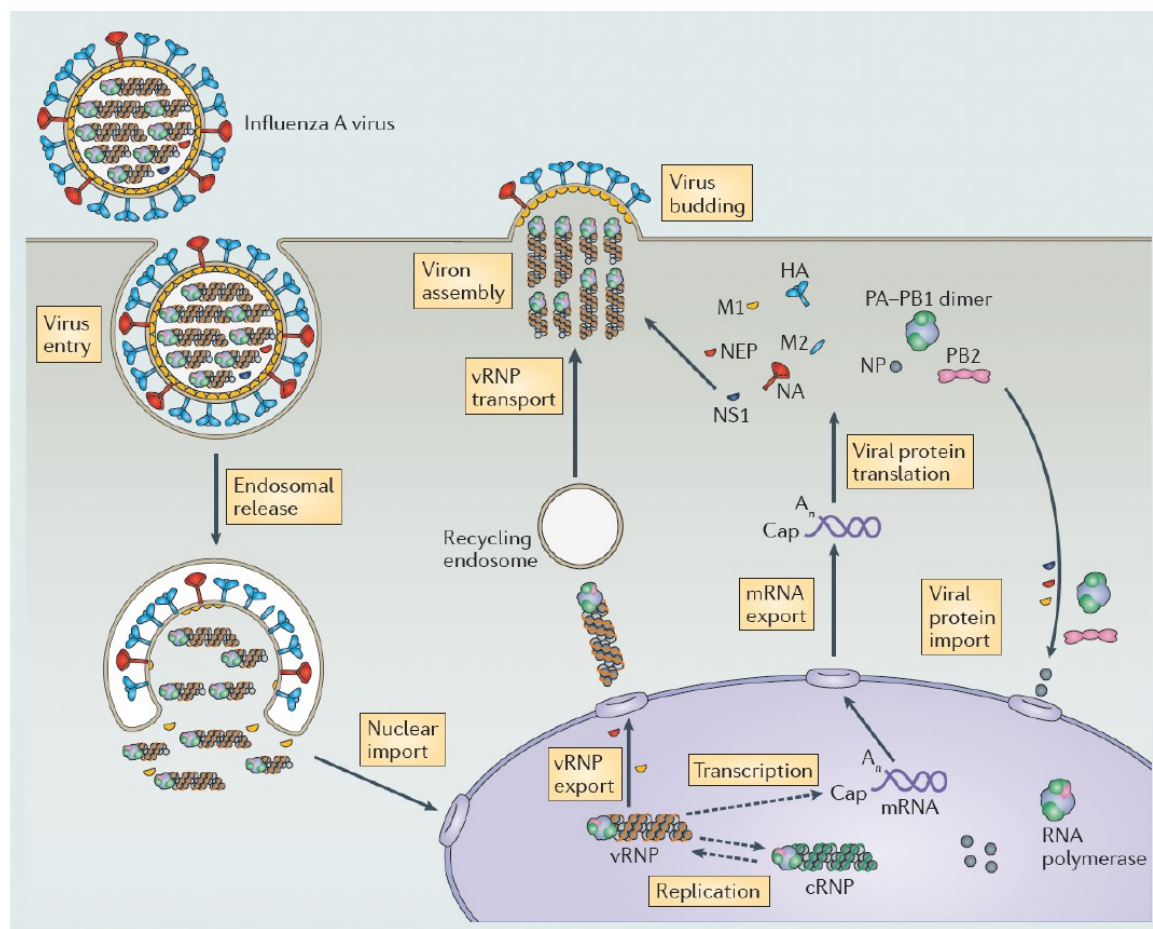


Figure 4: Overview of the influenza A virus life cycle.

Figure reproduced from te Velhuis and Fodor, 2016, with permission from Springer Nature. Infection commences when influenza particles bind sialic acid via the viral attachment protein, HA (Bouvier & Palese, 2008; Long, Mistry, et al., 2019). Following attachment, the virus is primarily internalised by clathrin-mediated endocytosis, although alternative endocytic entry mechanisms such as macropinocytosis may also be utilised (de Vries et al., 2011; Sempere Borau & Stertz, 2021). In early endosomes, the mildly acidic pH triggers opening of the M2 ion channel. An influx of protons and potassium ions acidifies the viral core, which is necessary to release vRNPs from the M1 capsid. As the endosome matures, the lower pH triggers a conformational change in HA, resulting in viral envelope and endosomal membrane fusion and release of the vRNPs into the cytosol (Sempere Borau & Stertz, 2021). Subsequently, the vRNPs are imported into the nucleus, the site of both viral transcription and replication (Section 1.3) via the host importin pathway (Hutchinson & Fodor, 2012). Following transcription, capped and polyadenylated viral

transcripts are exported to the cytoplasm for translation and further processing by the host cell (Eisfeld et al., 2015). Following replication and encapsidation, nascent vRNPs are exported via the host CRM1 export pathway in a mechanism requiring the viral M1 and NS2/NEP protein (Eisfeld et al., 2015). vRNP trafficking to the plasma-membrane occurs in a microtubule dependent manner, associated with Rab11-positive recycling endosomes (Amorim et al., 2011; Eisfeld et al., 2011; Momose et al., 2011). Intersegment vRNA-vRNA interactions are believed to coordinate the correct bundling of a single copy of each of the eight genome segments in each virion (Noda, 2021), although defective particles lacking one or more segments are produced abundantly during infection (Brooke, 2014). The current model of viral budding suggests that HA/NA intrinsically localise to lipid rafts enriched in cholesterol and sphingolipids, where they trigger a local change in membrane curvature that initiates budding (Scheiffele et al., 1999). M1 is recruited to sites of budding by interactions with the HA/NA cytoplasmic tails. Interactions with M1 are thought to recruit both M2 and the vRNP genome bundle. Finally, scission of the membrane is mediated by the M2 ion channel cytoplasmic tail. As the viral particle emerges, NA acts to cleave terminal sialic acids from host membrane proteins, preventing viral aggregation at the surface due to HA binding, and allowing the release of viral progeny (Rossman & Lamb, 2011). The final stage of the viral life cycle is onwards transmission to a new host. Influenza virus is believed to be spread through fomites/direct contact, large respiratory droplets generated by coughing and smaller droplets described as aerosols (C. C. Wang et al., 2021; T. P. Weber & Stilianakis, 2008).

1.3 THE INFLUENZA POLYMERASE (FLUPOL)

1.3.1 Introduction

The influenza RdRp (FluPol) is the single enzyme responsible for both viral transcription and genome replication, producing viral mRNAs, complementary RNAs (cRNAs) (the positive-sense genome intermediate, Section 1.3.4) and nascent vRNA genomes from each segment (te Velthuis & Fodor, 2016). Moreover, FluPol is an important determinant of host restriction (Naffakh et al., 2008), and a proven drug target (Furuta et al., 2017; Hayden et al., 2022; Kiso et al., 2010). Therefore, increasing our understanding of this vital component of the virus promises multiple translational benefits.

FluPol activity can be effectively studied using a range of approaches, ranging from *in vitro* biochemical analyses using purified proteins, through mini genome assays (also known as mini replicon systems or polymerase assays), to assessment of the accumulation of vRNA, cRNA and mRNA in full infections in cells (Table 1) (te Velthuis et al., 2018). All three FluPol activities (vRNA, cRNA and mRNA synthesis) are inherently interdependent: any reduction in replication reduces the amount of template for secondary transcription, whilst any reduction in transcription reduces the amount of nascent viral proteins for packaging vRNA/cRNA into RNPs. Consequently, reductive approaches such as mini genome and *in vitro* assays have proven instrumental in unpicking the precise activity that certain residues or host factors support (te Velthuis et al., 2018). Many of the insights discussed in the following sections have been obtained using these approaches. Nevertheless, it is important to caveat that the role of co-opted host factors is not captured in a standard *in vitro* assay. Although host factor interactions can be measured in mini genome assays, other viral proteins are implicated in replication that are not expressed in this system, and it is known that the dynamics differ from full infections (Robb et al., 2009). Therefore, studying full infectious virus remains essential for a complete understanding of replication.

Approach	Summary	Advantages	Disadvantages	References
<i>In vitro</i> replication assays	-Biochemical assays measuring purified FluPol activity	-Addition of different promoters (cRNA vs vRNA) and inclusion/exclusion of a capped primer source allow vRNA, cRNA and mRNA synthesis to be effectively measured in isolation -Non-infectious. FluPol from highly pathogenic strains can be studied without the need for high containment facilities -Can be used to study protein and promoter mutants that would be lethal in the context of full infectious virus	-Lose the effect of host factors and spatial and kinetic effects that occur in the context of the cell. Additional viral factors implicated in replication are not typically present in this system -Advanced biochemical assays requiring work with radioactivity, low-throughput	(te Velthuis et al., 2016, 2018)
Mini genome (mini replicon/ polymerase) assays	- <i>In situ</i> assays in which artificial viral-like reporter RNAs are co-expressed in cells alongside PB1/PB2/PA and NP to reconstitute a vRNP expressing the reporter protein of choice -Alternative versions of this assay can be undertaken in which FluPol is provided <i>in trans</i> by infection rather than expressed proteins	-Semi-high-throughput, reporters such as firefly luciferase or GFP can be read using a microplate reader -Reconstitution within the context of the cellular environment – captures the effects of host factors, and possibly spatial effects -Can be performed in cell types from many different species -vRNA, cRNA and mRNA outputs can be specifically measured using RT-qPCR or primer extension analysis (chapter two) -Non-infectious. FluPol from highly pathogenic strains can be studied without the need for high containment facilities -Can be used to study protein and promoter mutants that would be lethal in the context of full infectious virus -Short RNA reporters (≤ 76 nt) can be used to study FluPol activity in the absence of NP -Variations on the assay e.g., using known transcription/replication mutant proteins or RNA promoters, can be used to untangle the effect of specific conditions or mutations on each FluPol activity	-Overexpression assay - may not accurately reflect the spatial and kinetic effects at play during infections -Viral proteins excluded from the assay that are known to play a role in the regulation of replication. Kinetics differ from those seen in authentic infection	(T. S. Huang et al., 1990; Neumann et al., 1994; Nilsson-Payant et al., 2021; Robb et al., 2009; te Velthuis et al., 2018; Turrell et al., 2013)
cRNP stabilisation/ replication assays (see chapter two)	-Infectious assays in which conditions are manipulated so that transcription and replication are decoupled. In a cRNP stabilisation assay vRNA synthesis is blocked, whereas in a replication assay	-Replication assays allow transcription and replication to be decoupled within the context of a cell and authentic infection -cRNP stabilisation assays allow the pioneering round of cRNA synthesis to be studied in the absence of vRNA synthesis, within the context of a cell and authentic infection	-RNP components are over-expressed - may not accurately reflect the spatial and kinetic effects at play during infections -ActD treatment inhibits cellular transcription whilst CHX treatment	(Mänz et al., 2012; Nilsson et al., 2017; Vreede et al., 2004)

	multiple rounds of replication can occur. Cells are first pre-transfected with RNP components, then treated with either ActD (blocks viral transcription so only replication occurs) or CHX (blocks translation so primary transcription still occurs), followed by infection		inhibits cellular translation. May alter the way the cell interacts with the virus -Viral proteins that are known to be important for viral replication are not pre-expressed in this assay	
Full infections followed by RT-qPCR, primer extension or InVERT RNAseq analysis of viral RNA species (see chapter two)	-Accumulation of FluPol products at different timepoints during infection can be measured using either RT-qPCR or primer extension analysis targeting the vRNA, cRNA and mRNA of a specific segment, or using the InVERT RNAseq pipeline to quantify the vRNA, cRNA and mRNA of all eight genome segments simultaneously	-Most accurate recapitulation of a natural infection of a cell – all viral and host factors present, spatial and kinetic factors captured	-Requires access to containment facilities -Interdependence of FluPol activity complicates mechanistic understanding -Cannot study mutants that prevent production of viable virions, unless grown in cells that complement the deficiency	(Kawakami et al., 2011; Luo et al., 1992; Phan et al., 2021; te Velthuis et al., 2018)

Table 1: Key approaches for studying FluPol activity.

nt= nucleotide; ActD= actinomycin D; CHX= cycloheximide; InVERT= Influenza Virus Enumerator of RNA Transcripts

1.3.2 Structure of the trimeric FluPol

In the last decade, technical advances have allowed high resolution structures of the RdRps from several negative-sense single-stranded RNA viruses (NSVs) to be obtained, and both structural and conceptual parallels have been noted (te Velthuis et al., 2021). For influenza, the FluPol complex is an ~270 kDa heterotrimer consisting of the three largest viral proteins: PB2, PB1 and PA (Pflug et al., 2017). The structure of the FluPol from bat (H17N10 (Pflug et al., 2014; Wandzik et al., 2020)), human (H3N2 (Fan et al., 2019), H1N1 (Keown et al., 2022)) and avian (H5N1 (Fan et al., 2019)) isolates of IAV, IBV (Kouba et al., 2019; Reich et al., 2014, 2017; Thierry et al., 2016), ICV (Carrique et al., 2020; Hengrung et al., 2015) and IDV (Q. Peng et al., 2019) have been obtained in various configurations. There is significant structural similarity, despite sequence divergence.

The influenza polymerase adopts a globular structure, comprising a relatively invariable core catalytic region, attached to highly dynamic, flexible accessory domains (Figure 5A) (Pflug et al., 2017). The key catalytic domain is encoded by PB1 and adopts a canonical RdRp right-hand-like fold (Pflug et al., 2014; Reich et al., 2014). In addition, the thumb subdomain encodes a 'priming loop' which extends into the catalytic site, and likely supports the initiating nucleotide during terminal *de novo* initiation (Section 1.3.4) (Pflug et al., 2014; te Velthuis et al., 2016). The PB1 structure is stabilised by considerable interactions with the C-terminal and linker domains of PA (PA-C), plus the N terminal third of the PB2 subunit (PB2-N). When in complex, the vRNA promoter has been shown to adopt a 'hook' conformation, with a folded stem loop at the 5' end, a single stranded region at the 3' end and a base paired region between the distal parts of the 5' and 3' promoters (Figure 5B). The 5' stem loop binds tightly to a pocket between the PB1 and PA subunits, whilst the single stranded 3' region is inserted into the template entrance (Pflug et al., 2014; Reich et al., 2014, 2017). The flexible accessory domains consist of the N-terminal PA endonuclease, plus the remaining C-terminal portion of PB2 (PB2-C) encoding the cap binding, mid-link, 627 and nuclear localisation signal (NLS) domains (Figure 5A) (Pflug et al., 2017). Different orientations of the accessory domains have been observed in the available structures, which are thought to reflect different functional states of the enzyme (te Velthuis et al., 2021). Initially two key configurations were described: the transcriptionally active 'open' structure (Figure 5C) and the transcriptionally inactive 'closed' structure (Figure 5D). In the transcriptionally active grouping, the cap-binding domain and the endonuclease domain are favourably orientated to allow cap-snatching to occur (Section 1.3.3) (Pflug et al., 2014, 2017; Reich et al., 2014). In the transcriptionally inactive 'closed' structure, the endonuclease and cap-binding domain face away from each other, whilst a portion of the mid-link domain blocks the cap-binding pocket (Fan et al., 2019; Hengrung et al., 2015; Keown et al., 2022; Thierry et al., 2016). Thus, this structure is incompatible with cap snatching and therefore transcription (Hengrung et al., 2015). Recently, the transcriptionally inactive 'closed' structure has been implicated in replication (Carrique et al., 2020; Fan et al., 2019), and is increasingly referred to as a 'replicase' (Carrique et al., 2020; Keown et al., 2022).

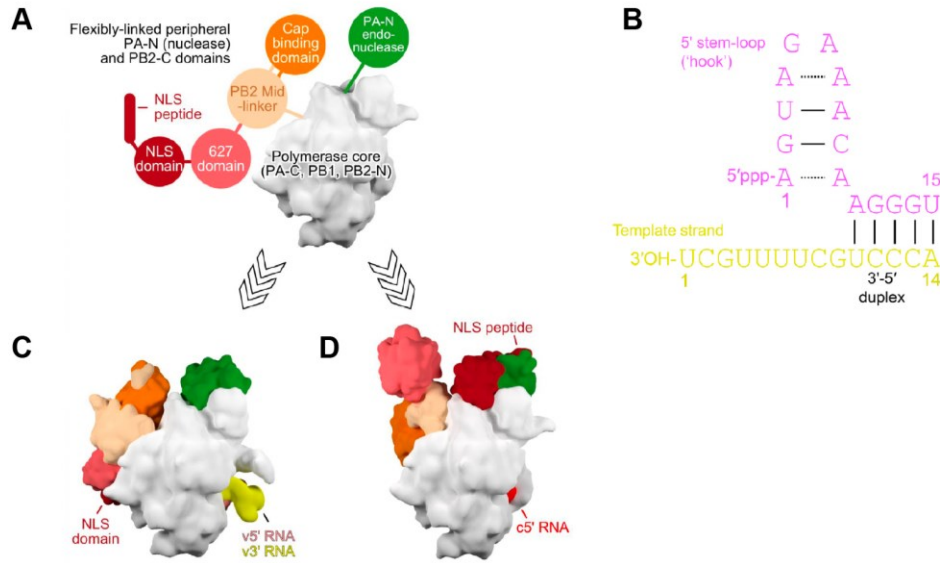


Figure 5: Key FluPol structures.

Figure adapted from Pflug et al., 2017. (A) The influenza polymerase adopts a globular structure and can be considered as an invariable core attached to highly dynamic, flexible accessory domains. The key catalytic domain is encoded by the PB1 segment, stabilised by considerable interactions with PA-C and PB2-N (Pflug et al., 2014; Reich et al., 2014; te Velthuis et al., 2021). All three subunits contribute to the four tunnels leading into the main catalytic site, which correspond to template entry, NTP entry, template exit and product exit (Pflug et al., 2017; te Velthuis et al., 2021). Core structure is IBV co-purified with the 5' end of the cRNA promoter (PDB:5EPI). (B) Crystal structures of the FluPol enzyme in complex with the vRNA promoter suggest that the promoter adopts a 'hook' confirmation. (C) The transcriptionally active 'open' FluPol structure, shown here as IBV FluPol co-crystallised with the full vRNA promoter (PDB:4WSA). The PA-N endonuclease domain (green) and PB2-C cap binding domain (orange) are orientated together in a structure presumed to be favourable for transcription. (D) The 'closed' replicase structure, shown here as IBV co-purified with the 5' end of the cRNA promoter (PDB:5EPI). The dramatic rearrangement of the flexible domains can be observed, with the cap binding domain (orange) now orientated away from the endonuclease domain (green).

Small angle X-ray scattering and crosslinking mass spectrometry demonstrate that many further orientations exist in equilibrium in solution (Thierry et al., 2016). Accordingly, recent publications have identified additional structures by stalling FluPol at different stages of transcription (Kouba et al., 2019; Wandzik et al., 2020). Other structural studies have highlighted that FluPol can form higher order oligomers (Carrique et al., 2020; Chang et al., 2015; Fan et al., 2019), as suggested by previous biochemical and imaging analysis (Huet et al., 2010; Jorba et al., 2008). The biological significance of a tetrameric structure (Chang et al., 2015) has not been established, however two different dimeric forms (Carrique et al., 2020; Fan et al., 2019) appear to be required for genome replication (Section 1.3.4). Interestingly, the two dimers are not mutually exclusive, and both dimers could combine to form a FluPol trimer (Carrique et al., 2020). Trimers can be detected during gel filtration analysis of purified FluPol (Jorba et al., 2008), however whether such structures form *in vivo* has not been confirmed.

1.3.3 Transcription

Following nuclear import, the negative-sense vRNA genome first undergoes primary transcription, to produce capped and polyadenylated (polyA) viral mRNAs. This process is necessarily independent of any viral proteins produced *de novo* and is driven by the packaged FluPol acting in *cis* (Eisfeld et al., 2015; te Velhuis & Fodor, 2016). The incorporation of a 5' N7-methyl guanosine (m⁷G) cap and 3' polyA tail results in viral mRNAs that resemble host transcripts, and can therefore enter endogenous pathways for processing, nuclear export, and translation (Walker & Fodor, 2019). Influenza does not encode enzymes for capping its own mRNAs, and instead steals caps from host transcripts in a process known as 'cap snatching' (Plotch et al., 1981). Host caps are bound by the PB2 cap binding domain and cleaved 8-15nt downstream by the PA endonuclease, preferentially after a guanine residue (Datta et al., 2013; Plotch et al., 1981). It has been suggested that small non-coding RNAs (snRNAs/snoRNAs) are preferentially snatched (Gu et al., 2015; Koppstein et al., 2015), although this may be due to abundance rather than selection (Sikora et al., 2017). Regardless, the resulting capped RNA fragments are subsequently used as primers to initiate viral transcription (te Velhuis & Fodor, 2016). Thus viral mRNAs contain a short host derived RNA sequence at the 5' end. These host sequences may provide an additional benefit to the virus through providing alternative open reading frames that expand the viral proteome (Ho et al., 2020). Cleaving host transcripts may also contribute to host transcriptional shut off (Bauer et al., 2018).

The process of cap-snatching and transcription relies on FluPol binding to the C-terminal domain (CTD) of host RNA polymerase II (Pol II) (Figure 6A) (Serna Martin et al., 2018; te Velhuis et al., 2021). The Pol II CTD is a regulatory domain consisting of 57 reversibly phosphorylated heptad repeats with the consensus sequence Y₁S₂P₃T₄S₅P₆S₇. The serine, threonine and tyrosine residues are differentially phosphorylated through host transcription, allowing different cellular factors to be recruited at specific times (Palancade & Bensaude, 2003). FluPol specifically binds to the serine-5 phosphorylated form (Ser5P CTD), which associates with host transcription initiation (Engelhardt et al., 2005). In IAV, two CTD binding sites have been identified that both localise to the PA C-terminal domain (Lukarska et al., 2017). Other host proteins/complexes, such as the RNA exosome and CDK9, may enhance targeting to Pol II transcription initiation sites (Rialdi et al., 2017; Walker & Fodor, 2019; J. Zhang et al., 2010). Binding Ser5P CTD appears to stabilise the transcriptionally active FluPol structure (Serna Martin et al., 2018); likely allows FluPol to snatch caps before they are sequestered by host cap binding proteins (Engelhardt et al., 2005; Fodor & Vreede, 2010); may aid access to other host factors such as splicing factors (Fodor & Vreede, 2010); and is thought to facilitate host transcriptional shutoff (Chan et al., 2006; Rodriguez et al., 2007; Vreede et al., 2010).

Recent publications have described the precise conformational changes that occur throughout transcription, from pre-initiation through to termination (Figure 6) (Kouba et al., 2019; Wandzik et al., 2020). As elongation progresses, the vRNA emerges from the template exit channel, and is sequestered by a secondary 3' binding site where it is protected from degradation by host exonucleases. The rest of the vRNA loops out of the polymerase as transcription continues (Wandzik et al., 2020). It has been

speculated that NP, which must peel off the vRNA as it enters the FluPol, may be transferred to the outgoing, transcribed template, as it loops out the other side, in a regulated manner (Wandzik et al., 2020). Such a mechanism is consistent with cryo-electron microscopy (cryo-EM) data of transcribing vRNPs, which show that a double helical structure is maintained either side of the FluPol whilst it appears to slide along the helix (Coloma et al., 2020). At termination, polyadenylation of transcripts is achieved by stuttering of the FluPol on a poly-U tract at the 5' end of the coding region (X. Li & Palese, 1994; Poon et al., 1999; Robertson et al., 1981; Wandzik et al., 2020). Eventually, the weak U-A template-product duplex dissociates, and the product mRNA is released (Wandzik et al., 2020).

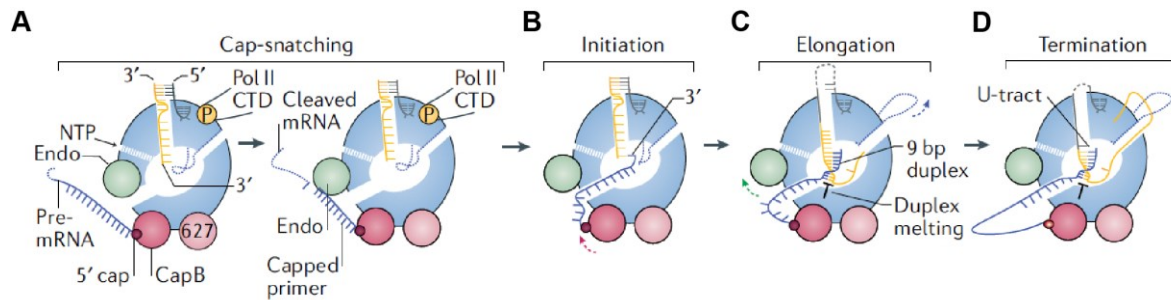


Figure 6: The structural basis of transcription.

Figure from te Velthuis, Grimes and Fodor, 2021. Reproduced with permission from Springer Nature. (A) Cap snatching. Following binding of the host cap by the PB2 cap binding domain (CapB), the PA endonuclease (Endo) cleaves the host transcript 10-15nt downstream of the 5' cap (Datta et al., 2013; Plotch et al., 1981). It is thought that primers with complementary ends to the 3' template (i.e., ending in AG) are preferentially selected (Pflug et al., 2017). Binding to serine-5 phosphorylated host Pol II CTD likely enables privileged access to newly synthesised host capped RNAs. (B) Initiation. Following cap snatching, CapB rotates 70° to insert the end of the cleaved capped primer into the active site (Reich et al., 2014). For the template vRNA to enter the active site, the priming loop must be partially extruded. Initial hybridisation between the primer and vRNA template can then occur, allowing incorporation of the first few nucleotides (Kouba et al., 2019). (C) Elongation. During the transition from pre-initiation to elongation, several key structural changes occur, including full extrusion of the priming loop from the active site to open the template exit channel, and melting of both the promoter and the initial primer-template duplexes, to allow translocation of the product and vRNA template through their respective exit channels. Separation of the primer-template duplex appears to be driven by an aromatic residue in the lid subdomain of PB2-N, which is present at the end of the active site and prevents growth of the duplex beyond 9 bp. Melting of the vRNA promoter is associated with structural changes that narrow the initial template entry channel, thus disfavours backtracking, reformation of the promoter and the production of abortive products (Kouba et al., 2019; Wandzik et al., 2020). (D) At termination, polyadenylation of transcripts is achieved by stuttering of the FluPol on a poly-U tract present starting at position 17 from the 5' end of the coding region (Poon et al., 1999; Robertson et al., 1981; Wandzik et al., 2020). Stuttering is facilitated by the 5' promoter hook remaining bound to the outside of the FluPol, resulting in tension in the template (Wandzik et al., 2020). Eventually, the weak U-A template-product duplex dissociates, and the product mRNA is released (Wandzik et al., 2020). Release of the cap from CapB is thought to occur earlier during elongation, potentially due to the force generated by the bulging of the nascent product as it exits the FluPol (Braam et al., 1983; Kouba et al., 2019). Multiple rounds of transcription can be driven from a single vRNP (Vreede et al., 2004). Therefore, the vRNP can recycle back into a pre-initiation state. However, following product release the template is trapped, bound at the 5' and 3' ends and looped through the template exit channel. The proposed model for recycling suggests that the template exit channel is opened through a dramatic conformational change of the PB1-C and PB2-N domains. This would allow the vRNA template to leave the channel without unbinding the 3' or 5' ends, again avoiding exposure to exonuclease degradation. The 3' template then transfers back to its initial binding site, potentially driven by the FluPol re-closing the template exit channel (Wandzik et al., 2020).

1.3.4 Genome replication

Genome replication occurs later in infection, once nascent RNP components have accumulated within the nucleus, and is a two-step process. First, negative-sense vRNA is copied into a full-length positive-sense genome intermediate known as the complementary RNA (cRNA), which is packaged into analogous replicative complexes known as complementary RNPs (cRNPs). cRNA is then copied back into full-length nascent vRNA (te Velthuis et al., 2021). Unlike transcription, both vRNA and cRNA synthesis use a primer-independent mechanism. Such an approach is inherently less efficient than primed RNA synthesis, and consequently replication is less efficient than transcription (Reich et al., 2017). The initiation strategy for cRNA synthesis and vRNA synthesis differ (Deng et al., 2006). cRNA synthesis undergoes *de novo* terminal initiation at position 1 and 2 at the 3' end of the vRNA template (Deng et al., 2006). This requires the first two nucleotides (ATP and GTP) to assemble in the catalytic site opposite the template, which is thought to be stabilised by the priming loop present in the active site (te Velthuis et al., 2016). In contrast, vRNA synthesis undergoes internal initiation at position 4 and 5 in the 3' end of the cRNA template, to generate a short dinucleotide primer which is subsequently realigned to the 3' end of the template in a mechanism described as primer-realignment (Deng et al., 2006). Internal initiation requires the 3' end of the template to extend further into the entry channel. This may be possible due to differences in the vRNA vs cRNA promoter secondary structure (Oymans & te Velthuis, 2018).

Accumulation of cRNA and vRNA does not occur in the absence of newly synthesised RNP components (Vreede et al., 2004). Moreover, electron micrographs of RNPs appear to show short branching intermediates, in which smaller RNPs bud from larger full-length RNPs (Moeller et al., 2012). Such images are suggestive of co-replicative encapsidation, a mechanism that is likely important to prevent the degradation of uncapped viral products (Eisfeld et al., 2015). Recently, a novel asymmetric FluPol dimer structure was solved by cryo-EM, which suggests a mechanism for co-replicative encapsidation (Figure 7A) (Carrique et al., 2020). In this structure, the leucine-rich repeat (LRR) domain of the essential pro-viral host factor ANP32 bridges two ICV FluPol complexes. The vRNA-promoter bound FluPol, described as the replicating polymerase (FluPol_R), adopts a replicase structure whilst the additional apo-enzyme adopts a novel configuration. Based on the alignment of the 5' cRNA binding site of the apo-enzyme with the product exit channel of FluPol_R – i.e., poised to bind the nascent RNA product as it is extruded – this configuration was described as an encapsidase (FluPol_E) (Carrique et al., 2020). A subsequent study has suggested that the flexible low-complexity acidic region (LCAR) domain of ANP32, which is unresolved in the structure, may act to recruit nascent NP to the progeny RNA, a role that is carried out by the virally encoded P protein in non-segmented NSVs (F. Wang et al., 2022). Consequently, the ANP32-stabilised dimer structure may represent a replication platform, which ensures efficient assembly of progeny RNA into RNPs. As both vRNA and cRNA synthesis require encapsidation, such a structure is likely significant for both vRNA and cRNA synthesis (Carrique et al., 2020). Further details of the host factor ANP32 and the asymmetric dimer are discussed in Section 1.4.

Primer realignment, and therefore vRNA synthesis, is dependent on a conformational change in the active site priming loop, which is mediated by binding of an additional regulatory FluPol (Figure 7B) (Fan et al., 2019; Oymans & te Velthuis, 2018). The resulting dimer is symmetric, with both the replicating and regulatory FluPol adopting a replicase-like configuration mediated by interactions between PA-C, PB2-N and the thumb subdomain of PB1 (Fan et al., 2019). Disruption of this dimer reduces vRNA, but not cRNA or mRNA, synthesis, and results in incorrectly primed, short replication products from a cRNA template, consistent with disruption of primer-realignment (Fan et al., 2019). Moreover, such a mechanism is supported by *in vitro* analysis demonstrating that purified cRNPs, but not vRNPs, require the presence of additional purified FluPol for replication. Importantly, this additional ‘*trans*-activating’ polymerase (FluPol_T) stimulates vRNA synthesis even if catalytically inactive, in agreement with a regulatory rather than enzymatic role (York et al., 2013).

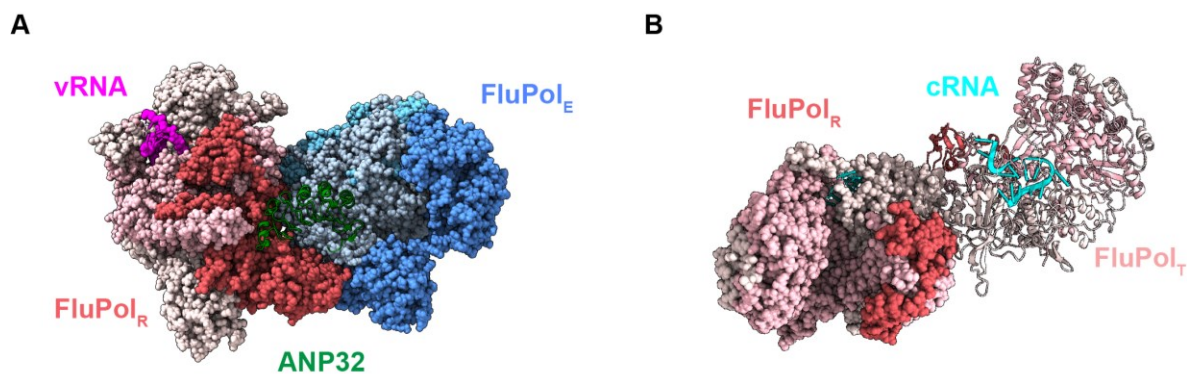


Figure 7: FluPol dimer structures.

(A) Cryo-EM structure of the chicken ANP32A-stabilised asymmetric ICV FluPol dimer structure with short 47 nt vRNA fragment (Protein Data Bank (PDB) accession code:6XZR) (Carrique et al., 2020). (B) Cryo-EM structure of the symmetric IAV (H3N2) dimer structure with cRNA promoter (PDB accession code:6QX8) (Fan et al., 2019). Both FluPol protomers adopt a replicase-like structure bound to cRNA, thus have been arbitrarily labelled FluPol_R and FluPol_T (*trans*-activating FluPol). Structural models were prepared with ChimeraX (Pettersen et al., 2021).

A summary of the known structural details of cRNA and vRNA synthesis is depicted in Figure 8. The symmetric and asymmetric dimer structures are compatible however it is unknown whether they combine to create a trimer during vRNA synthesis. Alternatively, it is equally unknown whether the roles of FluPol_T and FluPol_E are undertaken sequentially by the same protomer. Indeed, a detailed structural description of the conformational changes that occur as vRNA or cRNA synthesis move from initiation, into elongation, reaching termination and recycling back to initiation, has not yet been achieved. It is likely there is some overlap with transcription (te Velthuis et al., 2021). Equally, key differences must occur, for example to prevent polyadenylation of cRNA transcripts and ensure full replication including the 5' promoter. How such activity is regulated, and how hybrid mRNA/cRNA transcripts are avoided, requires further investigation.

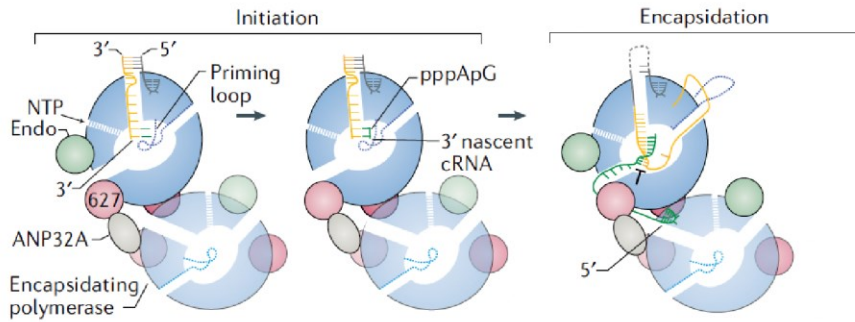
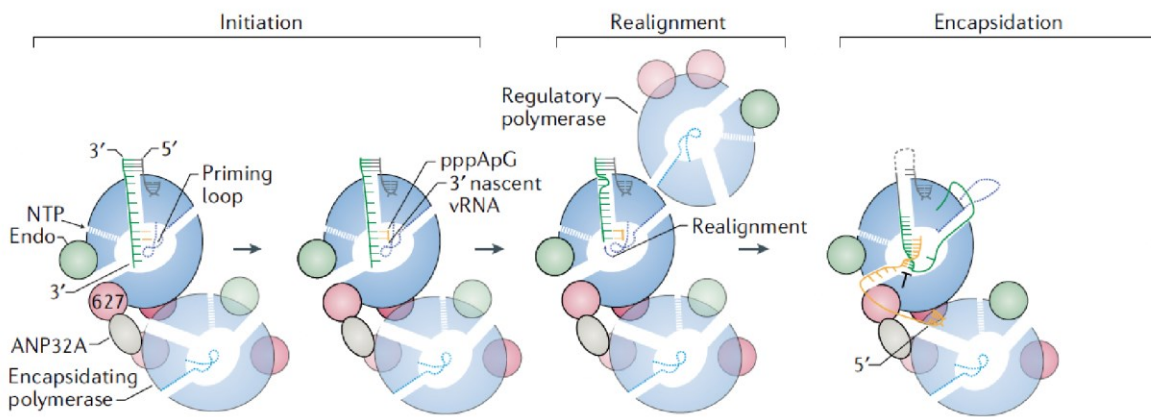
A**B**

Figure 8: The structural basis of replication.

Figure is adapted from te Velthuis, Grimes and Fodor, 2021, and reproduced with permission from Springer Nature. Replication is a two-step process in which the vRNA is first copied into a full-length positive-sense genome intermediate, the cRNA (A), then copied back into nascent negative-sense vRNAs (B). (A) Structural details of cRNA synthesis. cRNA synthesis involves *de novo* terminal initiation at position 1 and 2 at the 3' end of the vRNA template (Deng et al., 2006). The priming loop present in the FluPol active site is believed to stabilise the first two nucleotides (ATP and GTP) within the catalytic site opposite the template (te Velthuis et al., 2016). At some point, possibly during initiation, the encapsidating FluPol binds to the replicating FluPol in an asymmetric dimer structure stabilised by the host factor ANP32, which is thought to be required for co-replicative encapsidation into cRNPs. (B) Structural details of vRNA synthesis. Unlike cRNA synthesis, vRNA synthesis undergoes internal initiation at position 4 and 5 in the 3' end of the cRNA template, to generate a short dinucleotide primer. Internal initiation requires the 3' end of the template to extend further into the entry channel to align the 4-UC-5 with the active site, which may be possible due to differences in the cRNA promoter secondary structure (Oymans & te Velthuis, 2018). The dinucleotide primer is subsequently realigned to the 3' end of the template in a mechanism described as primer-realignment (Deng et al., 2006), which depends on a conformational change in the priming loop triggered by the binding of an additional *trans*-activating regulatory FluPol which forms a symmetric dimer structure (Fan et al., 2019). Formation of the asymmetric dimer stabilised by ANP32 is also thought to be required for vRNA synthesis, although this structure is yet to be observed on a cRNA promoter. Whether both dimers form simultaneously during vRNA synthesis as a trimer of FluPol heterotrimers remains an open question. Endo= endonuclease domain; 627= 627 domain.

1.3.5 Regulation of polymerase activity

The mechanisms of mRNA, cRNA and vRNA synthesis differ, yet are undertaken by the same FluPol enzyme. Structural evidence suggests this functional flexibility is possible due to dramatic rearrangements of the flexible accessory domains (te Velthuis et al., 2021). However, how these are regulated to ensure timely transcription and replication is not fully understood. Viral mRNA accumulates very early in infection, whilst replication products accumulate later (Olson et al., 2010; Shapiro et al., 1987; Vreede et al., 2004). A simple model has been proposed, in which transcription and replication occur stochastically from the start of infection, however in the absence of nascent encapsidating RNP proteins the naked cRNA is degraded. In contrast, the capped and polyadenylated viral mRNA is protected from host nucleases (Vreede et al., 2004; Vreede & Brownlee, 2007). Alternatively, FluPol may initially act in a transcription mode before a regulated switch to replication. The recent discovery that higher order FluPol complexes (dimers, and possibly trimers), are required for both cRNA and vRNA synthesis (Carrique et al., 2020; Fan et al., 2019; Nilsson-Payant et al., 2021) suggests a model in which replication is triggered when sufficient nascent viral proteins have accumulated in the nucleus to allow such complexes to form. Such a mechanism would presumably be advantageous through avoiding the production of naked RNA that could stimulate the host immune response and agrees with the observation that increasing FluPol concentration enhances replicative activity (Olson et al., 2010). Maintaining the correct balance of dimerization is certainly important for productive replication, as a reassortant virus demonstrating increased dimerization was found to be attenuated, and serial passage rapidly selected for destabilising mutations in the symmetric dimer interface (K. Y. Chen et al., 2019). It has also been suggested that saturation of Pol II Ser5P CTD sites, by the combined effects of an increasing concentration of trimeric FluPol alongside infection-driven Pol II degradation, may be an important factor in the transition from transcription to replication (Fodor & Vreede, 2010; Lukarska et al., 2017). Host transcriptional shut-off may also contribute through reducing the concentration of capped mRNAs present in the nucleus, another factor which enhances transcription (Olson et al., 2010).

Levels of vRNA and cRNA synthesis frequently do not correlate, whilst the eight genome segments demonstrate different transcription/replication dynamics over time - implying additional layers of regulation (Phan et al., 2021). Several viral factors including NP, M1, NS1, NS2/NEP and vRNA/short viral RNAs have been implicated (Hale et al., 2008; Hankins et al., 1990; Heldt et al., 2012; Olson et al., 2010; Perez et al., 2010, 2012; Portela & Digard, 2002; Robb et al., 2009). Moreover, different proviral host factors are specifically associated with either vRNA, cRNA or mRNA synthesis. For example, the splicing factor proline-glutamine rich (SFPQ) protein has been shown to support transcription through enhancing the polyadenylation of viral mRNAs (Landeras-Bueno et al., 2011), whilst the mini chromosome maintenance (MCM) complex promotes cRNA synthesis (Kawaguchi & Nagata, 2007) (for a full review of proviral host factors associated with FluPol activity see Peacock et al., 2019). How the virus co-ordinates these complex interactions is yet to be elucidated (Peacock et al., 2019), although it is likely that post-translational modifications, of both viral and host proteins play a role (Dawson et al., 2020; Günl et al., 2022; Hu et al., 2020). Segment-specific dynamics can, at least partially, be attributed to differences in the promoter region. Although the promoters are highly

conserved across strains and segments, there are two types – either a C or a U at position 4 of the 3' vRNA promoter (G or A in the 5' cRNA promoter) (Robertson, 1979). The C4 promoter is typically associated with the polymerase segments, although in some strains it is also found in the NA and M segment (K.-H. Lee & Seong, 1998). U4 promoters show enhanced transcription with delayed replication, whilst C4 promoter segments demonstrate the reverse pattern, and this correlates with the amount of viral protein detected in infected cells (K.-H. Lee & Seong, 1998). Moreover, additional segment- and strain- specific non-coding regions adjacent to the 5' and 3' promoter sequences alter the relative efficiency of transcription vs replication (Ferhadian et al., 2018; J. Wang et al., 2017). Finally, it has been proposed that short viral RNAs may act to promote vRNA synthesis in a segment-specific manner (Perez et al., 2012).

1.3.6 Host restriction

The influenza polymerase is an important determinant of host range (Long, Mistry, et al., 2019): FluPol from most avian influenza isolates replicates inefficiently in mammalian cells, and mutations that increase activity are a prerequisite for adaptation (Cauldwell et al., 2014). Adaptive mutations have been identified on all three polymerase proteins, as well as NP (Cauldwell et al., 2014; Naffakh et al., 2008). Adaptive mutations in NS2/NEP have also been shown to enhance replication of avian FluPol in mammalian cells, which may link to a regulatory role in replication (Mänz et al., 2012).

The dominant determinant of host restriction is the PB2 protein, and the archetypal adaptive mutation is from a glutamic acid to a lysine at position 627 (hereafter referred to as FluPol 627E or FluPol 627K) (Almond, 1977; Subbarao et al., 1993). This single amino acid switch is sufficient for otherwise unadapted avian FluPol to replicate to high titres in mammalian cells and is also associated with increased virulence (Fouchier et al., 2004; Hatta et al., 2001; Subbarao et al., 1993). Approximately one third of H5N1 strains isolated from infected humans contain this genetic signature (Long et al., 2013), as do the majority of human H7N9 isolates (Q. Liu et al., 2013; Quan et al., 2018) and all four pandemic strains of the 20th century (Cauldwell et al., 2014). Nevertheless, a proportion of mammalian isolates retain the avian-like PB2 627E signature, including the 2009 pandemic lineage, indicating that there are redundant strategies to overcome host restriction (Mänz et al., 2012; Mehle & Doudna, 2009). The 2009 pandemic lineage has been shown to contain two alternative mutations at position 590 and 591 (G590S, Q591R) on PB2, which locate close to the eponymous 627 residue of the 627 domain (Pflug et al., 2014). Examples of other mammalian-adapting mutations include PB2 D701N and T271A, PA T552S, PB1 N375S and NP N319K (Cauldwell et al., 2014). As with E627K, several of these mutations are associated with increased pathogenicity, as well as increased replication (C. Y. Lee et al., 2020; Nieto et al., 2017; Yamaji et al., 2015).

The precise mechanism(s) by which different mammalian-adapting mutations overcome restriction have not been fully defined. Within the cytoplasm, certain mutations are associated with decreased triggering of the pattern recognition receptor RIG-I (M. Weber et al., 2015), and the potent restriction factor MxA (Mänz et al., 2013). Other mutations appear to increase the efficiency of nuclear import of both nascent

RNP components and vRNPs, through adapting FluPol to efficiently bind mammalian-specific importin- α isoforms (Gabriel et al., 2008, 2011; Sediri et al., 2015). Once within the nucleus, restriction is thought to be imposed specifically at the level of viral replication, rather than transcription (Long, Mistry, et al., 2019), although it is worth noting that processes downstream of transcription such as splicing are suboptimal in zoonotic infections (Bogdanow et al., 2019; Calderon et al., 2019), potentially mediated by interactions with the human protein transformer 2 alpha homolog (TRA2A) protein (Zhu et al., 2020). Whether restriction is imposed specifically at the level of vRNA synthesis, or vRNA and cRNA synthesis, is contentious (Bi et al., 2019; Mänz et al., 2012; Nilsson et al., 2017; Nilsson-Payant et al., 2021). The FluPol PB2 E627K switch has been associated with differences in temperature sensitivity of the enzyme (Massin et al., 2001), and this may contribute to restriction at the lower temperatures of the mammalian upper respiratory tract (Aggarwal et al., 2011; Hatta et al., 2007; Massin et al., 2001). Moreover, it has been suggested that FluPol 627E has a decreased affinity for NP in mammalian cells, and that this may impact RNP assembly (Labadie et al., 2007; Mehle & Doudna, 2008; Ng et al., 2012; Rameix-Welti et al., 2009), although other reports contest this conclusion (Cauldwell et al., 2013; Paterson et al., 2014). Restriction of avian influenza virus (AIV) FluPol 627E in mammalian cells within the context of mini genome assays can be alleviated through the introduction of mutations at positions 3 and 8, or 3,5 and 8 of the 3' vRNA promoter (3-8 or 3-5-8 mutants), which has been interpreted as evidence host restriction is imposed at the level of promoter binding/initiation. One study found this effect to be specific to FluPol 627E (Crescenzo-Chaigne et al., 2002), whilst others have found both 627E and 627K to be enhanced (Nilsson-Payant et al., 2021; Paterson et al., 2014). Regardless, neither FluPol 627E or 627K are enhanced by 3-8 promoter mutants in avian cells, and indeed transcription appears to be reduced in this context (Paterson et al., 2014). Nevertheless, a recent report observed no difference in FluPol 627E or FluPol 627K promoter binding either using quantitative single-molecule Förster resonance energy transfer (smFRET) and purified FluPol, or RNA immunoprecipitation using transfected FluPol in mammalian cells (Nilsson-Payant et al., 2021). Finally, avian and mammalian FluPol show comparable activity *in vitro*, implying cell factors play a role in restriction *in vivo* (Aggarwal et al., 2011; Paterson et al., 2014). The 627 domain is solvent-exposed, and both E627K and Q591R increase the overall positivity of this region, suggesting they affect interactions with a host factor through a comparable mechanism (Long, Mistry, et al., 2019). Indeed, both mutations are thought to be adaptations to mammalian orthologues of the host factor ANP32 (Section 1.4) (Long et al., 2016; Long, Mistry, et al., 2019). Human importin- α 1 and importin- α 7 have also been shown to specifically enhance the activity of FluPol 627K (Hudjetz & Gabriel, 2012; Resa-Infante et al., 2008), in the case of importin- α 7 potentially as a co-factor of ANP32 (Yu et al., 2022). Meanwhile, other studies suggest that avian FluPol may be specifically inhibited by mammalian restriction factors, such as the dead box RNA helicase DDX17, Tu elongation factor, mitochondrial (TUFM) and butyrophilin (Bortz et al., 2011; Kuo et al., 2017; Maria Pinto et al., 2022; Mehle & Doudna, 2008).

It is possible different adaptive mutations use different strategies to overcome restriction. Whilst experimentally mutating PB2 E627K into a 2009 H1N1 background does not have an additive effect, addition of PB2 D701N does boost replication (Zhou et al., 2013). This suggests that adaptations at

PB2 position 627 and 591 use the same mechanism to overcome restriction, whilst position 701 uses a different strategy. Similar genetic studies using different combinations of mutations may shed further light on the pleiomorphic strategies available for adaptation of FluPol to mammalian hosts. It is important to note that many of the studies described here have compared isogenic viruses/FluPol constellations differing at position 627, and that the conclusions are therefore biased towards this adaptive strategy. In contrast, one comparative study of multiple PB2 adaptive mutations found two distinct classes – those that specifically enhanced replication in mammalian cells, and those that increased replication in both avian and mammalian cells (Cauldwell, Moncorgé, et al., 2013). Furthermore, although interactome analysis has identified numerous candidate interaction partners with FluPol, relatively few have been investigated as determinants of host range, and it is likely many currently unidentified host factors play a role (Long, Mistry, et al., 2019; Peacock et al., 2019). The precise mutations acquired are likely restricted by the context of the viral background and the presence of alternative selection pressures, as well as interactions with the host (Long et al., 2013; Mehle & Doudna, 2008).

1.3.7 A role for subnuclear localisation in FluPol activity and host restriction?

One element that has not been fully explored is the role of spatial localisation in FluPol activity and host restriction. Although not bounded by dividing membranes, the nucleus is organised into a highly compartmentalised, dynamic structure, which is essential for normal function. Firstly, chromosomes are divided into regions of highly condensed, transcriptionally inactive heterochromatin, and loosely packed, transcriptionally available euchromatin (Pongubala & Murre, 2021). Secondly, during interphase, individual chromosomes arrange in a non-random manner into distinct regions known as chromosome territories. The positioning of chromosomes in the nucleus varies depending on cell type, differentiation status and with environmental factors such as stress, however in general more transcriptionally active, gene-rich chromosomes locate to the nuclear interior, and more transcriptionally silenced, gene-poor chromosomes localise towards the nuclear periphery (Brickner, 2017; Pongubala & Murre, 2021; A. M. Wood et al., 2014). Moreover, transcriptionally silenced loci tend to localise within the chromosomal territories whilst transcriptionally active loci tend to extend out into the inter-chromosomal regions (Meldi & Brickner, 2011). Finally, several distinct subnuclear compartments known as nuclear bodies, are found in the inter-chromosomal regions. These regions are enriched in factors required for functions such as transcription, repression, splicing and DNA repair, and thus spatially divide important biochemical reactions. Nuclear bodies include such structures as the nucleolus, paraspeckles and Cajal bodies (Meldi & Brickner, 2011; Zakaryan & Stamminger, 2011). Environmental stimuli, including infection, can lead to widespread reorganisation of this structure (Zakaryan & Stamminger, 2011).

Considering the need of IAV to parasitise many of the functions of, and factors within, nuclear bodies, it follows that the virus must interact with these specialised regions, either through localisation or through reorganisation of the nucleus. Many early electron microscopy (EM) studies reported ultrastructural perturbations of unknown biological significance in the nucleus of influenza infected cells (Figure 9) (Anisimová et al., 1977; Anisimová et al., 1980; Compans & Dimmock, 1969; Herrero-Urbe et al., 1983; Josset et al., 2008; Kopp et al., 1968; Saito et al., 1970; Shaw & Compans, 1978). Both

NS1 (Murayama et al., 2007) and NP (Ozawa et al., 2007) contain nucleolar localisation signals, and several nucleolar and ribosomal proteins have been shown to interact with the vRNP including the main protein constituents, nucleolin (C23) and nucleophosmin (B23) (Mayer et al., 2007). Disruptions to SC35+ speckles, Cajal and PML bodies have also been reported during infection (Fortes et al., 1995; Sato et al., 2003).

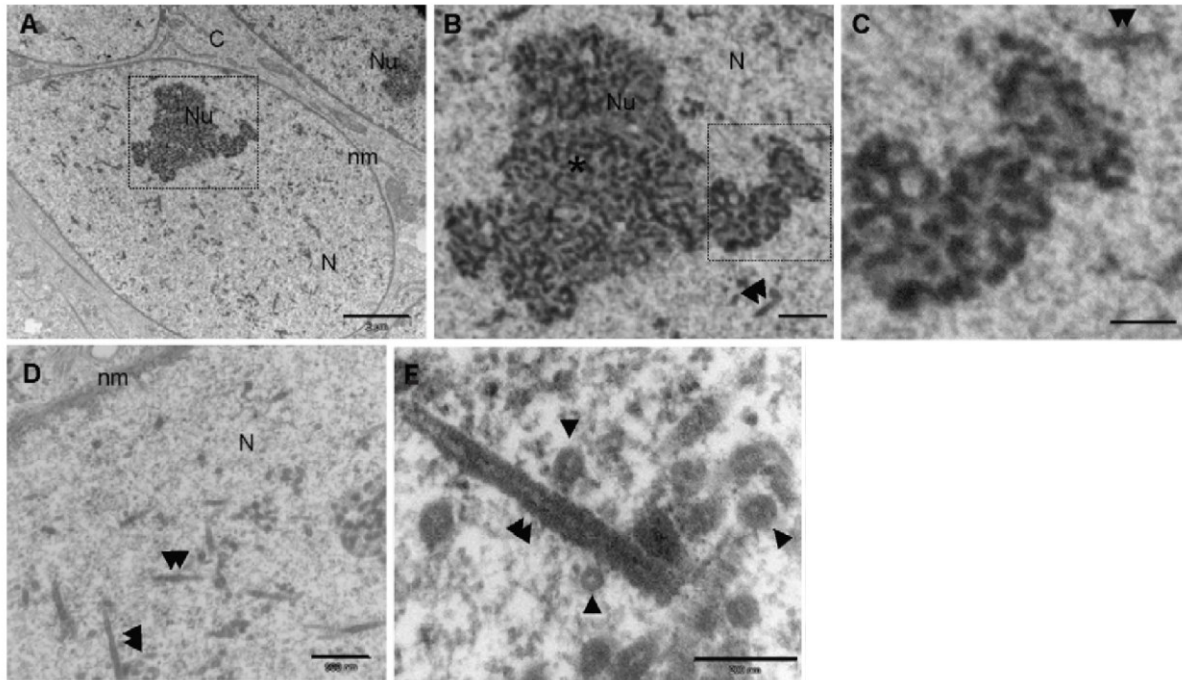


Figure 9: Ultrastructural perturbations visualised in influenza infected cells.

MDCK cells infected with IAV (A/Moscow/10/99 (H3N2)), 24hpi. Figure adapted from Josset et al., 2008, with permission from Elsevier. (A) Overview of modifications visible in the nucleus: disruption of the normal subdomains of the nucleolar compartment (fibrillar centres, dense fibrillar component, granular component) and replacement with a homogenous structure of electron dense inclusions, plus formation of elongated electron dense tubular structures in the nuclear matrix. Scale bar is 2 μ m. (B) Enlargement of the nucleolar region in A. Scale bar: 500 nm. (C) Enlargement of the nucleolar region highlighted in B. Scale bar: 200 nm. (D,E) Elongated tubular structures visualised in the nucleus (indicated with double-arrowheads). Single arrowheads indicate transverse sections of these structures. Scale bars: D: 500 nm, E: 200 nm. Nm= nuclear membrane; Nu= nucleolus; C= cytoplasm; N= nucleus.

Following nuclear import, vRNPs traffic from the nuclear pore to transcriptionally active euchromatin to directly interact with Pol II Ser5P CTD – the site of viral transcription (Engelhardt et al., 2005; te Velthuis et al., 2021). However, whether the switch to genome replication coincides with an accompanying movement to a new nuclear niche is not known. Maximal interaction with Ser5P CTD coincides with peak mRNA synthesis, and the virus initiates degradation of the large subunit of Pol II later in infection, suggesting the vRNP may detach (Rodriguez et al., 2007; Vreede et al., 2010). Previous reports have implicated both the nuclear matrix (López-Turiso et al., 1990; Takizawa et al., 2006) and, more recently, the nucleolus (Miyamoto et al., 2022), as potential locations for genome replication. Downstream elements of viral replication are also known to require nuclear trafficking. It has been shown that splicing to form M2 mRNA involves trafficking through SC35+ speckles - nuclear bodies involved in the storage

and maturation of splicing factors (Mor et al., 2016). Meanwhile, nascent vRNPs are thought to traffic through SC35+ speckles then localise to densely packed heterochromatin prior to nuclear export, in a process that depends on interactions with the host factor clustered mitochondria protein homolog (CLUH) (Ando et al., 2016; Chase et al., 2011). Influenza uses the CRM1-dependent nuclear export pathway for vRNPs, and the heterochromatin is the site at which CRM1 export complexes are regenerated by the Ran guanine exchange factor RCC1. Therefore, targeting to this compartment appears to enable the virus to gain preferential access to export factors (Chase et al., 2011). Finally, it has been shown that vRNP export from the nucleus in epithelial cells appears to specifically occur at the side of the nucleus facing the apical membrane. This asymmetric distribution may contribute to the specific budding of nascent virions from the apical plasma membrane at the surface of infected cells, although a detailed mechanism has not been described (Elton et al., 2005).

Whether nuclear localisation plays a role in host restriction is also unknown, although it seems probable that suboptimal host factor interactions would disrupt the complex trafficking patterns appreciated to be required for viral replication. Certainly, host restriction is associated with impaired nuclear import of both vRNPs and nascent FluPol components due to inefficient interactions with host-specific importin- α isoforms (Gabriel et al., 2008, 2011; Sediri et al., 2015). Once within the nucleus, fluorescence recovery after photobleaching (FRAP) analysis of transfected FluPol differing only at the residue at position 627 on PB2 has shown that PB2 627E demonstrates slower mobility compared to PB2 627K (Foeglein et al., 2011). Similarly, unpublished work from the Barclay lab observed that transfected avian FluPol forms 'speckles' in human cells, and that introduction of mammalian adaptations reduces this phenotype in a manner that is proportional to the increased level of replication. In contrast, neither avian nor mammalian FluPol form speckles in avian cells (Mistry, 2019). Combined, this data suggests that aberrant nuclear trafficking, potentially due to inefficient interactions with a pro-viral host factor or aggregation by a restriction factor, may contribute to the restriction of avian FluPol in mammalian cells.

1.4 THE ESSENTIAL HOST FACTOR ANP32

1.4.1 Introduction

The acidic leucine-rich nuclear phosphoprotein 32 KDa (ANP32) proteins are an important family of proviral host factors (Long, Idoko-Akoh, et al., 2019; Staller et al., 2019; Sugiyama et al., 2015; H. Zhang et al., 2019). Human ANP32 (huANP32) proteins were independently identified as proviral host factors for influenza A virus multiple times: firstly, in two different interaction screens (Bradel-Tretheway et al., 2011; Watanabe et al., 2014); and secondly, in a biochemical study in which huANP32A/B were identified as host factors present in nuclear extracts that specifically enhance vRNA synthesis in cell-free assays (Sugiyama et al., 2015). Subsequently, knockdown and knockout (KO) cell lines have demonstrated that at least one compatible ANP32 protein is required for robust IAV replication in both mammalian and avian cells, for all strains investigated thus far (Long et al., 2016; Long, Idoko-Akoh, et al., 2019; Staller et al., 2019; H. Zhang et al., 2019). ANP32 proteins are also essential to support IBV and ICV replication (Carrique et al., 2020; Staller et al., 2019).

In 2016, species-specific differences between ANP32 proteins were identified as the key underlying cause of restricted avian (precisely prototypical FluPol 627E) influenza virus polymerase activity in mammalian hosts (Long et al., 2016). Specifically, FluPol 627E cannot be supported by mammalian ANP32 proteins that lack a 33 amino acid (aa) insertion present in most avian ANP32A orthologues, and accordingly show restricted replication in human cells. In contrast, FluPol 627K can utilise mammalian ANP32 proteins that lack the avian insertion, and replicate to high titres (Long et al., 2016). Data published in a pre-print suggests that the alternative mammalian-adapting PB2 mutations, Q591R and D701N, are also adaptations to mammalian ANP32 proteins, whilst PB2 G158E, T271A and D740N are not (Peacock, Sheppard, et al., 2020). Whether other adaptive mutations in PB1 or PA, or indeed NS2/NEP, act in an ANP32-dependent or independent mechanism, is currently unknown.

The unique potency of ANP32 was illustrated in a comparative experiment in which individual proviral host factors associated with viral replication were ablated by CRISPR-Cas9. Whilst ablation of the other host factors investigated reduced polymerase activity and viral infectivity 2-10-fold, loss of ANP32 resulted in a dramatic 10,000-fold drop in both measures (H. Zhang et al., 2019). Therefore, understanding the mechanism by which ANP32 supports viral replication, and how this interaction can be disrupted, may lead to the development of potent antiviral therapeutics or even infection-resistant gene edited animals.

1.4.2 ANP32 proteins

The ANP32 family are a highly conserved group of small proteins (generally 130-290 aa, 14.8-32 kDa) present across the animal, plant and protist kingdoms. In vertebrates, there are three well-described members: ANP32A, ANP32B and ANP32E. ANP32D and ANP32C have been described but are intronless and believed to be pseudogenes (Matilla & Radrizzani, 2005; Reilly et al., 2014). All ANP32 proteins are characterised by two domains: a structured N-terminal LRR domain, followed by the unstructured C-terminal LCAR (Figure 10) (Matilla & Radrizzani, 2005; Reilly et al., 2014). The structure of the LRR from human ANP32A (huANP32A) (de Chiara et al., 2007; Huyton & Wolberger, 2007; Zamora-Caballero et al., 2015) and huANP32B (Tochio et al., 2010) are similar and consist of an N-terminal helix-loop-helix followed by five LRR repeats: the N-terminal and C-terminal repeats (LRR1 and LRR5) are both incomplete and cap the internal three repeats to form a stable structure, resulting in a curved surface as is characteristic for LRR domains (de Chiara et al., 2007; Huyton & Wolberger, 2007; Zamora-Caballero et al., 2015). In contrast, the LCAR is a highly negatively-charged intrinsically disordered domain (IDD) (Reilly et al., 2014). The 33 aa insertion present in avian ANP32A homologues lies between the LRR and LCAR domains and consists of a novel hexapeptide sequence (VLSLVK), followed by a 27 aa partial duplication of the preceding exon 4 (positions 149-175) (Domingues & Hale, 2017; Long et al., 2016). This insertion is also thought to be unstructured, thus extending the length of the IDD in avian ANP32A proteins (Camacho-Zarco et al., 2020).

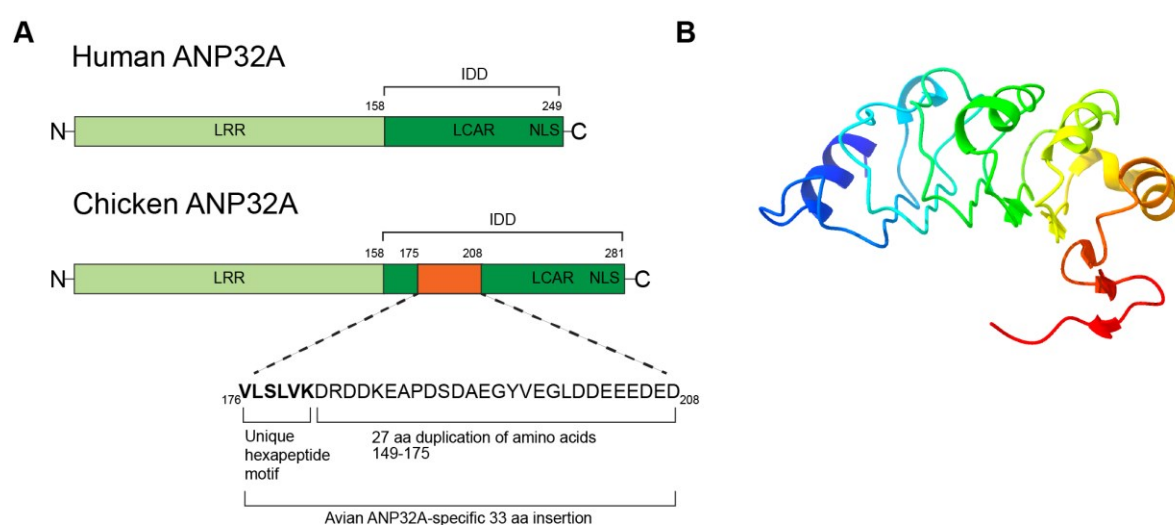


Figure 10: Key structural details of ANP32 proteins.

(A) Schematic comparing human ANP32A and chicken ANP32A. LRR= leucine-rich repeat domain; LCAR= low-complexity acidic region; IDD= intrinsically disordered domain; NLS= nuclear localisation signal. (B) The structured portion of human ANP32A as resolved when bound to ICV FluPol (PDB accession code: 6XZQ). The canonical curved surface of the LRR domain can be clearly observed, whilst the flexible LCAR domain is unresolved. Structural models were prepared with ChimeraX (Pettersen et al., 2021).

Both LRR and LCAR domains are associated with protein:protein interactions. LRR domains typically interact on the concave face of their curved structure, which shows an overall negative charge potential in ANP32A/B (Huyton & Wolberger, 2007). Meanwhile, the acidity and flexibility of the LCAR likely enables non-specific interactions with most positively charged surfaces (Reilly et al., 2014). ANP32 proteins can also be phosphorylated, further increasing the negative potential, and likely modifying activity (Reilly et al., 2014). Moreover, ANP32 proteins localise to both the nucleus and cytoplasm (Brennan et al., 2000; Huyton & Wolberger, 2007). Unsurprisingly, ANP32 proteins have been found to interact with numerous cytoplasmic and nuclear complexes and are implicated in an array of host functions (Huyton & Wolberger, 2007). These include transcription, protein phosphatase regulation, apoptosome activation and intracellular transport (Matilla & Radrizzani, 2005; Reilly et al., 2014). Moreover, ANP32 proteins are associated with chromatin remodelling: both huANP32A and huANP32B bind core histones and are associated with regulating histone acetylation (Munemasa et al., 2008; Saavedra et al., 2017; Seo et al., 2002; Tochio et al., 2010), whilst huANP32E is a chaperone of the unconventional H2A.Z histone variant (Obri et al., 2014). This diversity of function, combined with the evolutionarily conserved nature of the family, has been used to suggest that ANP32 proteins may represent a 'critical regulatory node', spanning multiple essential cellular pathways (Reilly et al., 2014).

Consistent with their proposed role in cellular regulation, ANP32 proteins have been implicated in several different cancers and neurodegenerative disorders (Matilla & Radrizzani, 2005; Reilly et al., 2014). Moreover, in addition to influenza virus, ANP32 proteins have been implicated in the lifecycles of several other viral families (Günther et al., 2020; Reilly et al., 2014). As might be expected for diverse viruses and a highly multi-functional protein, the mechanism of support differs: from export of viral mRNAs (retroviruses, adenoviruses) (Bodem et al., 2011; Higashino et al., 2005; Y. Wang et al., 2019); nuclear shuttling of viral proteins (paramyxoviruses) (Günther et al., 2020); to association with the chromatin chaperone complex TAF-1(INHAT)/SET (adeno-associated viruses) (Pegoraro et al., 2006).

1.4.3 ANP32 orthologues differ in influenza proviral activity

In most mammalian species investigated so far, ANP32A and ANP32B act redundantly as proviral host factors for influenza, whilst ANP32E does not support robust FluPol activity (Figure 11) (Peacock, Swann, et al., 2020; Staller et al., 2019; H. Zhang et al., 2019). Nevertheless, although ANP32A/B act redundantly, some viral strains have a 'preferential' paralogue which enhances replication to a greater extent (Staller et al., 2019). Some strains can inefficiently use huANP32E to achieve a low level of viral replication (H. Zhang et al., 2019; Z. Zhang et al., 2020; Goldhill et al., under revision) and the H5N1 strain Tky05 can rapidly adapt to efficiently use huANP32E in huANP32A/B KO cells (Goldhill et al., under revision). Whether other strains can achieve similar adaptation, or whether influenza can adapt to become ANP32-independent in cells lacking all three paralogues (huANP32A/B/E), is not yet known.

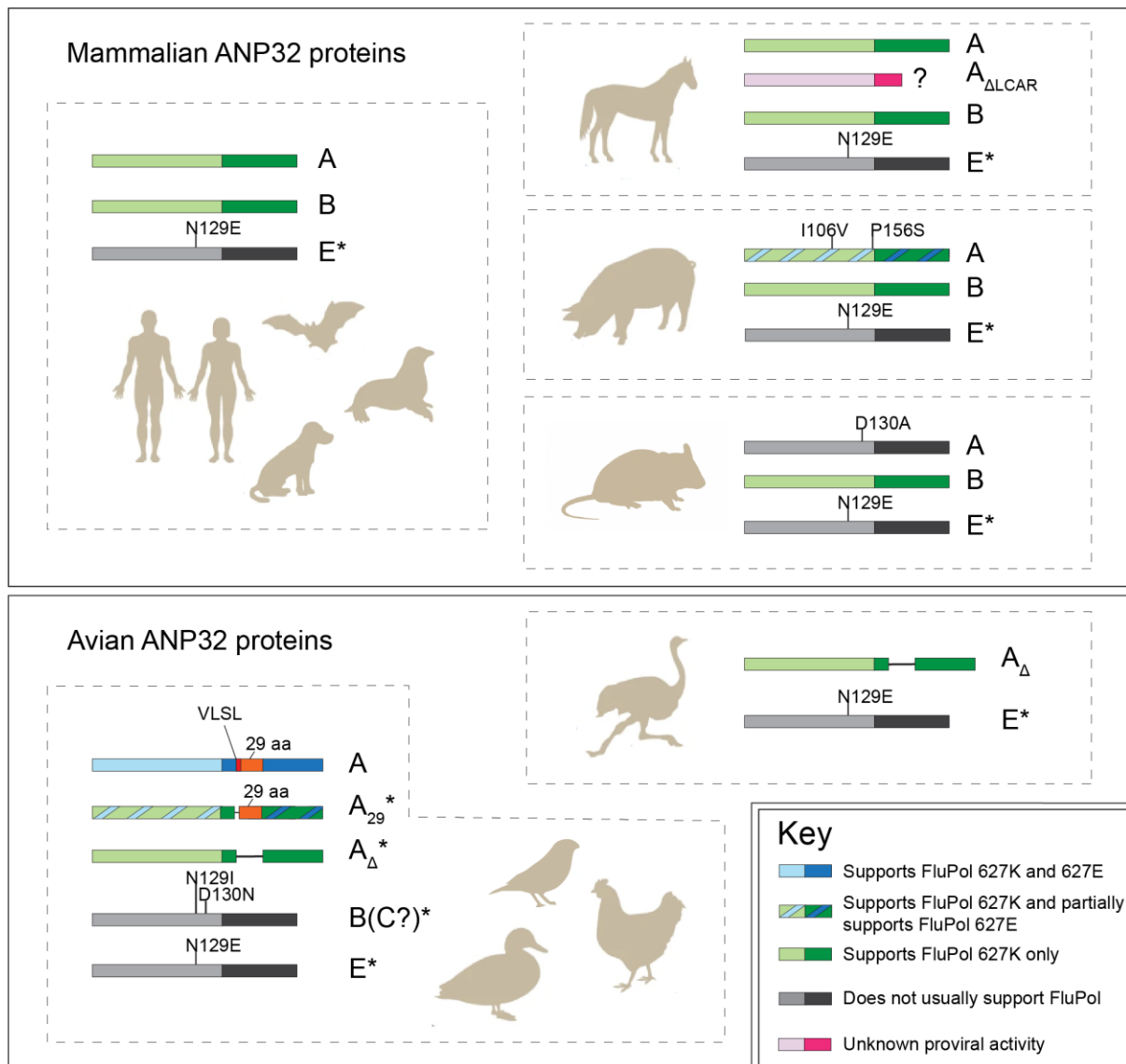


Figure 11: ANP32 orthologues and proviral activity in species relevant to IAV ecology.

All vertebrates express three ANP32 proteins: ANP32A, ANP32B and ANP32E. For mammalian and avian species, the general trend for proviral activity of ANP32 proteins and examples of species that demonstrate this pattern, are depicted in the boxes on the left. Interesting exceptions to these rules are depicted on the right. Significant residues linked to the level of proviral activity of the ANP32 protein are depicted. ANP32 proteins that have not been experimentally validated in all species depicted but are predicted to have the same activity based on sequence, are marked with an asterisk (*). For simplicity, ANP32 proteins apart from the splice variants are depicted as the same length. Full sequence alignments and shared amino acid identity for each group of ANP32 proteins are included in appendix A. In most mammals investigated (human, dog, bat, seal, horse, pig), ANP32A and ANP32B are essential but redundant proviral host factors (Peacock, Swann, et al., 2020; Staller et al., 2019; H. Zhang et al., 2019). HuANP32E does not usually support robust FluPol activity (although it can occasionally support a low level of replicative activity) (H. Zhang et al., 2019; Z. Zhang et al., 2020; Goldhill et al., under revision). Using IBV, the limited proviral activity of huANP32E has been mapped to a single residue at position 129 which is an asparagine in huANP32A but a glutamic acid in huANP32E (Z. Zhang et al., 2020), although this has not been validated for IAV. Horse are the only mammalian species with any evidence for alternatively spliced ANP32 variants, having been found to express a shorter truncated transcript lacking a portion of the LCAR (Domingues et al., 2019). The proviral activity of this variant, or even whether the protein is expressed, has not been demonstrated. Interestingly, pig ANP32A is the mammalian ANP32 protein with the strongest ability to support AIV polymerase activity, albeit still to a very low level relative to avian ANP32A (Peacock, Swann, et al., 2020; H. Zhang et al., 2020). This

increased support maps to two substitutions relative to most other mammalian ANP32A proteins, an isoleucine to a valine at position 106 and a proline to a serine at position 156, which also increase binding to FluPol. This data provides new evidence that pigs may be unusually susceptible to infection with both mammalian and avian IAV strains, and thus play an important ecological role as an intermediate species (Peacock, Swann, et al., 2020; H. Zhang et al., 2020). Interestingly, the P156S substitution is also present in pika, another mammalian species that is readily infected with minimally adapted avian IAV (Peacock, Swann, et al., 2020). Finally, in mice, only ANP32B is proviral. ANP32A does not support FluPol activity, due to a single substitution relative to huANP32A at position 130 from aspartic acid to alanine (Staller et al., 2019; H. Zhang et al., 2019). In avian species, only ANP32A supports robust FluPol activity. The inactivity of chANP32B has been mapped to two substitutions relative to mammalian ANP32B proteins at positions 129 and 130, from asparagine to isoleucine and aspartic acid to asparagine, respectively. Most avian species contain these substitutions (Long, Idoko-Akoh, et al., 2019; H. Zhang et al., 2019). Moreover, phylogenetic analysis demonstrates that avian ANP32B proteins cluster with *Xenopus* ANP32C in a separate clade from other vertebrate ANP32B proteins, suggesting avian and mammalian ANP32B proteins are not orthologues, which may help explain the differing pro-viral activity (Long, Idoko-Akoh, et al., 2019). To highlight this, avian ANP32B proteins have been labelled as B(C?) in the figure. ANP32B(C?) is not depicted for ostrich, as there is currently no sequence information available for this homologue. As with huANP32E, the limited proviral activity of chANP32E has been mapped to the substitution N129E relative to huANP32A and chANP32A, using IBV (Z. Zhang et al., 2020). Experiments using IBV suggest that avian ANP32E proteins are even less supportive of FluPol activity than mammalian ANP32E proteins, and this maps to a 10 aa deletion within the LCAR tail (Z. Zhang et al., 2020). However, whether these findings are translatable to IAV is not known. Avian species have also been found to express alternative splice variants of ANP32A, which are expressed at varying proportions in different species and cell types. These are the full-length ANP32A containing the 33 aa insertion (ANP32A₃₃), a slightly shorter ANP32A lacking the first 4 aa (VLSL) from the unique hexapeptide sequence at the start of the insertion (ANP32A₂₉) and a mammalian-like version that excludes the full insertion (ANP32A_Δ) (Baker et al., 2018; Domingues et al., 2019). The splice factor SRSF10 has been implicated in enhancing ANP32A splicing to generate the shorter ANP32A₂₉ variant in avian cells (Fang et al., 2020). Full-length chANP32A fully supports avian FluPol, chANP32A₂₉ is supportive but less potent than full-length chANP32A₃₃, and the mammalian-like ANP32A_Δ only supports mammalian-adapted FluPol. Species that express higher proportions of the shorter splice variants may select for mammalian FluPol adaptations (Baker et al., 2018; Domingues et al., 2019). Ostriches and other ratites only express ANP32A_Δ, and accordingly have been observed to select for typical mammalian FluPol adaptations (Long et al., 2016; Shinya et al., 2009). Comparisons across ANP32 proteins clearly highlight the importance of residues at positions 129/130 for proviral activity. The significance of this region can be explained by the structures of huANP32A and chANP32A in complex with IAV FluPol – in which these residues map to the interface between ANP32A and the encapsidating FluPol. Thus, substitutions in this region likely disrupt functional binding of ANP32 to the asymmetric dimer (Carrique et al., 2020). aa= amino acid, huANP32= human ANP32, chANP32= chicken ANP32. Animal silhouettes, apart from the zebra finch which was produced for this figure, were reproduced from those in Figure 1 (Long, Misty, et al., 2019).

In avian species, only ANP32A supports robust FluPol replication. Avian ANP32B and ANP32E lack the 33 aa avian insertion required by AIV FluPol. Moreover, insertion of the 33 aa sequence from chicken ANP32A (chANP32A) into chANP32B is insufficient to rescue proviral activity, and human adapted FluPol does not replicate in chicken cells in which chANP32A has been ablated, i.e., chANP32B/E contain additional mutations that render them non-supportive (Long, Idoko-Akoh, et al., 2019). Nevertheless, some avian strains have occasionally been observed to grow to low titres in ANP32A KO chicken cells (Long, Idoko-Akoh, et al., 2019), which may suggest chANP32B/E can inefficiently support viral replication in an analogous manner to huANP32E. Interestingly, avian ANP32A exists as three different splice variants, that are present in varying proportions in different species and cell types (Baker et al., 2018; Bi et al., 2019; Domingues et al., 2019). These splice variants include the full-length ANP32A containing the 33 aa insertion (ANP32A₃₃), a slightly shorter ANP32A lacking the first 4 aa (VLSL) from the unique hexapeptide sequence at the start of the insertion (ANP32A₂₉) and a mammalian-like version that excludes the full insertion (ANP32A_Δ) (Baker et al., 2018; Domingues et al., 2019).

al., 2019). chANP32A₂₉ is supportive of AIV polymerase, but less potent than full-length chANP32A₃₃. Meanwhile, the mammalian-like ANP32A_Δ supports mammalian-adapted, but not avian polymerase (Baker et al., 2018; Bi et al., 2019; Domingues et al., 2019). In competition experiments, both splice variants (chANP32A₂₉, chANP32A_Δ) select for mammalian adaptations in the polymerase protein, suggesting avian species that express high proportions of these variants, such as blackbirds, magpies and swan geese, may pose a particular risk for driving the emergence of new variants with pandemic potential (Baker et al., 2018; Domingues et al., 2019). Ostriches and other ratites only express ANP32A_Δ, explaining the anomalous presence of typical mammalian adaptations in the FluPol of isolates collected from these species (Long et al., 2016; Shinya et al., 2009). No evidence for alternatively spliced ANP32 variants has been found in mammalian species, apart from horse, which express a truncated ANP32A transcript lacking a portion of the LCAR (Domingues et al., 2019).

Baker and Mehle argue that the diversity and range in proviral activity of ANP32 proteins signifies a long evolutionary history of viral exploitation (Baker & Mehle, 2019). A summary of the different ANP32 variants discussed here is outlined in Figure 11. As further influenza strains and ANP32 proteins from different species are investigated, it is likely that additional insightful exceptions to these general rules will be uncovered.

1.4.4 Mechanism of proviral activity

Numerous interaction studies have demonstrated that ANP32A and ANP32B directly interact with the full trimeric polymerase, but not individual FluPol subunits, both in the context of infection and transfected proteins (Baker et al., 2018; Bradel-Tretheway et al., 2011; Domingues & Hale, 2017; Mistry et al., 2020; Sugiyama et al., 2015; Watanabe et al., 2014). Using a split-venus system, the ANP32A-FluPol interaction has been shown to occur in the nucleus of transfected cells (Mistry et al., 2020). The addition of either vRNA or cRNA enhances the interaction under conditions in which elongation is inhibited: either in the absence of NP or using a catalytically dead polymerase mutant (Baker et al., 2018; Mistry et al., 2020). In contrast, under conditions in which elongation is active, titrating in vRNA decreases the interaction between either huANP32A and FluPol 627K or chANP32A and FluPol 627E (Mistry et al., 2020). This suggests that ANP32 binds to vRNPs/cRNPs when FluPol is bound to the promoter, i.e., during initiation, but falls off once elongation starts (Mistry et al., 2020). A role for ANP32 prior to the elongation step of RNA synthesis aligns with previous studies suggesting that host restriction of FluPol is imposed at the point of initiation (Section 1.3.6) (Crescenzo-Chaigne et al., 2002; Paterson et al., 2014).

The cryo-EM structure of human and chicken ANP32A in complex with an asymmetric dimer of two ICV FluPol heterotrimers strongly suggests a role for ANP32 in coordinating co-replicative encapsidation of nascent RNA into RNPs (Section 1.3.4) (Carrique et al., 2020). In agreement with such a role, several studies have demonstrated that ANP32 is not required for primary transcription (viral mRNAs do not require encapsidation) (Nilsson-Payant et al., 2021; Staller et al., 2019; Sugiyama et al., 2015). In contrast, as both cRNA and vRNA require encapsidation into RNPs, this implies ANP32 is required for

both vRNA synthesis and cRNA synthesis. Binding of ANP32 appears to stabilise the replicase configuration of the 627 and NLS domains (Camacho-Zarco et al., 2020) and disruptive mutations introduced into the asymmetric dimer interface between FluPol_R and FluPol_E reduce cRNA accumulation, implying the dimer structure at least is required for cRNA synthesis (Carrique et al., 2020). Nevertheless, this conflicts with earlier *in vitro* data in which purified huANP32A stimulated RNA replication from a short cRNA, but not vRNA template, suggesting ANP32 is only involved in vRNA synthesis (Sugiyama et al., 2015).

Although only the LRR domain is fully resolved in the cryo-EM structure of the ANP32-stabilised asymmetric dimer, binding studies suggest that the flexible LCAR domain also directly binds FluPol (Camacho-Zarco et al., 2020; Domingues & Hale, 2017; Mistry et al., 2020; Staller, 2021). NMR analysis shows that the huANP32A LCAR, and the chANP32A avian insertion plus LCAR, are both IDD domains that form multiple, transient, low affinity interactions with the PB2 627-NLS domain. Nonetheless, relatively high avidity is achieved, due to the polyvalency of the interaction (Camacho-Zarco et al., 2020). Genetic analysis using truncation mutants has demonstrated an inverse correlation between FluPol binding and the length of the LCAR tail (Domingues & Hale, 2017; Mistry et al., 2020; Staller, 2021). It is unclear whether this is sequence-specific or a matter of protein length: although interactions occur along the length of the LCAR, binding is particularly focused on the region 25-35 amino acids downstream from the LRR (Camacho-Zarco et al., 2020). Speculatively, the interaction between ANP32 and the LCAR may stabilise the LRR-bridged dimer complex, as binding is completely ablated in full LCAR deletion mutants (Domingues & Hale, 2017; Mistry et al., 2020; Staller, 2021). Intriguingly, chANP32A mutants that lack the entire LCAR domain but retain the avian insertion (chANP32A₁₋₂₀₈) demonstrate only a moderate reduction in proviral activity, despite undetectable FluPol binding (Domingues & Hale, 2017; Mistry et al., 2020). This demonstrates a disconnect between FluPol binding and activity that is difficult to explain with our current understanding of ANP32.

A recent study has suggested an additional, mechanistic role for the LCAR tail in loading monomeric NP onto the nascent RNA, further implicating ANP32 in RNP assembly (F. Wang et al., 2022). The authors demonstrate that NP interacts with the LCAR from huANP32A, huANP32B and chANP32A via its two RNA binding grooves and RNA outcompetes this binding (F. Wang et al., 2022). Previous work has shown that host restriction occurs during replication of an NP-independent short viral-like template (Paterson et al., 2014) and the addition of chANP32A in such a system fully restores replication (Baker et al., 2018; Nilsson-Payant et al., 2021). Whilst this demonstrates the mechanism by which ANP32 supports FluPol is partially independent of NP, it does not exclude an additional NP-dependent role. Indeed, in the context of mini genome assays, FluPol replication is less sensitive to LCAR truncation mutants in an NP-independent system than in an NP-dependent system: suggesting an NP-specific role for this domain (F. Wang et al., 2022). Overall, the authors suggest the LCAR tail acts as a molecular whip to recruit monomeric NP to the nascent strand of RNA, in a mechanism reminiscent of the P protein expressed by non-segmented NSVs but absent from segmented NSVs. Due to the higher affinity of RNA, the NP is subsequently displaced from the LCAR, and added to the growing RNA.

Alternatively, as the LCAR could bind multiple NP monomers, it may act to increase the local concentration of NP surrounding the replicase complex (F. Wang et al., 2022). A schematic of our current understanding of the mechanism by which ANP32 proteins support FluPol activity is depicted in Figure 12A.

Intriguingly, under specific conditions, ANP32 proteins can inhibit influenza polymerase activity. Several studies have observed an optimal level of ANP32 expression, beyond which stimulation of FluPol activity is reduced (Bi et al., 2019; Long et al., 2016; Staller et al., 2019; H. Zhang et al., 2019) and ablation of huANP32A to leave just huANP32B expression increases the activity of some polymerase constellations (Staller et al., 2019). This suggests that the correct balance of ANP32 expression is crucial for optimal viral replication. Such a 'goldilocks effect' in supporting FluPol activity has also been described for importin- α 7, which may act as a co-factor of ANP32 (Yu et al., 2022). Overexpression of ANP32 paralogues that are not strongly proviral, including huANP32E, huANP32C and huANP32D, have also been shown to restrict FluPol replication (Park et al., 2020). However, the biological relevance of these overexpression studies is uncertain, particularly in the case of the suspected pseudogenes ANP32C/D. Confirmatory studies, for example using knockdown or KO of huANP32E in human cell lines, have not been performed. Nevertheless, introducing non-functional mutations such as N129I or D130A into either huANP32A or huANP32B by CRISP-Cas9 exerts a dominant-negative effect on viral replication i.e., replication is reduced despite the presence of an endogenous functional WT ANP32 paralogue (Staller et al., 2021; H. Zhang et al., 2019). One proposed mechanism for this effect is that the non-functional mutants compete with WT proteins for binding at the FluPol_R interface and block functional bridging of the ANP32-stabilised dimer due to the broken interaction at the FluPol_E interface (Figure 12B) (Staller et al., 2021). Interestingly, very rare individuals have been identified in genetic databases that carry natural single nucleotide variations (SNVs) resulting in missense mutations in their ANP32A/B genes, including homozygotes for huANP32B-D130A. Such individuals may have a natural resistance to influenza replication (Staller et al., 2021).

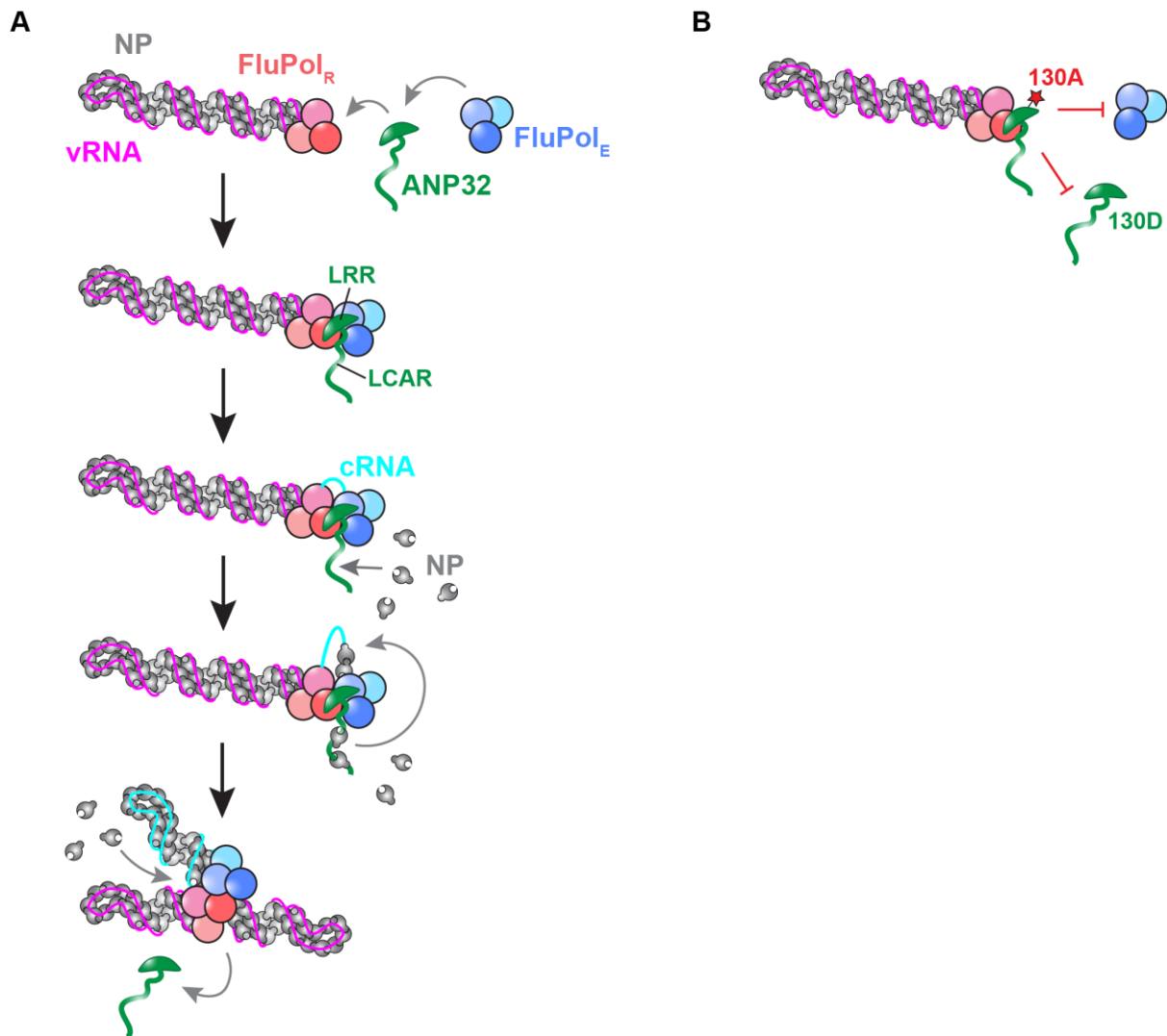


Figure 12: Proposed mechanism of ANP32 support/inhibition of FluPol activity.

(A) Proposed mechanism of proviral activity. ANP32 stabilises the replicase configuration of FluPol and coordinates co-replicative encapsidation through recruiting a nascent encapsidating polymerase (FluPol_E) and NP to coat the nascent strand of RNA (Camacho-Zarco et al., 2020; Carrique et al., 2020; F. Wang et al., 2022). As replication progresses from initiation to elongation, ANP32 is predicted to fall off FluPol (Mistry et al., 2020). This suggests further NP recruitment to the nascent RNA strand may occur in an ANP32-independent manner. The asymmetric dimer has only been resolved on a short vRNA, however is assumed to be required for both cRNA and vRNA synthesis. (B) Proposed mechanism for dominant negative effect of non-functional ANP32 proteins. ANP32 proteins bearing substitutions in the 129/130 region competitively bind the replicating FluPol at the functional N terminal end of the LRR, however are unable to stabilise functional dimers due to the disrupted interface with the encapsidating FluPol (Staller et al., 2021).

1.4.5 Mechanism of host restriction

Avian FluPol (627E) cannot be supported by mammalian ANP32 proteins that lack the 33 aa insertion present in most avian ANP32A orthologues (Long et al., 2016). To investigate the mechanism by which the 33 aa avian insertion supports AIV FluPol, many different chANP32A substitution and deletion mutants, as well as chANP32A/huANP32A chimeric mutants, have been generated. A summary of the ability of these mutants to support AIV FluPol is depicted in Figure 13.

The sequence of the insertion is significant, as chANP32A containing a scrambled 33 aa insertion does not support AIV FluPol activity (Long, Idoko-Akoh, et al., 2019). The unique hexapeptide motif at the start of the insertion (VL^SLVK) is particularly important, as substitutions/deletions in this region significantly decrease both the ability to support and bind FluPol 627E (Baker et al., 2018; Domingues et al., 2019; Domingues & Hale, 2017; Mistry et al., 2020). Switching the huANP32A residues at the equivalent positions to the unique 6 aa plus two additional downstream amino acids (176-EEEYDEDA-183 to 176-VL^SLVKDR-183), is sufficient to rescue FluPol 627E activity in mammalian cells (Carrique et al., 2020). Moreover, adding in the hexapeptide sequence alone is sufficient to fully rescue an H7N9 FluPol bearing PB2 627E, and provides partial but strong support to either an H9N2 or H1N1 FluPol bearing PB2 627E (Bi et al., 2019). It has been noted that this region resembles a SUMO interaction motif (SIM)-like sequence (Domingues & Hale, 2017). Although SUMO could not be shown to directly interact with the SIM-like sequence, SUMO interactions are typically low affinity and transient, and in a gain of function approach, insertion of known SIM-sequences into huANP32A partially enhanced the ability to support an AIV FluPol in human cells (Domingues & Hale, 2017). However, a specific SUMO-decorated interaction partner has not been identified. In contrast, inserting the 27 aa duplication into huANP32A alone is not sufficient to rescue avian FluPol activity (Bi et al., 2019). Nevertheless, inserting the 29 aa insertion from chANP32A₂₉ into huANP32A has been shown to partially rescue AIV polymerase activity in human cells, whilst stably expressing chANP32₂₉ restores viral replication almost to humanised levels (Baker et al., 2018). Moreover, deleting this region from chANP32A₂₉ further reduces activity and binding (Mistry et al., 2020).

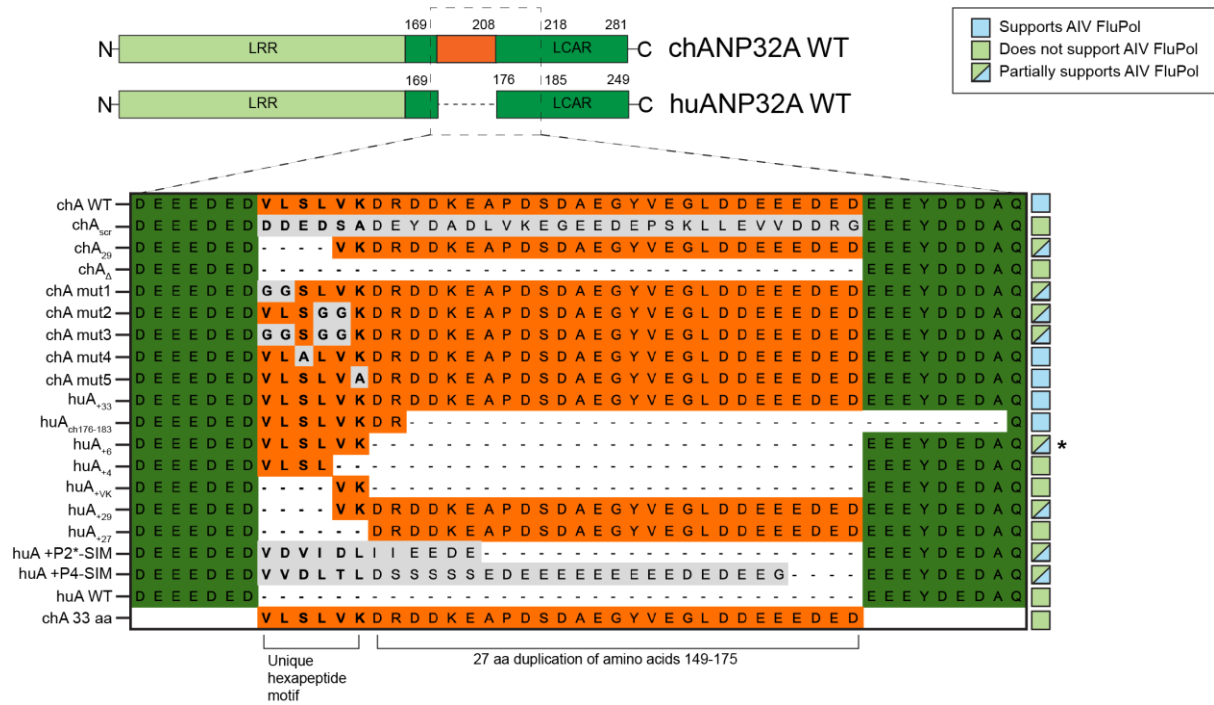


Figure 13: Sequence alignment of ANP32A mutants involving the 33 aa avian insertion.

The unique hexapeptide sequence at the start of the avian insertion is particularly important for the support of AIV FluPol, as substitutions/deletions in this region significantly decrease both activity and binding. In contrast, inserting the 27 aa duplication into huANP32A is not sufficient to support AIV FluPol, although the 29 aa of chANP32A₂₉ (27 aa duplication plus the final 2 aa of the hexapeptide sequence) can provide partial support. Exogenously expressing the 33 aa avian insertion, either alone or alongside huANP32A, has been shown to be insufficient to overcome host restriction. Note that no study has directly compared all mutants described here. 'Partial support' implies any significant increase in support of avian FluPol over that of WT huANP32, but that does not reach the same levels as either humanised FluPol 627K or FluPol 627E+chANP32A. Where mutants have been investigated in multiple studies, nomenclature may differ from that used here. Where multiple names exist, efforts have been made to use the nomenclature that most clearly describes the mutant. HuA₆ is marked with an asterisk as it demonstrated a strain-specific phenotype, with partial but strong support for two avian(-like) FluPol constellations investigated (H9N2 627E and H1N1 PR8 K627E) but full support for a further H7N9 FluPol. AIV= avian influenza A virus; aa= amino acids; chA WT= chicken ANP32A wild-type (Long et al., 2016); chA_{scr}= chicken ANP32A with a scrambled 33 aa insertion (Long, Idoko-Akoh, et al., 2019); chA₂₉= chicken ANP32A splice variant/deletion mutant lacking the first 4 aa of the insertion (Baker et al., 2018; Domingues et al., 2019; Mistry et al., 2020); chA_Δ= chicken ANP32A splice variant/deletion mutant lacking the 33 aa insertion (Baker et al., 2018; Domingues et al., 2019; Long et al., 2016); chA mut1-5= series of mutants introducing glycines into key positions of the hexapeptide motif (Domingues & Hale, 2017); huA₃₃= human ANP32A plus 33 aa avian insertion (Long et al., 2016); huA_{ch176-183}= human ANP32A with residues 176-183 replaced with the corresponding residues from chicken ANP32A (Carrique et al., 2020); huA₆= human ANP32A plus the unique hexapeptide motif at the start of the avian 33 aa insertion (Bi et al., 2019); huA₄= human ANP32A plus the first 4 aa from the unique hexapeptide motif at the start of the avian 33 aa insertion (Bi et al., 2019); huA₁₋₄= human ANP32A plus the final 2 aa from the unique hexapeptide motif at the start of the avian 33 aa insertion (Bi et al., 2019); huA₂₉= human ANP32A plus the 29 aa insertion present in the chicken ANP32A₂₉ splice variant (Baker et al., 2018); huA₂₇= human ANP32A plus the 27 aa duplication present in the 33 aa avian insertion (Bi et al., 2019); huA+P2*-SIM/ huA+P4-SIM= human ANP32A plus established SIMs (Domingues & Hale, 2017); huA WT= human ANP32A wild-type (Long et al., 2016); chA 33 aa= chicken ANP32A 33 aa insertion (Bi et al., 2019).

ChANP32A binds FluPol with greater affinity than huANP32A, and this increased interaction maps to both the avian insertion and the FluPol 627 domain (Baker et al., 2018; Domingues & Hale, 2017; Mistry et al., 2020). Surprisingly, initial binding studies suggested that these interactions are independent of the 627 domain signature i.e., both FluPol 627E and 627K show equally strong binding to chANP32A and equally reduced binding to huANP32A (Baker et al., 2018; Domingues & Hale, 2017; Mistry et al., 2020). Nevertheless, careful analysis using sensitive NMR assays suggest that interactions between the LCAR tail and 627-NLS domain are destabilised in the host restricted scenario (FluPol 627E plus huANP32A) (Camacho-Zarco et al., 2020). Camacho-Zarco et al. demonstrate that the 627K domain forms an exposed basic surface across which the acidic huANP32A LCAR can sample, resulting in a dynamic, polyvalent interaction with moderately high binding affinity. The presence of glutamic acid in the 627 domain disrupts this basic surface and the polyvalency of the interaction, resulting in a weakened binding affinity. Importantly, many of the disrupted interactions are observed in regions of PB2 corresponding to known mammalian-adapting residues. In contrast, the additional length of the chANP32A IDD (avian insertion plus LCAR) allows recruitment of additional contacts to FluPol 627E, that compensate for the disrupted charged surface and restore overall binding affinity. Significantly, compensating contacts are particularly focused within the unique hexapeptide sequence in the insertion (Camacho-Zarco et al., 2020). Such a mechanism is further supported by the cryo-EM structure of the ANP32-stabilised replicase complex (Carrique et al., 2020). Although the LCAR is primarily unresolved, additional density is observed in a groove formed between the two opposing 627 domains of FluPol_R and FluPol_E in the chANP32A structure. The flexibility of this region prevents accurate assignment, however the authors estimate the density places an additional 20-30 aa (~residues 155-190) in contact with the two polymerases. This would appear to position the unique hexapeptide motif directly onto the 627 domain of FluPol_R, again suggesting this sequence functions to stabilise the interaction between the asymmetric dimer and ANP32A (Carrique et al., 2020). The current model for ANP32-mediated host restriction is summarised in the schematic in Figure 14.

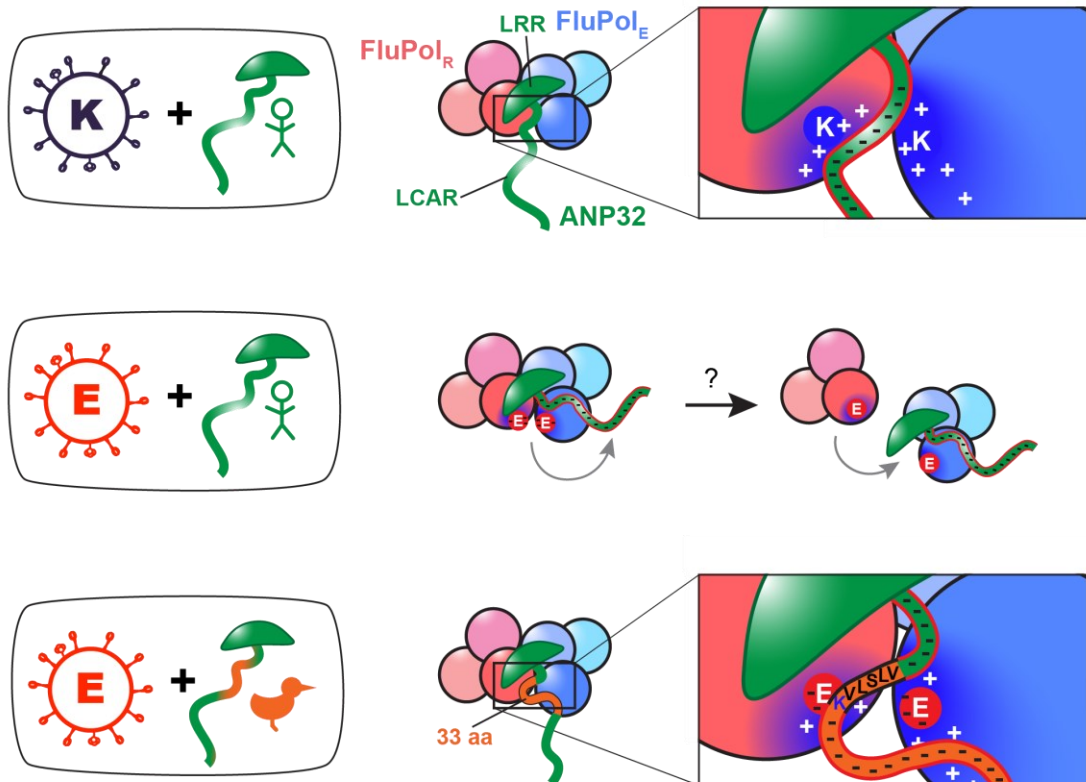


Figure 14: Proposed model for ANP32-mediated host restriction.

Mammalian-adapted FluPol 627K presents a continuous basic surface across the PB2 627 domain to which the acidic ANP32A LCAR can form multiple, low affinity, transient interactions resulting in an overall moderately high binding affinity. On avian FluPol 627E, this basic surface is disrupted by the presence of acidic glutamic acid at position 627, disrupting the polyvalent interaction (Camacho-Zarco et al., 2020). Speculatively, this could destabilise the asymmetric dimer, causing it to break apart. Nevertheless, huANP32A remains bound to at least one FluPol, as interactions are still detected (and for many binding assays are indistinguishable from the functional interaction with FluPol 627K) (Baker et al., 2018; Domingues & Hale, 2017; Mistry et al., 2020). Alternatively, huANP32A could remain bound to the full dimer, but fail to stabilise the correct conformation. In contrast, the addition of the 33 aa avian insertion in chANP32A allows recruitment of additional contacts with FluPol 627E that compensate for the disrupted charged surface. Cryo-EM data hints that the hexapeptide sequence (which unlike the rest of the LCAR contains a basic residue) may be positioned directly over the 627 domain – thus speculatively could interact productively with the acidic glutamic acid present in FluPol 627E (Carrique et al., 2020). huANP32A= human ANP32A; chANP32A= chicken ANP32A.

The precise significance of the additional LCAR and 627 domain interaction, and therefore the mechanism of host restriction, has not been elucidated. The most obvious explanation is that the additional stability provided by the LCAR-627 interaction is essential for proviral activity. Whilst interactions can still be detected in the host restricted scenario (Baker et al., 2018; Domingues & Hale, 2017; Mistry et al., 2020), it is plausible that this is between ANP32 and one half of the FluPol dimer, or an incorrectly configured dimer. In the host restricted combination of huANP32A and FluPol 627E, a decrease in interaction is not observed when vRNA is titrated in, unlike the functional combinations of FluPol 627K and huANP32A, or FluPol 627E and chANP32A (Mistry et al., 2020). This implies that the lack of a compatible LCAR-627 interaction somehow prevents correct onwards processing to elongation. Alternatively, although a recent study found FluPol 627E to be restricted in vRNA and cRNA

synthesis (Nilsson-Payant et al., 2021), several other reports have demonstrated that avian FluPol can synthesise and stabilise cRNPs in mammalian cells (Mänz et al., 2012; Nilsson et al., 2017). This could suggest that ANP32 is not required for cRNA synthesis at all, as implied in the first set of *in vitro* cell-free assays (Sugiyama et al., 2015), or that ANP32 is required for cRNA synthesis but that an incompatible 627-LCAR interaction can be tolerated in this step. The latter explanation suggests the 627-LCAR interaction is required for something specific to vRNA synthesis. Nevertheless, although cRNPs can be produced by avian FluPol in mammalian cells, it has been suggested that these may be aberrant, and therefore further replication from these templates does not occur (Mänz et al., 2012). Speculatively, as ANP32 is implicated in encapsidation, an incompatible 627-LCAR interaction might allow cRNA synthesis but result in incorrect packaging into cRNPs. Interestingly, in mini genome assays, 3-8/3-5-8 promoter mutants (Section 1.3.6) appear to overcome host restriction through allowing avian FluPol to utilise huANP32 proteins, despite the mismatched LCAR-627 domain. 3-5-8 promoter mutants remain dependent on ANP32 as replication is completely ablated in human cells lacking ANP32A/B expression, and exogenous expression of huANP32A/B restores activity (Mistry et al., 2020). However, unlike WT RNA, titrating in 3-5-8 RNA disrupts the interaction between FluPol 627E and huANP32A, suggesting functional processing to elongation (Mistry et al., 2020). Finally, titrating in exogenous chANP32A to FluPol 627E mini genome assays using 3-8 mutants either does not enhance replication further (Baker et al., 2018), or has a very small additive effect (Nilsson-Payant et al., 2021). Nevertheless, the mechanism of how RNA promoter mutants can compensate for a mismatched LCAR-627 interaction is currently unclear.

1.4.6 Summary and outstanding questions

ANP32 proteins are potent pro-viral host factors that are essential for influenza virus replication, but not transcription. In summary, our current understanding of the mechanism by which ANP32 supports FluPol replication is as follows:

1. Binding of a pro-viral ANP32 orthologue stabilises the replicase configuration of FluPol (Camacho-Zarco et al., 2020)
2. ANP32 coordinates co-replicative encapsidation:
 - a. The LRR domain of ANP32 bridges a dimer of two FluPol heterotrimers, recruiting an additional FluPol for encapsidation of the nascent RNP (Carrique et al., 2020)
 - b. The LCAR domain recruits NP. NP binding is outcompeted by RNA, allowing transfer to the nascent RNA strand (F. Wang et al., 2022)
3. During the transition from initiation to elongation, ANP32 dissociates (Mistry et al., 2020)
4. Additional interactions between the 627 domain and LCAR stabilise FluPol-ANP32 binding. Incompatibilities between the 627 domain of unadapted avian FluPol and the huANP32 LCAR destabilise this interaction and underly host restriction of AIV FluPol in mammalian cells (Camacho-Zarco et al., 2020; Carrique et al., 2020).

Nevertheless, several questions remain, including clarifying whether ANP32 does indeed play a role in both vRNA and cRNA synthesis, and if so whether host restriction is also imposed on both vRNA and cRNA synthesis. Such investigations may help clarify the precise role of the 627-LCAR interaction (Section 1.4.5). Moreover, how ANP32 fits into the wider context of the cell, and in particular other host factor interactions, is not yet known. On the other hand, it has been increasingly appreciated that ANP32 orthologues that were previously thought to be virologically inert can be co-opted by the virus in the absence of a preferred paralogue (Goldhill et al., under revision). It will be interesting to see whether FluPol can adapt to become completely ANP32-independent, potentially through the use of other LRR or LCAR containing proteins. If this is the case, this could significantly reduce the impact of future translational outputs, for example drugs designed to disrupt this virus-host interaction.

1.5 THESIS OBJECTIVES

One of the most important host range barriers limiting the emergence of novel pandemic IAV strains is adaptation of the RdRp (FluPol) for efficient activity in a new host cell environment. Moreover, pharmacologically targeting FluPol activity is a validated, effective therapeutic approach for treating influenza infection. Despite the translational opportunity, much remains unknown about this stage in the viral life cycle, including how the virus regulates the different roles of the vRNP to enable transcription, replication and export to occur in a timely manner. Therefore, our overarching aim in this work was to increase our understanding of IAV polymerase activity and host restriction.

The precise aims of each results chapter are outlined below:

1. Chapter Two – Visualising and quantifying influenza A virus polymerase activity:
 - ◇ To optimise an approach for bulk quantification of IAV vRNA, cRNA and mRNA
 - ◇ To develop an imaging approach that can distinguish different IAV RNA species, suited to dissecting the subnuclear localisation of FluPol activity, and investigate FluPol activity at a single-cell level
2. Chapter Three – Human ANP32 proteins enhance influenza A virus cRNA synthesis:
 - ◇ To clarify the role of huANP32A and huANP32B in cRNA synthesis
 - ◇ To investigate the role of huANP32E in cRNA synthesis
3. Chapter Four – Avian influenza A virus polymerase can utilise human ANP32 proteins to support cRNA synthesis:
 - ◇ To investigate whether avian FluPol (PB2 627E) is specifically restricted in vRNA synthesis, or vRNA and cRNA synthesis, in mammalian cells, and interpret this within the context of ANP32
 - ◇ To identify an ANP32 mutant that can support cRNA synthesis, but not vRNA synthesis

CHAPTER TWO: VISUALISING AND QUANTIFYING INFLUENZA A VIRUS POLYMERASE ACTIVITY

2.1 INTRODUCTION

Traditionally, FluPol activity has been primarily studied using bulk assays such as primer extension, mini genome assays, or through biochemical assays using purified polymerase protein (Section 1.3.1) (te Velthuis et al., 2018). Such analyses have been instrumental in informing our current understanding of how FluPol functions. Nevertheless, in recent years there has been an increasing appreciation of the value of single cell approaches, that reveal detail that is masked at the population level (Ciuffi et al., 2016; Müller et al., 2019; D. Wang & Ma, 2017). Single cell approaches have revealed the contribution of cell-to-cell variation, even within clonal populations of cells, as well as the role of viral quasispecies to the outcome of infection (Heldt et al., 2015; Russell et al., 2018, 2019; D. Wang & Ma, 2017).

Imaging approaches inherently capture data at the single cell level. Moreover, they capture spatial detail, which is essential to grasp the full interplay between the virus and highly organised, densely packed host cell (Müller et al., 2019; D. Wang & Ma, 2017). Traditionally, viral imaging of IAV has been complicated by multiple factors. Although well suited to the scale of IAV particles, electron microscopy (EM) has been hampered by a lack of readily discernible morphological structures formed during IAV replication (Müller et al., 2019). Although ultrastructural changes have been reported in the cytoplasm and nuclei of IAV infected cells since the 1960s (Figure 9, Section 1.3.7), the biological significance has never been elucidated (Josset et al., 2008). Meanwhile, live-cell imaging is challenging as the compact genome of IAV poorly tolerates fluorescent tags (Müller et al., 2019; Touizer et al., 2021). Discerning individual viral particles requires single molecule resolution, whilst the multifunctional nature of viral proteins makes it difficult to associate localisation with a specified function. Nevertheless, several advances have eroded these challenges, including the development of new, minimally-invasive tagging techniques, improved live-cell imaging, correlative light and electron microscopy (CLEM) and the increased availability of super-resolution approaches. Such advances have driven novel insights into many areas of the IAV life cycle (Müller et al., 2019; Touizer et al., 2021). However, very few have focused on events in the nucleus (Touizer et al., 2021).

Within the nucleus, FluPol transcription localises to sites of active host transcription (Engelhardt et al., 2005). Later in infection, vRNP export requires transport through SC35+ speckles, followed by localisation to the heterochromatin (Ando et al., 2016; Chase et al., 2011). However, the precise nuclear localisation of cRNA and vRNA synthesis, as well as the role of spatial localisation in FluPol regulation and host restriction, is unknown. Therefore, at the start of this project, we initiated the development of several imaging approaches with the aim to visualise viral replication products within the nucleus and gain insight into the spatiotemporal details of FluPol activity. The RNA FISH assay RNAscope proved to be most tractable and is presented here, alongside optimisation of a complementary tagged reverse

transcription-quantitative PCR (RT-qPCR) approach for quantifying vRNA, cRNA and mRNA at the population level. We established that RNAscope can detect IAV vRNA and cRNA/mRNA at early timepoints and low MOIs and is compatible with indirect immunofluorescence (IIF) co-staining. Although RNAscope probes cannot distinguish IAV cRNA and mRNA under standard infection conditions, combining RNAscope with *in situ* cRNP stabilisation/replication assays enabled specific visualisation of the cRNA – which can be used as a marker for the site of replication. These approaches are used in subsequent chapters to explore the mechanism by which the host factor ANP32 supports FluPol (chapter 3), and host restriction of avian polymerase in mammalian cells (chapter 4). Finally, in collaboration with Dr Lucy Collinson and Dr Alana Burrell at the Electron Microscopy (EM) Science Technology Platform at the Francis Crick Institute, we established an RNAscope protocol compatible with CLEM. We hope future work with this approach will allow the localisation of IAV vRNA and +RNA (cRNA/mRNA) at the ultrastructural level to be explored.

Tagged RT-qPCR primers targeting segment 6 were designed by this author, Dr Ecco Staller and Dr Thomas Peacock. Tagged RT-qPCR primers targeting segment 4 were designed and optimised by a master's student, Amalie Rasmussen, under the supervision of this author. The CLEM data presented in Figure 29A was collected by Dr Alana Burrell. The RT-qPCR data presented in Figure 29E,F,G was collected by Amalie Rasmussen. Figure 24 and Figure 25, and accompanying text, are adapted from Swann et al., in press.

2.2 RESULTS

2.2.1 Establishing a tagged RT-qPCR approach for bulk quantification of vRNA, cRNA and mRNA

The primary aim of this chapter was to develop a single cell, imaging assay suited to studying the spatiotemporal details of FluPol activity. Nonetheless, we required a quantitative bulk assay as a complementary tool to characterise replication on a population level. For this, we optimised a tagged RT-qPCR approach using primers that are specific to both the genome segment and RNA species (vRNA/cRNA/mRNA), thus enabling either transcriptional or replicative activity of the FluPol to be resolved (te Velthuis et al., 2018). Although RT-qPCR has traditionally been plagued with issues of background from non-specifically primed products (identified using reverse transcription (RT) controls in which the primer is omitted (-RT primer controls)), the introduction of a tagged methodology, based on an approach developed for hepatitis C virus (Lanford et al., 1994), has significantly improved specificity (Kawakami et al., 2011). In this approach, sequence-specific primers used in the RT step are designed with an additional 20 nucleotide ‘tag’ on the 5’ end. This tag is subsequently targeted by the qPCR forward primer, combined with a segment-specific reverse primer (Figure 15). In combination, this approach prevents PCR amplification of non-specifically primed products (Kawakami et al., 2011).

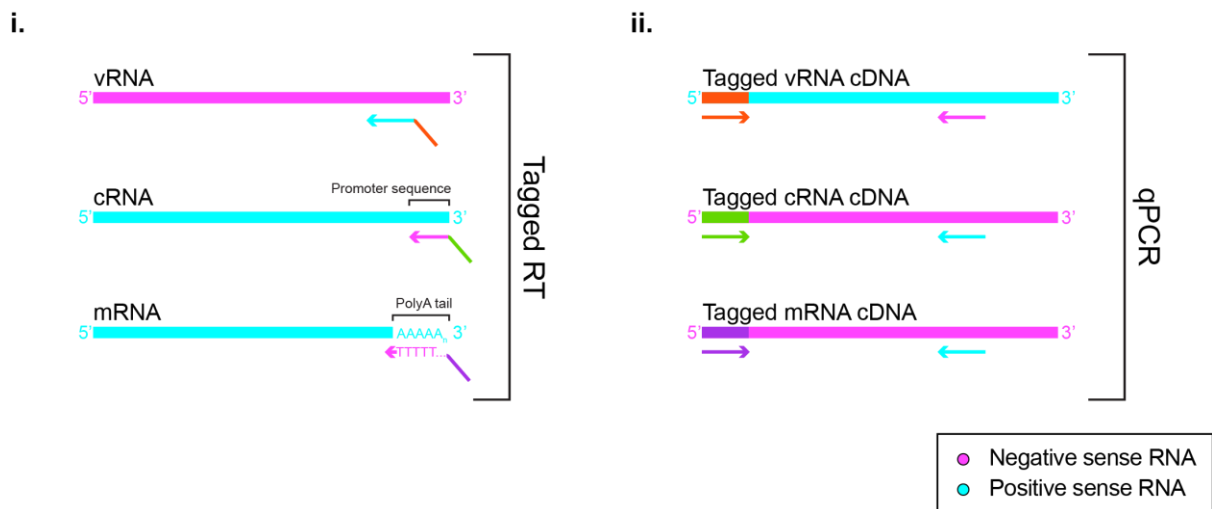


Figure 15: Schematic outlining the tagged vRNA/cRNA/mRNA RT-qPCR approach.

(i) Sequence-specific primers for RT are designed targeting the unique portions of the three RNA species: any portion of the negative-sense vRNA, the unique 16 nt promoter region of the positive-sense cRNA and the polyA tail of the positive-sense mRNA. In addition, RT primers include an additional unique 20 nt ‘tag’ on the 5’ end. (ii) Incorporation of tagged RT primers results in cDNA molecules labelled with unique 5’ tags. Forward primers for the qPCR reaction target the introduced tags, whilst reverse primers target a segment-specific sequence. In combination, this approach prevents PCR amplification of non-specifically primed products. RT= reverse transcription; nt= nucleotide.

To establish a tagged RT-qPCR approach, tagged primers based on those published by Kawakami et al. were designed to target segment 6 (NA) of the common lab-adapted H1N1 strain, PR8. The NA segment was chosen as viral strains in use in the lab are frequently rescued as either 6:2 or 5:3 reassortants with the internal genes from the viral strain of interest combined with either the HA/NA or HA/NA/M structural genes (whichever is more stable) from PR8. Such reassortants allow replication driven by different polymerase proteins to be examined independent of differences driven by viral entry (i.e., by the strain-specific HA/NA). Reassortants with PR8 external proteins are also considered safer than the full wild-type strains and allow polymerase constellations to be studied at containment level 2 (CL2) that would otherwise be categorised at CL3 or be regulated under the Specified Animal Pathogen Order (SAPO) system of containment. Thus, targeting the external glycoproteins from PR8 allows RT-qPCR primer sets to be standardised across several different viruses in use in the lab. This eliminates variation in primer efficiency when comparing across strains as well as having obvious practical benefits. Although most approaches design the mRNA RT primer to cross both the polyA tail and a small portion of the upstream mRNA sequence, we found this approach resulted in cross-reactivity with cRNA. Therefore, for NA mRNA reactions we converted total cellular mRNA into cDNA using a tagged-polydT reaction, then used the internal NA-specific reverse primer during the qPCR step to specifically detect NA mRNA.

To confirm whether tagged primers reduce background from non-specifically primed products, cells were either infected with PR8 (MOI=3) or mock infected, and total RNA extracted 7hpi. Viral RNA was then quantified using an untagged set of primers that were previously in use in the lab targeting HA vRNA, cRNA and mRNA, alongside the new tagged primer sets targeting NA vRNA, cRNA and mRNA. The Ct values resulting from both full reaction conditions (+RT primer), and conditions in which the RT primer was omitted (-RT primer) were compared (Figure 16A,B,C). In the absence of RT primers, the untagged HA primer set resulted in high non-specifically primed background in the infected, but not mock infected, samples. In contrast, using the tagged NA primer set abrogated this non-specific signal, confirming the effectiveness of the tagged approach. It is interesting to note, despite the large difference in Ct values obtained with the two primer sets in infected -RT controls, a modest difference in Ct values was observed in the full reactions. This may be due to the primers targeting different segments/sequences and thus not being directly comparable. Nevertheless, it could also suggest that the non-specific background is caused by viral RNAs that are alternatively primed only in the absence of a specific primer. In agreement with this interpretation, mock infected samples quantified with the untagged primers did not show high levels of background signal, suggesting the background is specifically viral. Following optimisation of the tagged RT-qPCR targeting segment 6, an additional set of tagged primers targeting segment 4 (HA) were designed and optimised by a master's student, Amalie Rasmussen, so that observations could be compared across two different segments.

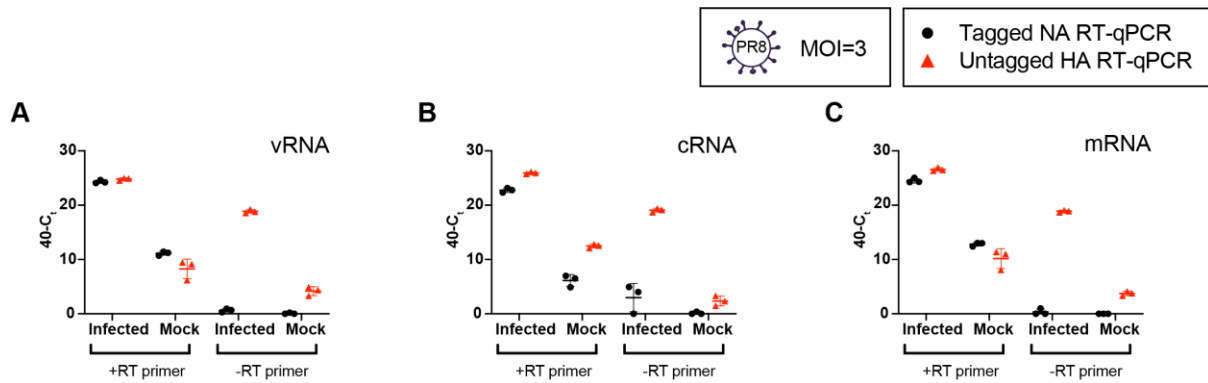


Figure 16: -RT background is abrogated by tagged primers.

(A-C) eHAP WT cells were (mock) infected with PR8 (MOI=3), and total RNA collected 7hpi. (A) vRNA, (B) cRNA, or (C) mRNA RT-qPCR analysis was performed using tagged primer sets targeting neuraminidase (tagged NA RT-qPCR), or untagged primer sets targeting haemagglutinin (untagged HA RT-qPCR). RT reactions were performed either including (+RT primer) or omitting (-RT primer) the RT primer. The resulting C_t values are plotted as $40-C_t$. $n=3$ biological replicates, plotted with mean $\pm$ s.d.. MOI= multiplicity of infection; hpi= hours post-infection; RT= reverse transcription.

Although a dedicated analysis to assess cross-reactivity was not performed, the data from our subsequent experiments (chapters three and four) implies cross-reactivity is minimal, as we observed multiple examples of, for example, cRNA accumulation in the absence of any detected mRNA accumulation. The only cross-reactivity we cannot comment on is that of the cRNA primers for vRNA, as there was no scenario in which only vRNA was present, however this is also the least likely source of cross-reactivity. A further disadvantage associated with RT-qPCR is background contamination due to its high sensitivity (te Velthuis et al., 2018). We did note higher than expected background signal in vRNA and mRNA reactions for mock infected samples ($C_t \sim 28-30$) that was likely due to low level environmental contamination and was persistently present despite careful precautions. Nevertheless, as our method of quantitation was comparative and always relative either to a mock or 0hpi timepoint, we assumed this background equally affected all samples and was accounted for using these controls.

2.2.2 Visualisation of IAV NA vRNA using RNAscope

To explore the spatiotemporal details of IAV replication, we optimised an RNA fluorescence *in situ* hybridisation (RNA FISH) approach, targeting the RNA products of FluPol activity. Our preliminary work investigating click chemistry as an approach (see discussion) suggested that visualising influenza RNA species, particularly at early timepoints, requires sensitive techniques that can amplify low levels of signal. Fortunately, several variants of RNA FISH now exist in which the signal of the original RNA molecule is sufficiently amplified to achieve single molecule sensitivity (Pichon et al., 2018). This includes the commercial RNA FISH assay, RNAscope (F. Wang et al., 2012), which we chose to use in this work. In RNAscope, pairs of z-shaped oligonucleotide probes are designed that bind along the target RNA of interest. When bound in tandem, each pair of probes cooperates to form a landing platform to which a series of branching probes can bind, eventually resulting in binding sites for up to 400 fluorescent probes/z-pair. By spreading ~ 20 Z-pairs along the target of interest, RNAscope generates a massively amplified signal from the original detected sequence. Simultaneously, preventing

amplification unless both Z-shaped probes are bound in parallel reduces off-target signal. In combination, this design ensures a strong signal-to-noise ratio (F. Wang et al., 2012). A schematic of the approach is illustrated in Figure 17.

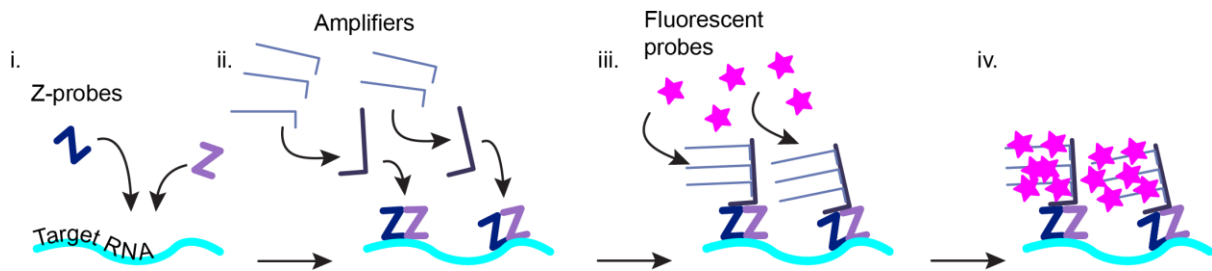


Figure 17: The RNAscope assay.

(i) RNAscope works through binding pairs of Z-shaped complimentary oligonucleotide probes in tandem along a target RNA sequence of interest. (ii) When correctly bound to the target sequences, probe pairs form a 28 base pair binding platform to which multiple rounds of amplifying branching probes can bind. (iii) In combination, these branching probes provide up to 400 binding sites for fluorescent probes. (iv) Through tiling up to 20 Z-pairs along a target sequence of interest, up to 8,000 fluorescent labels can bind to each detected molecule, massively amplifying the signal. Through only allowing the initial amplifying probe to bind when both Z-probes are present, the assay reduces off-target effects. In combination, this results in an assay with a very high signal-to-noise ratio and single molecule sensitivity (F. Wang et al., 2012).

Initially, an RNAscope probe was designed targeting the vRNA for segment six (NA) of PR8. As with the RT-qPCR primers, this segment and strain were chosen due to cross-compatibility with 6:2 and 5:3 reassortants in use in the lab, as well as for use with complete PR8. In an initial experiment, eHAP WT cells were either mock infected or infected with PR8 at a high MOI (MOI=3). eHAP cells were chosen as they are the background used in the Barclay lab for CRISPR knockout lines, and thus likely to be used in further experiments investigating the role of host factors. 8hpi, cells were fixed and hybridised either to the new segment 6 vRNA probe (NA vRNA), or to established positive control (PPIB) or negative control (DapB) probes, provided with the assay. PPIB (Peptidylprolyl Isomerase B) is a (human) host transcript that is ubiquitously expressed at low levels, thus acts as a quality control for RNA integrity in the sample. DapB (dihydrodipicolinate reductase) is the universal negative control provided by RNAscope, and targets a transcript from the soil bacterium, *Bacillus subtilis* strain SMY (Advanced Cell Diagnostics (ACDBio), 2022). By 8hpi, very high levels of NA vRNA are expected to be present throughout the nucleus and cytoplasm (Lakdawala et al., 2014). In agreement, extremely bright staining was observed throughout infected cells stained with the NA vRNA probe (Figure 18). A low level of diffuse background fluorescence was observed in the mock infected sample. Both positive and negative control probes showed expected staining.

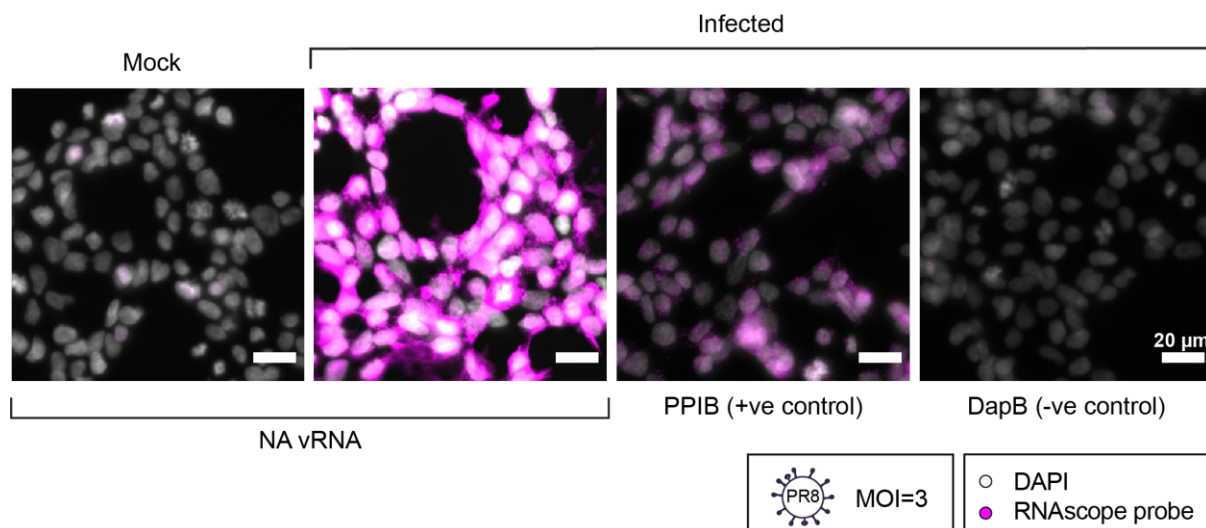
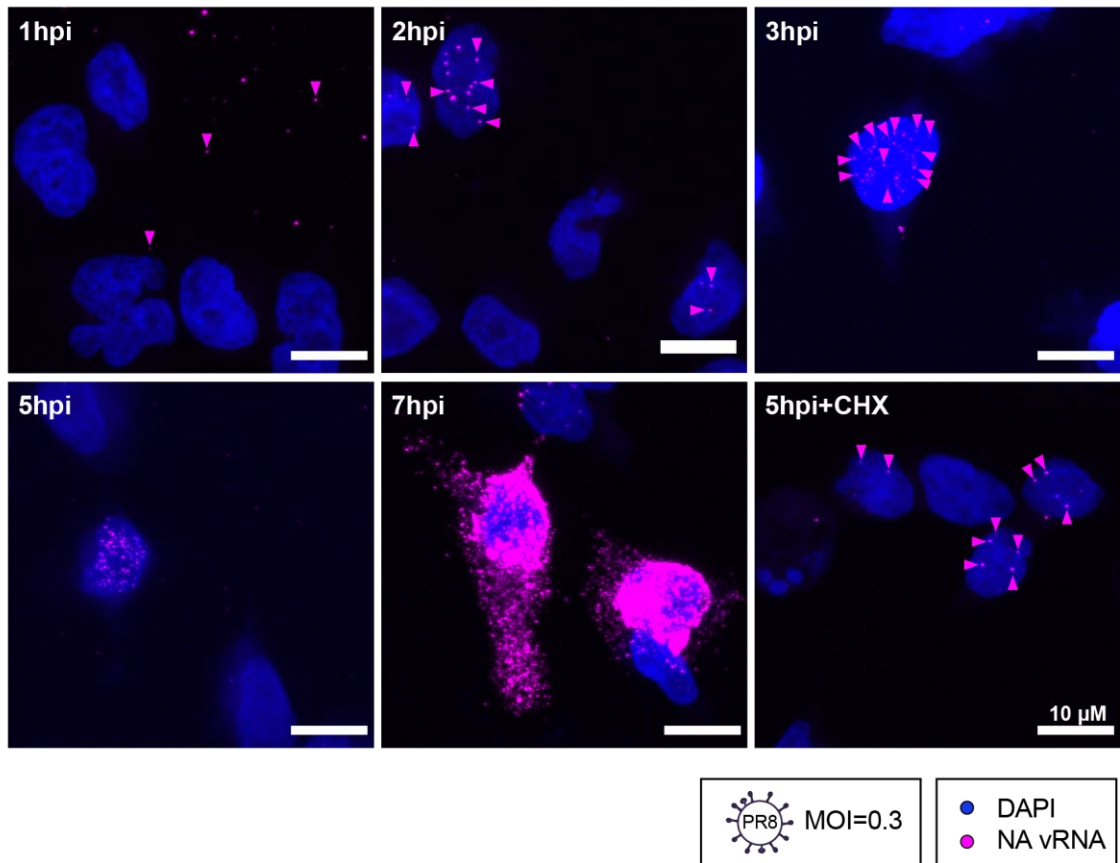


Figure 18: RNAscope detects segment 6 vRNA.

Preliminary characterisation of RNAscope probes (n=1 experiment) showing human eHAP cells (mock) infected with PR8 (MOI=3), 8 hours post-infection, prior to hybridisation with probes against the indicated RNA species and visualisation using a Zeiss Cell Observer Widefield microscope and 40x objective. All scale bars are 20 μ m. MOI= multiplicity of infection.

In a further experiment to characterise the NA vRNA probe, an infection time-course was undertaken in eHAP WT cells using PR8 at a low MOI (MOI=0.3). As a control, one sample was treated with the translation inhibitor cycloheximide (CHX), which inhibits viral replication by preventing the translation of RNP components required to encapsidate nascent RNPs (Vreede et al., 2004). All samples were stained using the NA vRNA probe and counterstained with DAPI. Encouragingly, incoming NA vRNA could be visualised as early as 1hpi (Figure 19A). At this timepoint, genomes were not yet visible within the cell nucleus, and appeared to be predominantly present outside the cells on the coverslip surface (see magenta arrowheads). By 2hpi, NA vRNA was primarily present in the cell nucleus. 2/3 NA vRNA puncta were observed in most infected nuclei (e.g. cell bottom right). Interestingly however, despite the early timepoint and low MOI, some nuclei already contained >8 NA vRNA puncta at this timepoint (see nucleus top left). Speculatively, these could be super-infected cells, as have been described using single cell transcriptomics (Russell et al., 2018). However more detailed analysis is required to clarify whether these cells have already initiated replication, or if they coincidentally received more infecting genomes. By 3hpi, the number of NA vRNA puncta present in infected cell nuclei significantly increased, suggesting replication has initiated by this time point. It is unlikely the increase in NA vRNA is caused by additional incoming genomes trafficking to the nucleus between 2 and 3hpi, as very few NA vRNA genomes were detected outside the nucleus 2hpi. Moreover, infections undertaken in the presence of CHX (5hpi+CHX), in which replication is blocked but vRNP import is unaffected, more closely resembled the 2hpi timepoint than 3hpi, with <10 NA vRNA spots present per infected nucleus. In the absence of CHX, further replication was observed by 5hpi. By 7hpi, replication had largely finished, and most NA vRNA molecules had been exported to the cytoplasm (Figure 19A,B).

A



B

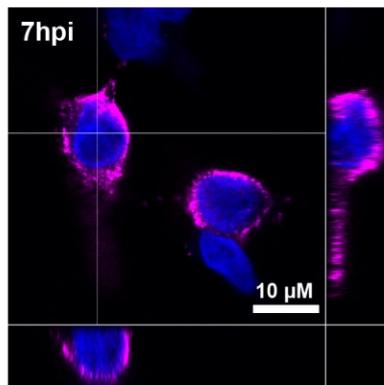


Figure 19: RNAscope can detect segment 6 vRNA at early timepoints and low MOIs.

(A) Representative maximum intensity projections (n=2 experiments; ~50 cells analysed/ sample) showing human eHAP cells infected with PR8 (MOI=0.3), 1, 2, 3, 5 and 7 hpi, prior to hybridisation with the RNAscope probe targeting NA vRNA. An additional sample was infected in the presence of CHX and fixed 5 hpi. Images were visualised using a Leica SP5 inverted confocal microscope and 63x objective. (B) Orthogonal view of the 7 hpi timepoint in (A). All scale bars are 10 μm, hpi= hours post-infection, MOI= multiplicity of infection, CHX= cycloheximide.

2.2.3 RNAscope is compatible with indirect immunofluorescence co-staining

During the RNAscope protocol, samples are first treated with protease, prior to hybridisation to RNAscope probes. This step is undertaken both to permeabilise cell membranes, as well as expose target RNAs which may be blocked by binding proteins. The length of protease treatment is optimised for the cell type in use, and in some cases may be omitted all together (ACDBio tech support, personal communication, June 2019). Considering vRNA is packaged into vRNPs, we assumed the protease treatment would be essential for exposing and visualising NA vRNA. Simultaneously, we were concerned this treatment would render RNAscope incompatible with immunofluorescence co-staining, through digestion of epitopes important for antibody recognition. To investigate whether this was the case, we undertook an RNAscope and immunofluorescence co-staining experiment on cells infected with PR8 (MOI=1), 7hpi, using three different protease treatment conditions: 10 minutes (standard), 2 minutes, and no protease treatment. Samples were then stained using the RNAscope assay, followed by IIF against NP. Surprisingly, no notable difference was observed for either RNAscope or antibody staining across the three different protease treatments: NA vRNA staining does not appear to be dependent on protease stripping of the NA vRNP, whilst antibody detection of NP does not appear to be disrupted by protease digestion (Figure 20). It has been reported that NP binds viral RNA via the phosphate backbone, leaving the base pairs exposed (Baudin et al., 1994), which could explain why protease treatment is not required. Regardless, the clear NA vRNA staining in the absence of protease was particularly encouraging as it suggests this step could be omitted should other antibodies prove to be more sensitive to protease digestion.

Initially we were surprised to find that the NP and NA vRNA staining showed limited co-localisation. In most cells visualised, NA vRNA was largely exported to the cytoplasm whilst NP staining was strongest in the nucleus (examples highlighted with white arrowheads). Moreover, in some rare cells, only yellow staining (IIF against NP) was detected (highlighted with yellow arrowheads). The reverse scenario, of cells that were positive for NA vRNA but not NP protein, was not observed. In the case of NP, it is not surprising to see staining in the absence of NA vRNA, as NP is present both as free monomers and in RNPs coating the other seven segments. It has previously been observed that NP and PB1 co-staining only partially overlap during infection, despite both being present in RNPs (Engelhardt et al., 2005). Moreover, it is known that IAV produces high numbers of defective interfering particles (DIPs), which do not contain a full complement of genome segments (Dou et al., 2017; Russell et al., 2018). DIPs are capable of initiating infection but are unable to generate functional virions for onward infection, unless in the context of co-infection. In tissue culture, the number of DIPs often exceeds the number of infectious particles (Brooke, 2014; Dou et al., 2017). Thus, infection with a DIP lacking the NA segment may explain instances in which cells are positive for NP but not NA vRNA. It was more unexpected to see NA vRNA in the absence of NP, as we would expect all vRNA to be encapsidated. One potential explanation is that the NP antibody may bind an epitope that is masked when bound to vRNA. The monoclonal NP antibody 3/1 has been described to specifically bind NP in vRNP complexes (Eisfeld et al., 2011), i.e., the opposite scenario, and it would be interesting to repeat this co-staining with this antibody. Alternatively, the lack of co-localisation appears to be partially caused by an imaging artefact,

as the imaging settings have been optimised to avoid saturation of the regions of highest signal (the nucleus). Increasing the brightness and contrast reveals that there is far more staining in the cytoplasm than is immediately apparent in these images, and a portion of this staining co-localises with NA vRNA. It is also worth noting that these are preliminary observations as the images were captured as small Z-stacks on a widefield microscope without deconvolution and have not been optimised for co-localisation analysis.

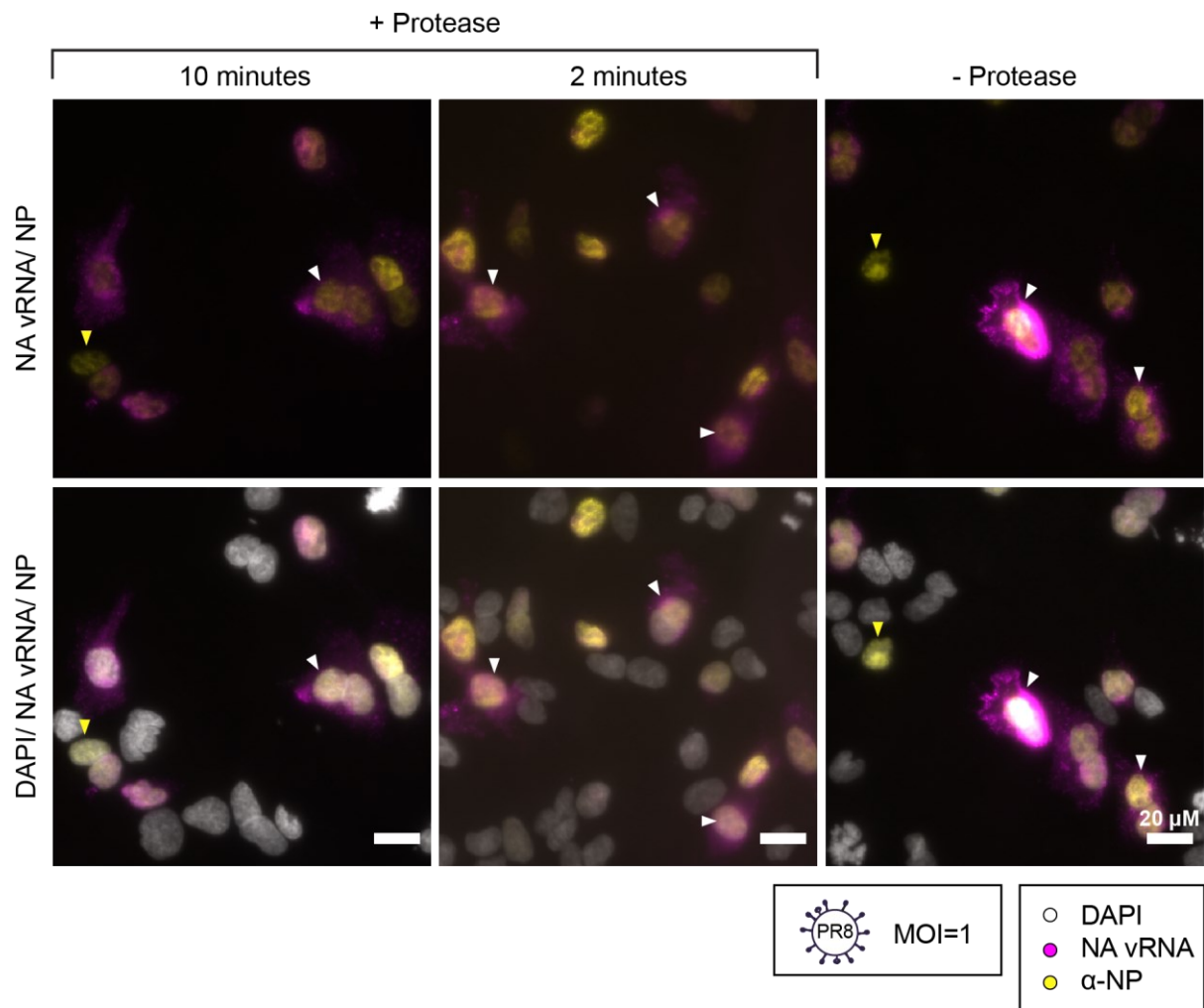


Figure 20: RNAscope is compatible with antibody co-staining.

Representative maximum intensity projections (n=1 experiment; ~100 cells analysed/ condition) showing human eHAP cells infected with PR8 (MOI=1), fixed 7 hours post-infection. Samples were treated with protease III for the indicated length of time, prior to hybridisation to the NA vRNA RNAscope probe followed by indirect immunofluorescence targeting NP. Yellow arrowheads highlight rare cells in which only NP staining was detected. White arrowheads highlight examples of cells in which both NP and NA vRNA are detected, but limited co-localisation is observed. Images were visualised using a Zeiss Cell Observer Widefield microscope with a 40x objective. Scale bars are 20 μ m. MOI= multiplicity of infection.

vRNPs co-localise with serine-5 phosphorylated host Pol II CTD (Ser5P CTD) during transcription (Section 1.3.3) (Engelhardt et al., 2005). Thus, to explore whether vRNPs can be co-localised with a known interaction partner, RNAscope and IIF co-staining was undertaken using an antibody targeting Ser5P CTD (Figure 21). At all timepoints investigated, extensive diffuse Ser5P CTD staining with stronger punctate speckles occurred throughout the nucleus, except the nucleolus from which staining was excluded. Unfortunately, this extensive staining made it difficult to judge co-localisation. Although occurrences of co-localised NA vRNA and strong Ser5P CTD puncta could be observed (indicated with white arrowheads), many other NA vRNA molecules localised to regions of more diffuse staining. In the original study demonstrating NP and Ser5P CTD co-localisation using IF, this issue was overcome through demonstrating a significantly higher degree of co-localisation between NP and Ser5P CTD than NP and a control protein (PCNA, that associates with DNA replication and repair but does not co-localise with active Pol II transcription sites) (Engelhardt et al., 2005). It is possible that such an approach could be used in future. Moreover, despite the early timepoints, many cells showed signs of early replication (compare number of vRNA spots/nucleus at 2.5hpi with 5hpi+CHX, in which replication is inhibited), suggesting our samples show mixed populations of vRNA undergoing transcription, replication and possibly even the start of packaging for export. As it is unknown whether vRNPs remain associated with Ser5P CTD during replication, this further confounded any analysis. It would be interesting to undertake a time-course including very early timepoints and quantify whether co-localisation decreases as replication initiates. Alternatively, examining host factors with lower expression levels, or focusing on markers of known nuclear bodies, might prove more feasible.

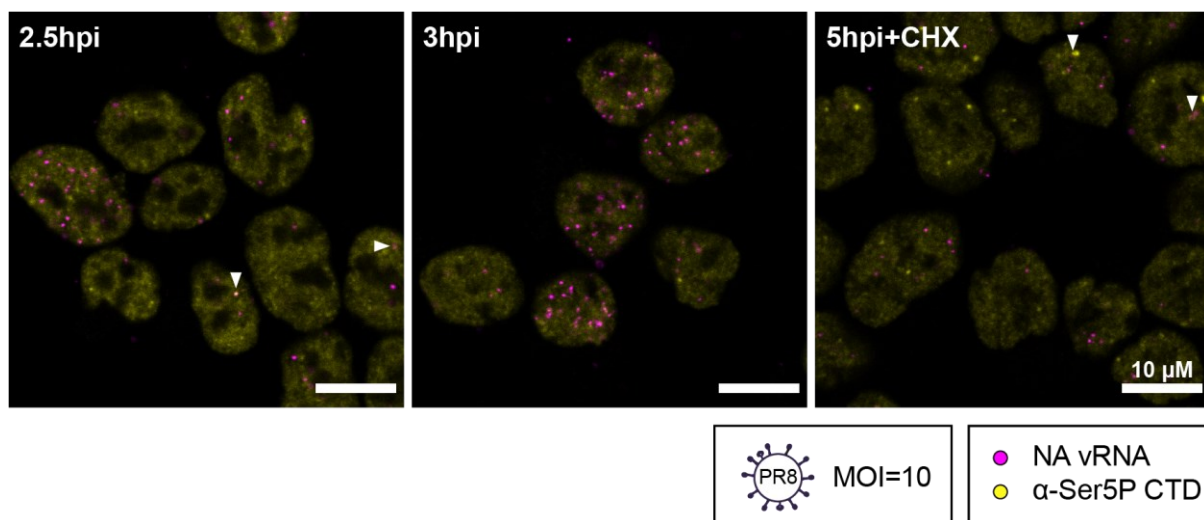


Figure 21: Preliminary investigations into co-localisation analysis using RNAscope and indirect immunofluorescence co-staining. Representative single Z-slices (n=1 experiment; ~50 cells analysed/ sample) from full Z-stacks showing eHAP cells infected with PR8 (MOI=10), 2.5 and 3hpi, prior to hybridisation with the RNAscope probe targeting NA vRNA and indirect immunofluorescence against Ser5P CTD. An additional sample was infected in the presence of CHX and fixed 5hpi. White arrowheads indicate instances of apparent vRNA and Ser5P CTD co-localisation. Images were visualised using a Leica SP5 inverted confocal microscope and 63x objective. Scale bars are 10 μ m. hpi= hours post-infection; MOI= multiplicity of infection; CHX= cycloheximide; Ser5P CTD= host RNA polymerase II serine-5 phosphorylated carboxy-terminal domain.

2.2.4 RNAscope probes cannot distinguish cRNA and mRNA

Both IAV cRNA and mRNA are positive-sense RNA species, differing only by short sequences located at the 5' and 3' ends of the molecules (Figure 22). As a full-length complementary product to the vRNA, the cRNA retains the full promoter sequences required for replication, and thus contains a unique 16 nucleotide sequence at the 3' end relative to the matched segment mRNA. Moreover, the mRNA differs from the cRNA in containing short heterogenous host-derived sequences at the 5' end of the molecule due to cap snatching, as well as a polyA tail at the 3' end (te Velthuis & Fodor, 2016). Whilst cRNA and mRNA can be distinguished by RT-qPCR by using a short primer targeting the promoter sequence or a polydT primer respectively (Kawakami et al., 2011), RNAscope probes have a minimum sequence requirement of 300bp to allow sufficient amplification for detection. Consequently, any RNAscope probe designed to target a particular segment cRNA will cross-react with the matched segment mRNA. We therefore refer to probes targeting cRNA/mRNA as a '+RNA probe'.

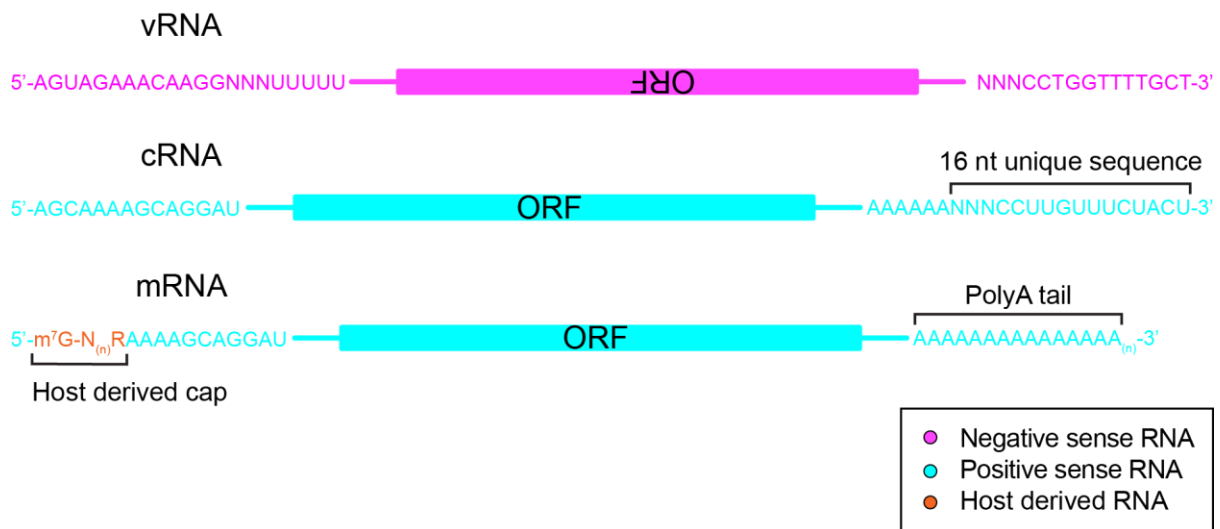


Figure 22: Schematic illustrating the key differences between IAV vRNA, cRNA and mRNA.

m⁷G= 7-methylguanylate cap; ORF= open reading frame; nt= nucleotide; N indicates any nucleotide; R indicates A/G.

To detect IAV cRNA/mRNA, an RNAscope probe was designed targeting NA +RNA. To characterise the probe, infections were undertaken in eHAP WT cells at a low MOI (MOI=0.3) and timepoints collected at one early (2hpi) and one later (6hpi) timepoint. As with the NA vRNA probe, +RNA transcripts could be detected in the nucleus at early timepoints following infection at low MOI (Figure 23). At 2hpi, most of the sparse punctate NA +RNA staining localises to the nucleus. At this timepoint, in a low MOI infection, most +RNA staining likely relates to NA transcripts. The lack of staining in the cytoplasm supports this assumption, as this suggests nascent viral transcripts are yet to be exported and translated. As replication relies on the accumulation of nascent RNP components following transcription, translation and import into the nucleus, this suggests only primary transcription is occurring at this timepoint. Nevertheless, we cannot exclude that mRNAs for other segments that we have not stained for here have been exported and translated. By 6hpi, the +RNA staining likely corresponds to both cRNA and mRNA, as we would expect both replication and transcription to have

initiated by this timepoint. Indeed, the number of NA vRNA puncta has clearly increased and some NA vRNA localises to the cytoplasm. This confirms both genome replication and export has started. No background staining was observed in mock infected cells.

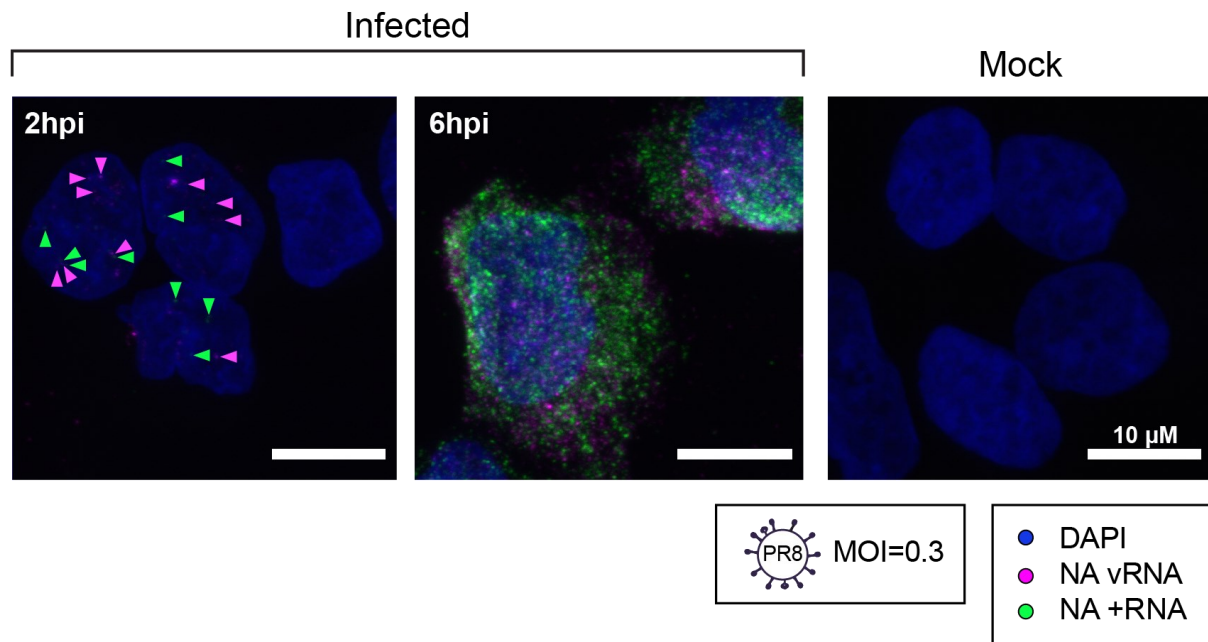


Figure 23: Preliminary characterisation demonstrating RNAscope probes cannot distinguish cRNA and mRNA. Maximum intensity projections showing human eHAP cells infected with PR8 (MOI=0.3), 2 and 6hpi, prior to hybridisation with RNAscope probes targeting NA vRNA and NA +RNA (n=1 experiment). Images were visualised using a Leica SP5 inverted confocal microscope and 63x objective. Scale bars are 10 μ m. hpi= hours post-infection; MOI= multiplicity of infection.

2.2.5 cRNA can be specifically visualised using cRNP stabilisation and replication assays

As the genome intermediate, specifically visualising cRNA is desirable, as it can act as a marker of ongoing genome replication. This is unlike the vRNA, which at any point in infection may undergo nuclear import, transcription, replication or nuclear export. Thus, visualising the cRNA allows key questions, such as whether replication occurs in a specific replicative niche, to be probed. However, due to the cross-reactivity of the RNAscope NA +RNA probe, infection needs to take place under conditions in which viral transcription is inhibited if we are to specifically visualise cRNA. This can be achieved using Actinomycin D (ActD)-treated cRNP stabilisation and replication assays. In these assays, conditions are manipulated so that only the primary round of cRNA synthesis (cRNP stabilisation assays, Figure 24A) or multiple rounds of replication (replication assays, Figure 24B) can take place (Mänz et al., 2012; Nilsson et al., 2017). Thus, positive-sense staining in these assays can be attributed to cRNA. In brief, both assays first require exogenous expression of RNP components (FluPol/NP) that allow encapsidation of nascent cRNA/vRNA in the absence of viral transcription. In the case of a cRNP stabilisation assay, the exogenously expressed FluPol must be a catalytically dead mutant (PB1 D446Y) that will allow stabilisation of nascent cRNA into catalytically inactive cRNPs, thus inhibiting further rounds of replication. In contrast, replication assays require wild-type FluPol to be pre-expressed. Cells are then treated with the cellular transcriptional inhibitor ActD, and infected. Under

these conditions, viral transcription is indirectly blocked due to its dependence on active host Pol II and host mRNAs for cap snatching. Under standard infection conditions, blocking transcription also indirectly blocks replication, due to the requirement of nascent FluPol/NP to stimulate replicative activity and for encapsidation. However, supplying FluPol/NP exogenously decouples the processes. A schematic outlining the method is illustrated in Figure 24C.

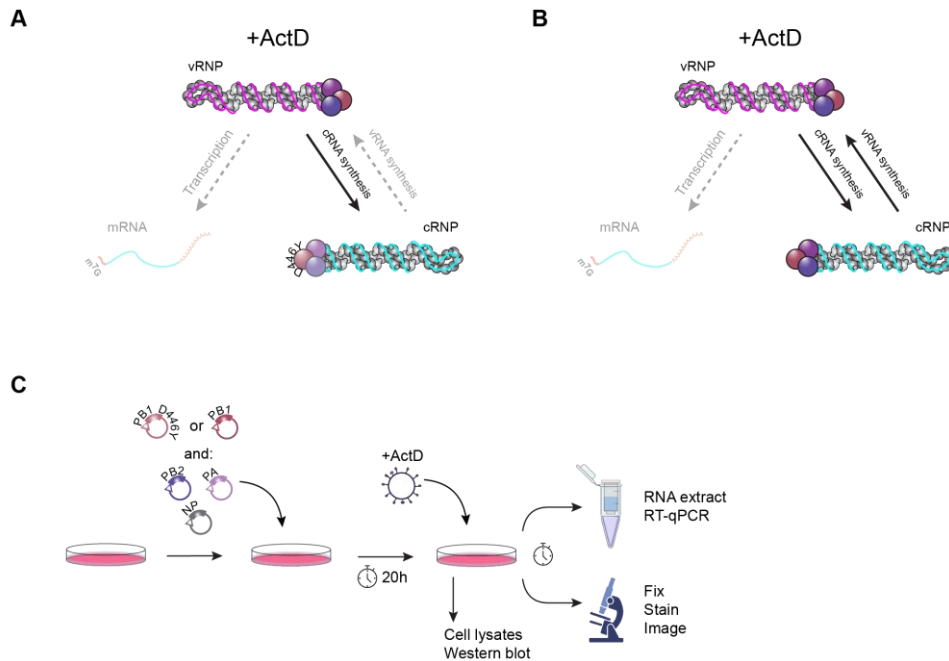


Figure 24: cRNP stabilisation and replication assays decouple transcription and replication.

(A) Schematic outlining FluPol activity during a cRNP stabilisation assay in the presence of ActD. Incorporation of a catalytically dead FluPol (PB1 D446Y) into the cRNP inhibits further replication following primary cRNA synthesis. (B) Schematic outlining FluPol activity during a replication assay in the presence of ActD. Incorporation of wild-type FluPol allows multiple rounds of replication to occur in the absence of transcription. (C) Outline of the experimental approach for cRNP stabilisation/replication assays. 20h= 20 hours. ActD= actinomycin D. A and B reproduced from Swann et al., in press. Icon of microscope and RNA-extraction column from BioRender.com.

To validate using cRNP stabilisation and replication assays to specifically visualise cRNA *in situ*, both assays were performed simultaneously in HEK-293T cells. Samples either pre-transfected with WT FluPol but infected in the absence of ActD (-ActD,WT), or pre-transfected with a plasmid mix lacking PB1 in the presence of ActD (+ActD,-PB1), were also included as positive and negative controls, respectively. Characterisation of the assays using RT-qPCR, 6hpi, confirmed that vRNA and cRNA significantly accumulate over background (-PB1 control) during a replication assay (+ActD,WT), whilst only cRNA significantly accumulates during a cRNP stabilisation assay (+ActD,D446Y) (Figure 25A,B). Although some residual transcription was observed in the replication assay (+ActD,WT), this was <0.2% untreated levels (greater than 550 fold reduction) (Figure 25C).

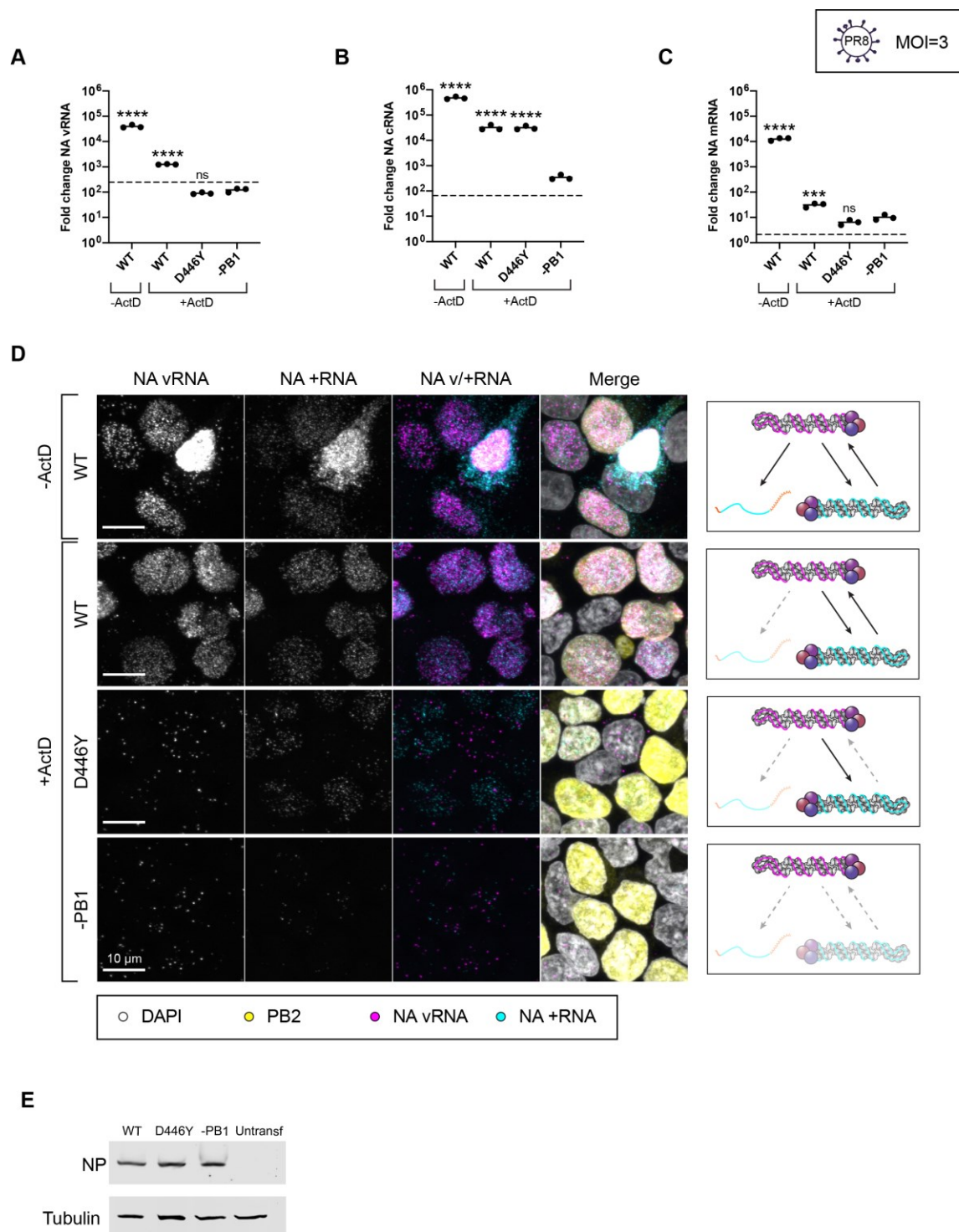


Figure 25: cRNA can be specifically visualised using cRNP stabilisation and replication assays.

Validation of ActD-treated replication and cRNP stabilisation assays analysed by RNA FISH. Assays were performed in HEK-293T cells, following infection with PR8 (MOI=3). Pre-transfected PR8 polymerase mixes were: WT= PB2, PB1, PA, NP (replication assay); D446Y= PB2, PB1 D446Y, PA, NP (cRNP stabilisation assay); -PB1= PB2, PA, NP (control). (A-C) RT-qPCR analysis of segment 6 (A) vRNA (B) cRNA and (C) mRNA accumulation, 6hpi. Pre-transfected polymerase mixes are indicated on the x-axis. Dotted line indicates input levels of RNA (0hpi). Fold change was calculated over mock infected cells. n=3 biological replicates, plotted as mean $\pm$ s.d.. Significance compared to -PB1 was assessed using one-way ANOVA with Dunnett's multiple comparison test, following log transformation. (D) Accumulation of NA vRNA and +RNA, 3hpi, analysed by RNAscope with indirect immunofluorescence against PB2. Pre-transfected PR8 polymerase mixes are indicated on the left-hand side. A schematic

depicting FluPol activity under each condition is depicted on the right-hand side. Images are representative maximum intensity projections, visualised using a Leica SP5 inverted confocal microscope and 63x objective. All scale bars are 10 μ m. (G) Matched western blot confirming equal transfection of different polymerase mixtures. ActD= actinomycin D; MOI= multiplicity of infection; hpi= hours post-infection. Ns= not significant; ***= $p<0.001$; ****= $p<0.0001$. Figure adapted from Swann et al., in press.

For *in situ* analysis, samples were fixed 3hpi, and co-stained with NA vRNA/+RNA probes followed by IIF against PB2, to act as a marker for transfected cells (Figure 25D). In control samples infected in the absence of ActD (-ActD,WT), significant accumulation of vRNA and +RNA was observed in the nuclei and cytoplasm of infected and transfected cells. In a replication assay (+ActD,WT), strong NA vRNA/+RNA staining was also detected in transfected and infected cells, however this staining was restricted to the nucleus. This confirms efficient inhibition of viral transcription, as vRNP export is dependent on the expression of additional viral proteins (M1, NS2/NEP) that are not pre-expressed in these assays. Moreover, we expect some NA transcripts to be exported by 3hpi, as seen in the untreated control (-ActD,WT), suggesting the +RNA staining present in the replication assay primarily relates to cRNA. In a cRNP stabilisation assay (+ActD,D446Y), +RNA was also detected throughout the nuclei of transfected cells. This staining was less abundant than for the replication assay, as expected in the absence of amplifying rounds of replication. Sparse NA vRNA staining was also detected in the nuclei of cells, which likely corresponds to input genomes, as a similar level of staining was detected in control samples lacking pre-expressed PB1 in which nascent vRNPs cannot be stabilised (+ActD,-PB1). The minimal NA +RNA staining detected in the -PB1 control (+ActD,-PB1) indicates the background that corresponds to residual viral transcription and non-specific staining, and confirms the majority of +RNA staining in cRNP stabilisation and replication assays can be attributed to cRNA. Equal transfection efficiency of the three pre-expressed plasmid mixes was confirmed by IIF against PB2 (Figure 25D) and western blot against NP (Figure 25E).

Interestingly, in RNAscope replication assays we found multiple instances where cRNA and vRNA appear to co-localise, which could be evidence for ongoing replication (Figure 26A,B). At both timepoints, cRNA was found co-localising with vRNA (white arrowheads), however unexpectedly cRNA was also observed in isolation at the earlier timepoint (3hpi) (cyan arrowheads). Speculatively, at earlier timepoints lower concentrations of RNP components may have accumulated in the nucleus. This could limit the formation of FluPol dimers (and possibly trimers) that are required for vRNA synthesis and thus cRNA is detected in the absence of vRNA as replication is not yet able to occur continuously. At later timepoints, higher concentrations of RNP components may enable constant replication, and thus cRNA is always found co-localising with a nascent vRNA. Nevertheless, as RNP components are pre-expressed in this assay it is probable that a high concentration is present from the start of infection. Alternatively, the co-localisation of cRNA and vRNA at later timepoints may be coincidental due to the high density of staining. In future, it will be interesting to see whether these replicative foci can be co-localised with other factors associated with replication, such as ANP32, or any specific subnuclear domains.

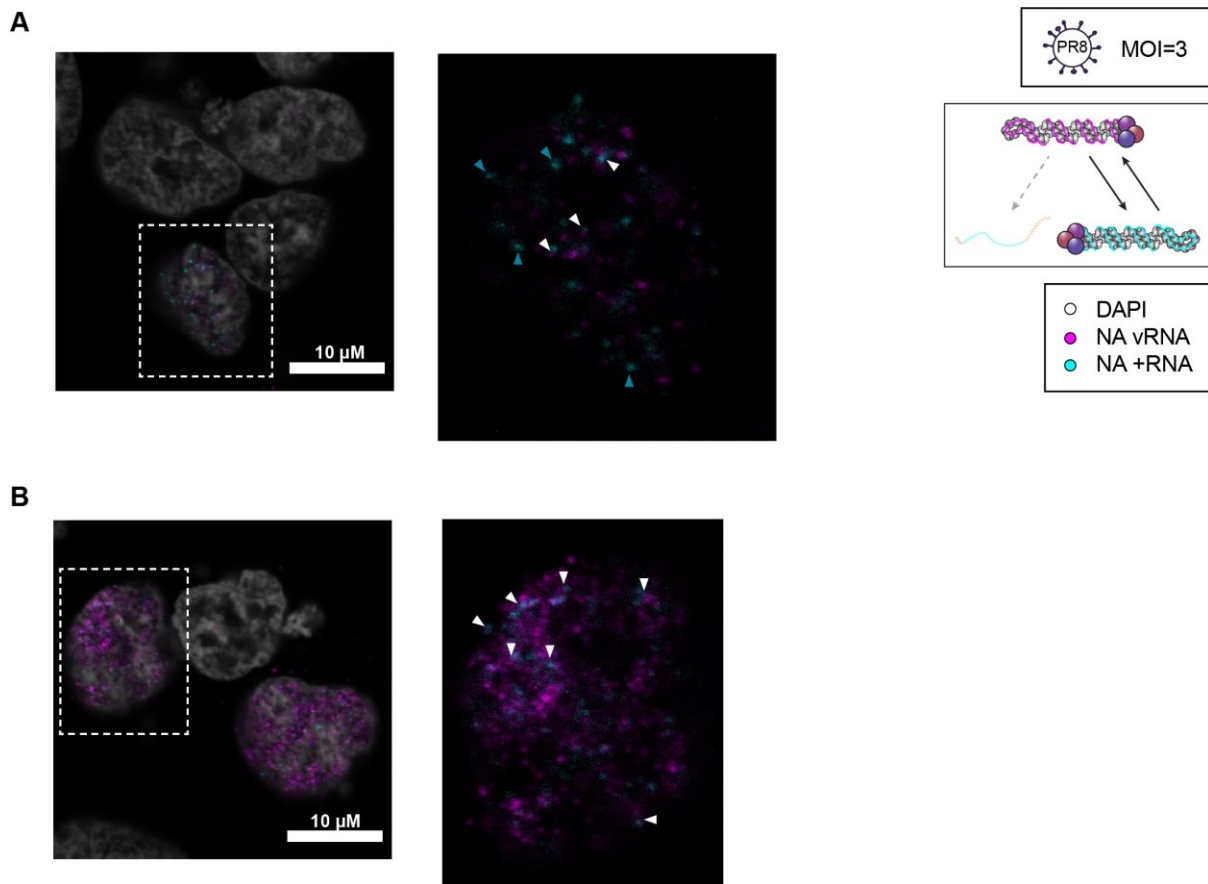


Figure 26: NA vRNA and cRNA co-localise in replication assays.

Replication assays in the presence of ActD in eHAP WT cells, (A) 3hpi, and (B) 6hpi. Cells were infected with PR8 (MOI=3). Inserts on the right are enlarged details from the images on the left, indicated by dotted boxes. Cyan arrowheads highlight instances of NA cRNA found in isolation, white arrowheads highlight instances of NA vRNA and NA cRNA co-localisation. Images are representative single slices from full Z-stacks, visualised using a Leica SP5 inverted confocal microscope and 63x objective. All scale bars are 10 μ m. hpi= hours post-infection, MOI= multiplicity of infection, ActD= actinomycin D.

A major drawback to using a host transcriptional inhibitor such as ActD to indirectly inhibit viral transcription is the resulting cellular side-effects. It is known that transcription inhibition results in major rearrangements in nuclear structure, including disruption of the nucleolus and other nuclear organelles, dramatic relocation of various nuclear RNAs/proteins, as well as alterations to histone ubiquitylation and phosphorylation (Bensaude, 2011). Many of these changes are relevant to FluPol replicative activity. Thus, studying the nuclear localisation of replication in ActD-treated samples may not reflect authentic cellular infection. A cleaner chemical approach could be directly inhibiting IAV transcription. Baloxavir marboxil is a recently licensed antiviral, which has been shown in clinical trials to be effective both in treating and preventing influenza infection (Hayden et al., 2018; Ikematsu et al., 2020; Ison et al., 2020). Following metabolism to the active form, baloxavir acid (BXA), the mechanism of action is selective inhibition of endonuclease activity of PA (Noshi et al., 2018). Previous reports have suggested BXA specifically inhibits FluPol transcription (Noshi et al., 2018).

As introduced earlier (Section 1.3.1), mini genome assays are *in situ* assays in which an artificial viral-like reporter RNA is co-expressed in cells alongside the PB1/PB2/PA and NP from a strain of interest, to reconstitute a vRNP-like complex encoding the reporter gene of choice (Figure 27A). Expression of the reporter is typically analysed using a microplate reader and gives a combined measure of both transcriptional and replicative activity of the polymerase, since FluPol transcription is required to express the reporter whilst replication increases the number of vRNP-like complexes from which transcription can occur (te Velthuis et al., 2018). To investigate the potential application of BXA as an inhibitor of FluPol transcription that would allow specific visualisation of cRNPs using RNAscope, we first tested the inhibitory activity against FluPol through performing mini genome assays in the presence of an increasing BXA concentration. We tested BXA against two polymerase constellations from two different strains: PR8, the H1N1 lab-adapted strain used throughout this chapter (Figure 27B); and 5092K, a version of the highly pathogenic avian influenza virus strain (A/Turkey/England/50-92/1991 (H5N1)) that has been engineered to contain the humanising mutation PB2 E627K, so that it undergoes robust replication in human cells (Figure 27C). As expected, FluPol activity decreased with increasing concentration of BXA, both in the case of PR8 and 5092K. Complete inhibition of 5092K was observed at 5 nM, whilst PR8 FluPol retained ~10% activity relative to DMSO control at this concentration and was fully inhibited at 50 nM. It is not surprising that 5092K was more readily inhibited, as the polymerase is from an avian background and likely less adapted to replication in a human cell, despite the PB2 E627K mutation. Moreover, it is known that the overall genetic background of influenza FluPol influences the type of mammalian adaptations that can be acquired and artificially introducing E627K may have resulted in a suboptimal polymerase. Indeed, earlier work has suggested that E627K is poorly tolerated by 5092 in the context of full virus (Long et al., 2013). In contrast, the PR8 polymerase is lab adapted and grows to very high titres in human cells.

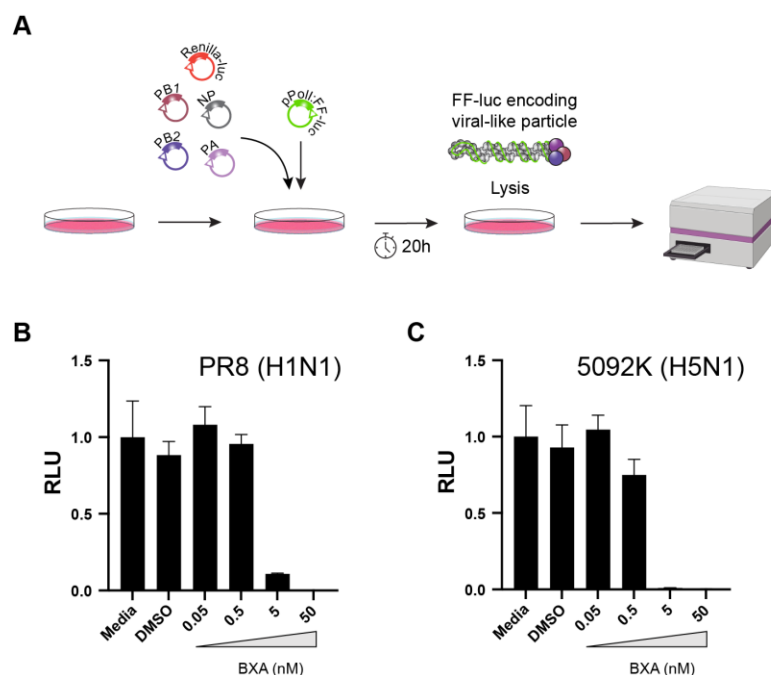


Figure 27: Preliminary assessment of baloxavir activity against FluPol.

(A) Schematic overview of a mini-genome assay. Cells are transfected with expression plasmids encoding the RNP proteins (FluPol/NP) of the strain of interest, as well as a reporter protein (renilla-luc) as a control for cell expression levels, alongside a plasmid encoding the antisense orientation of a reporter gene (here, FF-luc) flanked by the influenza promoter sequences under the control of a host Pol I promoter. The Pol I plasmid results in expression of a viral-like RNA that assembles into a viral-like particle in the presence of the expressed RNP components. Replicative and transcriptional activity of the reconstituted FluPol can then be measured through the levels of accumulated reporter gene, normalised to the control reporter expression, typically measured 20-24 hours after transfection. Renilla-luc= renilla luciferase; FF-luc= firefly-luciferase; h= hours. (B,C) Polymerase assay in eHAP WT cells (n=1 experiment) measuring the activity of the polymerase from (A) PR8 or (B) 5092 bearing the humanising 627K mutation (5092K), in the presence of increasing concentrations of BXA. Data is normalised to media control. n=3 biological replicates, plotted as mean $\pm$ s.d BXA= baloxavir acid; RLU= relative light units. Icon of microplate reader from BioRender.com.

In addition to measuring the reporter gene output in mini genome assays, which captures the combined replicative and transcriptional activity of the reconstituted FluPol, it is possible to extract and measure individual viral RNAs (vRNA/cRNA/mRNA) to separate transcription vs replication. This can be achieved in an identical manner as for infections, i.e., using RT-qPCR or primer extension analysis (te Velthuis et al., 2018). Primers can be designed that specifically target the vRNA/cRNA/mRNA of the viral-like reporter RNA, or alternatively the Pol I reporter plasmid can be replaced with a Pol I plasmid expressing a viral gene to which vRNA/cRNA/mRNA primers have already been designed, such as (in our case) PR8 segment 6 (Figure 28A). These types of assays are useful as they decouple replication from transcription (formation of reconstituted vRNPs is dependent on the expression plasmids rather than expression of the reporter of choice), allowing the specific effect of either mutations or inhibitors on replication to be investigated independent of their effect on transcription. Consequently, this assay was chosen to investigate the specific activity of BXA against transcription, and whether it could therefore be used to allow specific visualisation of cRNPs in *in situ* cRNP stabilisation/replication assays, as a cleaner approach than the indirect inhibitor ActD. We chose to characterise the activity of BXA using the polymerase from PR8, as the strain used for previous validation work, and therefore chose a 50 nM concentration of BXA for complete transcriptional inhibition based on the mini genome data presented in Figure 27. Unexpectedly, at a concentration of 50 nM RT-qPCR analysis demonstrated that the reconstituted PR8 polymerase was completely inhibited in both transcription and replication (Figure 28B,C,D).

BXA is an endonuclease inhibitor, that directly binds the PA endonuclease active site (Noshi et al., 2018; Omoto et al., 2018). It is not immediately obvious why this binding would inhibit replication, as the endonuclease domain localises on the flexible PA-N appendage, away from both the core enzyme and the symmetric and asymmetric dimer interfaces. Speculatively, binding of BXA might lock the polymerase in a transcriptionally active structure that is incompatible with replication, or have some other allosteric effect. Based on this data, we concluded BXA inhibits both transcription and replication and is therefore unsuitable for our intended application as a tool for specifically inhibiting FluPol transcription in cRNP stabilisation/replication assays.

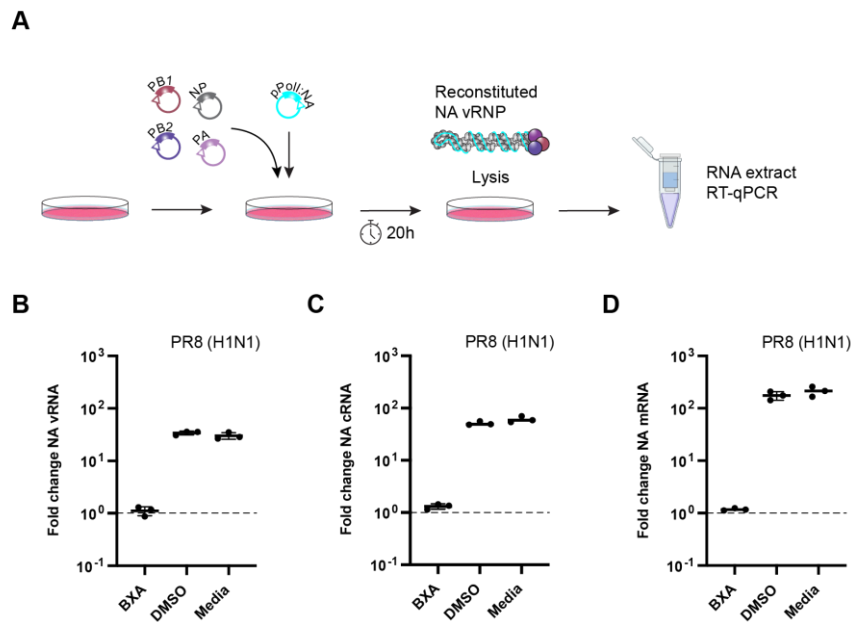


Figure 28: Preliminary data suggests baloxavir inhibits FluPol replication, as well as transcription.

(A) Schematic overview of the mini genome assay variation in which viral RNA products are individually quantified using a tagged RT-qPCR approach. Cells are transfected with expression plasmids encoding the RNP proteins (FluPol/NP) of the strain of interest, alongside the NA segment under the control of a host Pol I promoter. The Pol I plasmid results in expression of an NA vRNA, that can assemble into vRNPs in the presence of the expressed RNP components. The reconstituted NA vRNP can then undergo transcription and replication within the cell nucleus. 20-24 hours post transfection, total RNA is extracted and accumulation of NA vRNA, cRNA and mRNA, as measures of viral replication and transcription, quantified using the tagged RT-qPCR approach introduced at the start of this chapter. h= hours. (B,C,D) RT-qPCR analysis of NA (B) vRNA (C) cRNA and (D) mRNA accumulation, in the context of a polymerase assay, in the presence of 50 nM BXA, DMSO or media control, 20 hours post transfection (n=1 experiment). Polymerase constellation used was PR8. Fold change was calculated over background levels of RNA (samples lacking PB2 in the transfection mixture), indicated as dotted line on graph. N=3 biological replicates, plotted as mean $\pm$ s.d.. BXA= baloxavir acid. Icon of RNA-extraction column from BioRender.com.

However, further literature scanning has since highlighted that the concentration investigated here (50 nm) may have been too high to observe specific inhibition of transcription. In the original characterisation of BXA, Noshi et al. use *in vitro* assays to investigate the endonuclease activity, endonuclease/polymerase activity and polymerase activity alone of purified polymerase from an alternative lab-adapted H1N1 strain, WSN. They conclude selective endonuclease inhibition based on an IC_{50} of 2.5 nM for endonuclease activity and >40 nM for polymerase activity (Noshi et al., 2018). Nevertheless, this suggests that the concentration used here was close to the IC_{50} for polymerase activity, assuming a similar value for PR8 and that the results from *in vitro* assays can be extrapolated to mini genome assays. This may explain the broad inhibition observed, with vRNA and cRNA accumulation inhibited, as well as mRNA. In retrospect, basing the concentration on complete inhibition in a mini genome assay, in which both replication and transcription are captured, may have been misleading (although the reporter signal does require at least some viral transcription to occur for the reporter to be expressed). Although our initial investigations suggested some transcriptional activity would occur at a lower concentration, this was with the lab-adapted PR8 polymerase, which is highly adapted to growth in cell-culture and might not be representative of less adapted, more biologically

relevant strains. Indeed, our preliminary data using 5092K suggested a lower concentration would be effective with this strain. It is also possible that lower BXA concentrations could be used to bias FluPol towards a replicative, rather than transcriptional, state, and that the resulting enrichment for cRNA might be sufficient for our aims. Indeed, validation of ActD-treated replication assays demonstrated a low level of background transcription (Figure 25C,D), and we concluded that measuring this background in controls was a sufficient solution. Therefore, it is possible with further characterisation that BXA could yet prove to be a useful tool for specifically inhibiting FluPol transcription, which could be exploited to achieve our aim of visualising cRNPs *in situ*.

2.2.6 Establishing RNAscope correlative light and electron microscopy (CLEM)

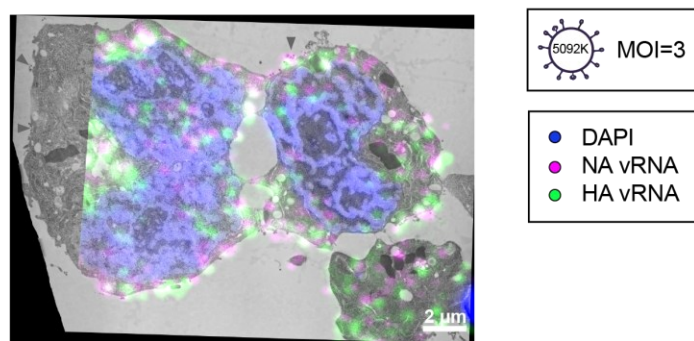
A key question driving this project was whether we could use imaging techniques to improve our understanding of the subnuclear localisation of vRNPs during replication. Consequently, having established RNAscope for visualising vRNA and cRNA/mRNA, we hoped to adapt this method for CLEM, which would allow us to visualise viral RNAs within the context of the nuclear ultrastructure. To do this, we established a collaboration with the EM science technology platform based at the Crick Institute, led by Dr Lucy Collinson. Optimisation of RNAscope staining for CLEM was led by Dr Alana Burrell.

For optimisation of the CLEM protocol, we decided to infect at high MOI (MOI=3) and collect samples 6hpi to ensure large amounts of viral RNA were available for detection. Infections were undertaken under standard conditions (as opposed to using ActD treatment as in cRNP stabilisation/replication assays) to avoid disrupting the cell ultrastructure. Eventually, we planned to use this approach to directly compare the localisation of mammalian-adapted vs. avian like strains and investigate whether any differences could be observed. To do this, we planned to use a pair of reassortant isogenic viruses containing the internal genes from the highly pathogenic H5N1 avian strain 5092 (A/England/turkey/50-92(H5N1)) and structural genes from PR8, that differ only in the residue present at position 627 on PB2: either the wild-type avian-like glutamic acid (5092E) or the mammalian adapting lysine (5092K). 5092E replicates poorly in mammalian cells, whilst 5092K undergoes robust replication. Thus for our preliminary optimising experiment, we chose to undertake infections using the mammalian-adapted half of the pair, 5092K. Samples were initially stained with RNAscope probes targeting NA and HA vRNA. We expected genomic RNA to be more abundant than cRNA, and thus an easier target for optimisation, and as we undertook infections without ActD treatment the +RNA probe would detect both cRNA and mRNA. Moreover, we were interested to localise two different RNA segments simultaneously.

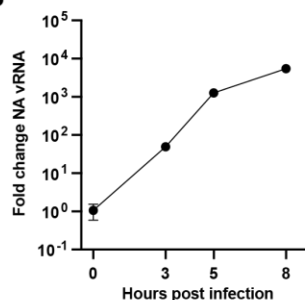
In this preliminary experiment, evidence of viral budding could be observed at the cell membrane (indicated with grey arrowheads), indicating that a full replication cycle had been completed and new virions have formed at the surface of the infected cell (Figure 29A). In agreement, most NA vRNA and HA vRNA staining is present in the cytoplasm, indicating replication has mostly finished and vRNPs have been exported. A previous report has described that vRNPs are exported from the nucleus both as individual segments, and as bundles with other segments. However significantly more vRNPs are

exported alongside other segments than individually (Lakdawala et al., 2014). Similarly, we found foci containing individual segments (just HA or NA), as well as foci containing both HA and NA vRNA, could be visualised in the cytoplasm. On a population level, RT-qPCR analysis of NA (Figure 29B,C,D) and HA (Figure 29E,F,G) vRNA, cRNA and mRNA suggest that transcription has plateaued for both segments by 6hpi. Replication is ongoing but approaching its plateau. It seems likely the remaining nuclear RNA represents a mixed population undergoing replication, or preparation for export. HA and NA vRNA remaining in the nucleus were found both in electron dense and more lucid regions but did not appear to localise to the nucleolus (Figure 29A). However, at the resolution collected here it is difficult to confidently assign localisation. With future work, it is hoped that super-resolution data will be collected using an integrated Light Microscopy-Scanning Electron Microscopy (LM-SEM) system available at the Crick which, alongside increased throughput and quantification, will allow localisation to be confidently described.

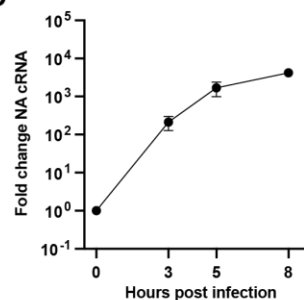
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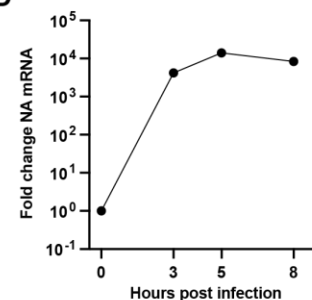
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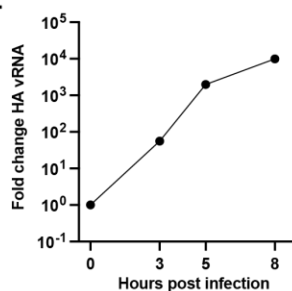
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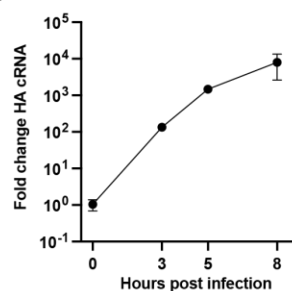
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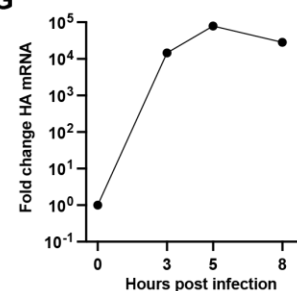


Figure 29: Establishing RNAscope CLEM.

(A) RNAscope stained CLEM (n=1 experiment). eHAP WT cells were infected with 5092K (MOI=3) and fixed in 4% formaldehyde, 6hpi. Samples were embedded in LR White resin and ultrathin sections (90nm) deposited on Formvar film. Samples were stained with RNAscope probes targeting the NA vRNA and HA vRNA and imaged using an Axio Observer 7 (Zeiss) microscope. Samples were subsequently washed and stained in aqueous uranyl acetate followed by Reynold's lead citrate, and electron microscopy performed using a JEM-1400Flash (JEOL) transmission electron microscope. Samples were infected and fixed by this author. Processing, staining and imaging was performed by Dr Alana Burrell. (B,C,D,E,F,G) RT-qPCR analysis of (B) NA vRNA (C) NA cRNA (D) NA mRNA (E) HA vRNA (F) HA cRNA (G) HA mRNA accumulation over time in eHAP WT cells following infection with 5092K (MOI=3). Fold change calculated over input (0hpi), n=3 biological replicates, plotted as mean $\pm$ s.d.. HA RT-qPCR analysis (E,F,G) was performed by Amalie Rasmussen.

2.3 DISCUSSION

2.3.1 Population level analysis of viral RNAs

In this chapter our main objective was to develop an RNA imaging approach capable of visualising replicating viral RNAs at the single cell level. First however, we optimised a complementary approach for quantifying viral RNAs (vRNA, cRNA and mRNA) at the population level, to both inform and support single cell investigations. For this, we chose to use a tagged RT-qPCR approach. Although RT-qPCR quantification of RNA viruses has been traditionally plagued with issues of cross-reactivity and self-primed background (Andersson et al., 2012; Craggs et al., 2001; Kawakami et al., 2011; Lanford et al., 1994; Tuiskunen et al., 2010), we found that the introduction of a tagged approach eliminated the background from self-primed products, as previously reported (Figure 16) (Kawakami et al., 2011). We also used a high temperature (50°C) during the RT step, which has been reported to improve strand-specificity (Lanford et al., 1994).

An alternative method for bulk quantification, that avoids many of the pitfalls of RT-qPCR, is primer extension analysis. This approach contains a size separation step using denaturing polyacrylamide gel electrophoresis, which allows accurate distinction of cRNA and mRNA, despite initial identification with the same primer. Nevertheless, primer extension analysis comes with its own caveats, including a practical limitation on the number of samples that can be run in each experiment (te Velthuis et al., 2018). More recently, an RNA-seq pipeline termed influenza virus enumerator of RNA transcripts (InVERT) has been developed for quantifying the vRNA, cRNA and mRNA for all eight genome segments in parallel, independent of primer bias. This pipeline was used to describe the kinetics of PR8 replication over the course of infection (Phan et al., 2021). We were encouraged that the overall NA/HA kinetics observed using our tagged RT-qPCR closely mirrored those described using InVERT (Figure 29). Consequently, we deemed the low-cost, simplicity and semi-high throughput nature of the RT-qPCR approach, combined with the ability to circumvent its caveats, well-suited to the aims of this project. Nevertheless, it would be reassuring to confirm some of the results described in this work, particularly in chapter 4 which is heavily reliant on RT-qPCR, using an alternative approach such as primer extension analysis. The key advantages and caveats of the three approaches for bulk RNA analysis described here are summarised in Table 2.

Approach	Advantages	Disadvantages	References
Segment- and strand-specific tagged RT-qPCR	-Semi-high throughput -Highly sensitive -Low cost and simple approach -Modifications to protocol reduce/eliminate cross-reactivity and self-primed background -Primers bind at 3' end of RNA, so will not detect degraded or abortive RNA products	-Risk of cross-reactivity between segments -Background signal due to high sensitivity -Requires a separate RT and qPCR reaction for each RNA species and segment	(Kawakami et al., 2011; te Velthuis et al., 2018)
Primer extension analysis	-Accurate strand- and segment-specific detection -Low background	-Low throughput, complex -Radioactive -Requires specialised costly equipment -Limited sensitivity -Primers typically bind close to the 5' end of the RNA (within 200bp), so may detect abortive or degraded RNA products	(Luo et al., 1992; te Velthuis et al., 2018)
InVERT	-Simultaneous identification of all eight segments vRNA, mRNA and cRNA without primer bias	-Costly and complex processing: depends on RNA-seq -Requires familiarity with bioinformatics analysis	(Phan et al., 2021)

Table 2: Comparison of the main approaches available for bulk quantification of IAV vRNA, cRNA and mRNA.

2.3.2 Approaches for studying influenza replication *in situ*

The primary aim of this chapter was to establish an *in situ* imaging approach suited to studying the spatiotemporal details of FluPol activity within the nucleus. Specifically, we became interested in visualising the genome intermediate, the cRNA, as it can be used as a marker for ongoing replication. Unfortunately, identifying IAV cRNA in full infections is technically challenging, as it is almost completely identical in sequence to the matched segment mRNA, which is vastly more abundant during infection (Hatada et al., 1989).

To circumvent this technical difficulty, we established *in situ* versions of cRNP stabilisation and replication assays (Figure 25). These assays continue to use authentic virus for infection however manipulate cellular conditions to inhibit transcription but allow cRNA synthesis (cRNP stabilisation assay) or full genome replication (replication assay) to occur. Thus, all positive-sense staining can be attributed to cRNA. Nevertheless, such assays involve treatment with the host transcriptional inhibitor ActD, to indirectly block viral transcription, which is known to disrupt the nuclear architecture and could lead to erroneous, artefactual conclusions (Bensaude, 2011). Presumably, directly inhibiting viral transcription using an antiviral compound would be less damaging to the host cell (although this would require validation). Our preliminary characterisation of BXA as a specific transcriptional inhibitor was unpromising (Figure 28), however having revisited the data we realise the BXA concentration used may have been too high. Therefore BXA could yet prove to be a useful tool for unpicking viral transcription and replication.

Alternatively, a cleaner approach would be to utilise well-characterised FluPol mutants that are specifically impaired in transcription, but not replication (Mänz et al., 2012; Rehwinkel et al., 2010; Robb et al., 2009). These mutants cannot be rescued as full virus (as transcription/replication are essential) however can be reconstituted in the nucleus in a mini genome-style assay using expression plasmids for the RNP components and a viral genome segment under the control of a host Pol I promoter. Unfortunately, although we attempted this approach, we found it to be undermined by persistent background staining (data not shown), which appeared to be caused by binding of the RNAscope probes to the Pol I promoter plasmid encoding the genome segment. Attempts to optimise the approach were not successful within the timeframe of this project, however this approach could be revisited in future. A summary of the potential approaches for studying the cRNP *in situ*, and the associated advantages and disadvantages, is presented in Table 3.

Overall, it is hoped that simplifying infections through cRNP stabilisation/replication assays, although artificial, will allow novel insights into the spatial details of FluPol replication that are not readily observed in the more crowded environment of a standard infection. Such insights could then be cross-referenced with the spatiotemporal details under standard infection conditions. For example, if cRNA was found to associate with a particular nuclear body during replication assays, an obvious question would be whether the presence of +RNA could be detected in the same nuclear body from the point at which replication initiates under standard infection conditions.

Approach	Advantages	Disadvantages
Authentic infection	-Most closely resembles natural infection	-Transcription and replication both occur – requires downstream imaging approach to be able to distinguish cRNA from mRNA, or if staining both cRNA/mRNA as +RNA results in data that is difficult to interpret
<i>In situ</i> cRNP stabilisation/replication assays	-Inhibits transcription - allows cRNA to be visualised even if downstream imaging approach cannot distinguish cRNA and mRNA -Uses infectious virus for input vRNP – may more closely reflect spatiotemporal details of a natural infection compared to reconstituted RNP -In the future - specific inhibitors of viral transcription (such as BXA, small molecule inhibitors or nanobodies) might overcome caveats associated with the use of host transcriptional inhibitors in these assays	-Requires drug treatment to inhibit transcription from the incoming vRNP. Currently only characterised with indirect inhibition using host transcriptional inhibitors which are known to disrupt nuclear architecture -Requires pre-expression of RNP components: (1) very high concentrations of RNP proteins might breakdown the requirement for spatial localisation; (2) overexpression of RNP proteins prior to infection may alter the nuclear landscape the virus first encounters (e.g., overexpression of polymerase is known to trigger degradation of host Pol II; (3) concentrations of FluPol proteins regulate FluPol activity - overexpression could alter the way the virus behaves
Mini genome assays	-Compatible with well-characterised FluPol mutants that are specifically impaired in transcription or replication. Allows cRNA to be visualised even if downstream imaging approach cannot distinguish cRNA and mRNA	-Visualisation of mini genome assays (at least using the RNAscope approach) has thus far proven unsuccessful due to high background staining -Overexpression assay: (1) very high concentrations of RNP proteins might breakdown the requirement for spatial localisation; (2) concentrations of FluPol proteins regulate FluPol activity - overexpression could alter the way the virus behaves

Table 3: Summary of the different approaches available to study influenza replication *in situ*.

2.3.3 Alternative imaging approaches for *in situ* analyses of FluPol activity

Targeting viral RNAs to study the spatiotemporal details of IAV replication is advantageous as they are specific markers of both the viral genome and FluPol activity. Nevertheless, relatively few studies have investigated IAV infection using RNA labelling approaches (Dou et al., 2017). There are several different RNA labelling methods that could have been explored (see Table 4): each of these approaches present their own advantages and disadvantages and can be useful for different applications. At the start of this project, preliminary investigations were made into three: RNAscope, click chemistry and padlock probes.

Ultimately, we found RNAscope to be the most tractable approach investigated. RNAscope can be used to detect IAV genomes and replicative products from very early timepoints and at low MOIs using standard confocal microscopy (Figure 19, 23), and is compatible with IIF staining (Figure 20), providing the opportunity for co-localisation studies. RNAscope can also be adapted for CLEM, enabling the viral genome to be placed in the context of the cell ultrastructure (Figure 29). Nevertheless, a significant disadvantage of RNAscope is the inability to distinguish cRNA from mRNA. In contrast, padlock probes (PLPs) are an alternative sequence-specific FISH approach that have a very short sequence requirement. In theory, PLPs can be used to specifically detect cRNA under standard infection conditions using the unique promoter sequence, thus avoiding many of the pitfalls discussed in Section 2.3.2. Indeed, specific detection of the cRNA of Crimean-Congo haemorrhagic fever virus (CCHFV) has previously been reported using PLPs (Andersson et al., 2012). A schematic of the approach is illustrated in Figure 30A. Unfortunately, although we successfully established PLPs as an approach for detection of NA vRNA, detection of NA cRNA was undermined by equivalent staining in -RT controls (Figure 30B), reminiscent of the -RT background seen in untagged RT-qPCRs. An initial attempt to remove this background using an analogous tagged approach to that used in the RT-qPCR eliminated all signal for both NA vRNA and cRNA (Figure 30C).

Technique	Summary	Advantages	Disadvantages	References
RNAscope	-Commercial single molecule RNA FISH approach -Segment- and sense-specific -Labels incoming and progeny RNAs	-Commercial approach – simple to establish, easy to use kits available, technical support provided -Highly sensitive – can detect IAV molecules at early timepoints and low MOIs -Highly specific approach -Specific detection of cRNA can be achieved under conditions in which transcription is inhibited (cRNP stabilisation/replication assays) (see Section 2.2.5) -Protocol optimised for CLEM	-Cannot distinguish cRNA and mRNA under standard infection conditions -Variation in the number of fluorescent probes bound to each branching structure prevents accurate quantification of the number of RNA molecules/fluorescent spot – instead can calculate ranges -Incompatible with live cell imaging	(F. Wang et al., 2012)
Classic single molecule FISH (smFISH)	-FISH approach in which multiple fluorescent probes are designed to tile along the RNA of interest, allowing single molecule detection -Segment- and sense-specific -Labels incoming and progeny RNAs	-Highly sensitive – can detect IAV molecules at early timepoints and low MOIs -Commercial kits available -Previously used for visualisation of IAV vRNA -Better suited to accurate quantification of individual RNA molecules – less variation in the number of fluorescent probes bound/molecule than in RNAscope -Although not investigated here, as with RNAscope, specific detection of cRNA likely possible under conditions in which transcription is inhibited	-Cannot distinguish cRNA and mRNA under standard infection conditions -Incompatible with live cell imaging	(Y. ying Chou et al., 2013; Y.-Y. Chou et al., 2012; Femino et al., 1998; Lakdawala et al., 2014)
Padlock probes (PLPs)	-Alternative RNA FISH approach -Segment- and sense-specific -Labels incoming and progeny RNAs	-Short sequence requirement should allow cRNA and mRNA to be distinguished under standard infection conditions -Highly sensitive approach – can detect IAV molecules at early timepoints and low MOIs -Can be designed to distinguish single nucleotide polymorphisms (SNPs)	-Cannot be used for accurate quantification of number of RNA molecules – unable to predict how many fluorescent probes incorporated/RNA molecule -Can be affected by self-primed background -Incompatible with live cell imaging	(Andersson et al., 2012; Dou et al., 2017)
Click chemistry	-Directly labels RNA with the alkyne-tagged uridine analogue 5-EU -Labels all viral segments	-Specific labelling of incoming viral genomes through growth of viral stocks in 5-EU followed by purification -Can also be used for co-immunoprecipitation of incoming viral genomes with interacting host factors -Can also be used to label total cellular RNA (host and viral)	-No amplification steps – lacks sensitivity -Incompatible with live cell imaging	(Müller et al., 2019; Senkevich et al., 2017)

	-Can be used to specifically label incoming vRNPs			
RNA tags e.g., PP7 and MS2	-Introduce tags into target RNAs that can be detected by a binding partner protein	-Compatible with live cell imaging -Can also be used to purify tagged RNAs via the protein binding partner	-for fluorescent imaging requires tandem repeats of tags (for PP7 >24 repeats for single molecule imaging) – large tags unlikely to be tolerated by authentic virus	(Pichon et al., 2018; Urbanek et al., 2014; York et al., 2013)
RNA aptamers e.g., Mango, Spinach	-Introduce aptamers into the target RNA that can bind and trigger fluorescence of a small fluorescent ligand	-compatible with live cell imaging -have also been used to purify target RNAs	-typically requires tandem arrays for single molecule resolution – large tags unlikely to be tolerated by authentic virus	(Pichon et al., 2018)
Molecular beacons	-short oligonucleotide probes with a quencher on one end and a fluorophore on the other	-only fluoresce when bound to the target RNA – thus increases signal to noise -compatible with live cell imaging -does not require the virus to be tagged -previously established for IAV	-laborious to design -must be exogenously supplied to the cell	(Pichon et al., 2018; W. Wang et al., 2008)
CRISPR-Cas9 based approaches	-family of approaches based on the CRISPR-Cas9 system in which a fluorescently tagged catalytically dead Cas9 (or Cas13) is targeted to an RNA of interest using a guide RNA	-compatible with live cell imaging -does not require virus to be tagged	-relatively new approach – currently quite laborious, requires multiple guide RNAs to achieve sufficient signal	(Nelles et al., 2016; Pichon et al., 2018)

Table 4: Summary of RNA imaging approaches that could be applied to understanding the spatiotemporal details of IAV replication.

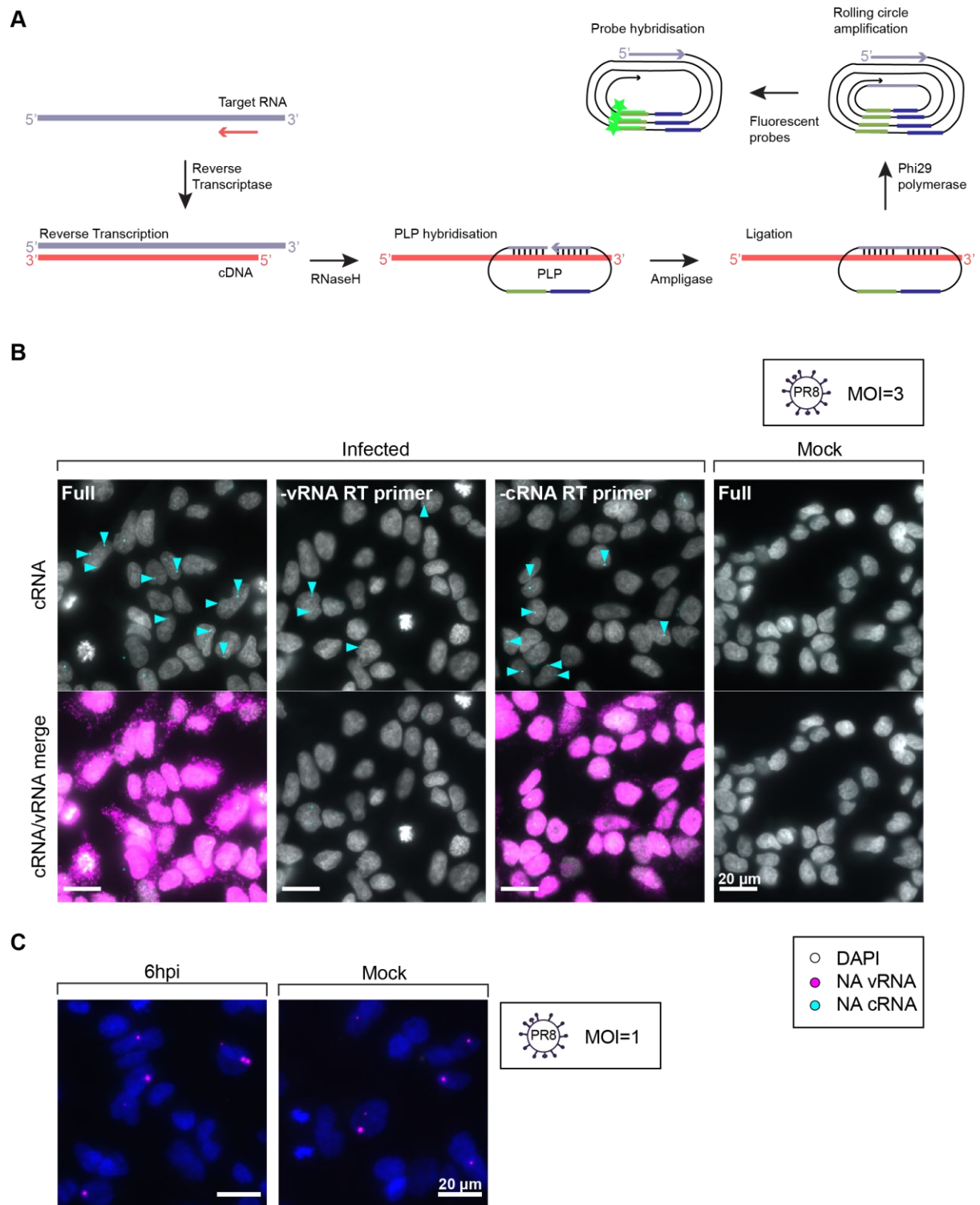


Figure 30: Exploring padlock probes as an approach for visualising cRNA under standard infection conditions.

(A) Schematic depicting the padlock probe (PLP) approach. Following infection and fixation, target RNA is first converted into cDNA *in situ* using a localised reverse transcription reaction and a sequence-specific primer. Nascent cDNA is fixed in place using PFA treatment, and the original RNA molecule removed by RNaseH treatment. The resulting single stranded cDNA is available to bind to a complementary PLP which contains two hybridising arms that bind to the target cDNA nose-to-tail, bringing the 5' and 3' ends of the probe next to each other and enabling enzymatic ligation by ampligase (Crosetto et al., 2015; Larsson et al., 2010). The resulting circularised DNA molecule can be used as a template by an isothermal polymerase for rolling circle amplification, resulting in thousands of localised copies of the PLP sequence, including the barcode tags built into the non

hybridising portion of the PLP. The amplified tags can then be detected with complementary short fluorescent probes (Crosetto et al., 2015; Gyarmati et al., 2008). (B) NA vRNA/cRNA padlock probe co-staining using untagged primer sets. eHAP WT cells were (mock) infected with PR8 at an MOI of 3. 8hpi cells were fixed in PFA and stained using padlock probes targeting segment 6 vRNA and cRNA. Nuclei were counterstained with DAPI. At the RT step, samples were either incubated in the full reaction mix required for cRNA and vRNA cDNA synthesis (Full) or in a control mix lacking either the vRNA RT primer (-vRNA RT primer) or the cRNA RT primer (-cRNA RT primer) to check for specificity of the two padlock probes. To aid visualisation of cRNA puncta, images are displayed as cRNA plus DAPI (top row) then merged with vRNA (bottom row). Images are single Z-slices. Cyan arrowheads identify a subset of segment 6 cRNA-stained puncta. (C) NA vRNA/cRNA padlock probe co-staining using tagged primer sets. eHAP WT cells were (mock) infected with PR8 (MOI=1), and fixed 6hpi. Nuclei were counter-stained with DAPI. Images are max intensity projections of Z-stacks. All images were collected using a Zeiss Cell Observer Widefield microscope and 63x objective. All scale bars are 20 μ m. MOI= multiplicity of infection, hpi= hours post-infection.

In contrast to RNAscope and PLPs, click chemistry involves non-specific labelling of RNA using a uridine analogue (5-ethynyluridine (5-EU)), that contains a biologically unreactive chemical group that can be conjugated to reporter molecules under the conditions of the copper-catalysed azide-alkyne cycloaddition (CuAAC) reaction (Fantoni et al., 2021; L. Li & Zhang, 2016). Through growing viral stocks in the presence of 5-EU and then purifying the labelled virus, click chemistry can be used to specifically label incoming vRNPs, and thus allows initiating genomes to be identified and tracked through replication (Figure 31A) (Müller et al., 2019). In contrast, with a method such as RNAscope, incoming genomes and nascent vRNPs are indistinguishable. Click chemistry is also useful as it can be used to biotinylate labelled molecules for purification and subsequent proteomics analysis (Senkevich et al., 2017). Unfortunately, although we could detect 5-EU incorporation in viral stocks (Figure 31B), we were unable to visualise click-labelled vRNPs, despite successful visualisation of a click-labelled HSV control (Figure 31C). This may suggest the individual IAV segments are too short for sufficient 5-EU incorporation to be distinguished above background.

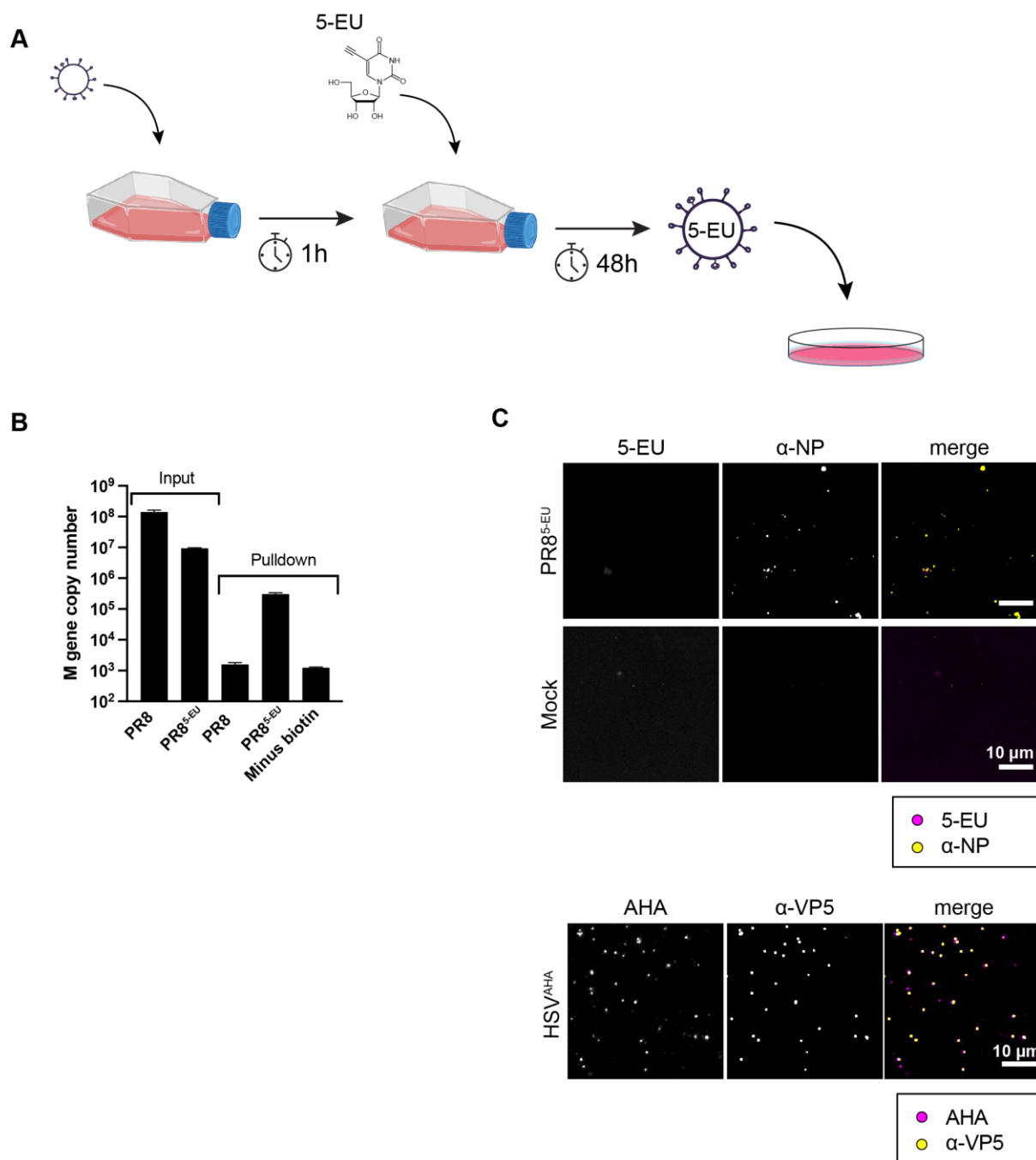


Figure 31: Exploring click chemistry as an approach for visualising incoming vRNPs.

(A) 5-EU staining is non-specific and will label cellular as well as viral RNA. However, by growing viral stocks in the presence of 5-EU, harvesting, and then using those 5-EU labelled virions to infect untreated cells, incoming vRNPs are specifically labelled and can be visualised throughout infection. (B) Click chemistry pulldown for assessing 5-EU incorporation into viral stocks. Viral RNA was clicked to azide-biotin and precipitated using streptavidin beads using the click-iT nascent RNA capture kit (ThermoFisher). Viral RNA pre- and post- pulldown in either treated or untreated stocks was quantified using RT-qPCR targeting the viral M gene. Minus biotin = 5-EU labelled stock in which the click reaction was performed in the absence of azide-biotin. (C) Representative images showing PR8^{5-EU} (~4000 pfu), mock (PBS) or HSV^{AHA} (~4000 pfu) spotted directly onto glass coverslips, fixed, clicked in the presence of azide-fluor 488 (PR8^{5-EU}/mock) or alkyne-fluor 488 (HSV^{AHA}) and co-stained with either α-NP (PR8^{5-EU}/mock) or α-VP5 (HSV^{AHA}) antibody. Images were collected using a Zeiss Axiovert 135 TV microscope and 40x objective. All scale bars are 10 μm. pfu= plaque forming units; 5-EU= 5-ethynyluridine; AHA= L-azidohomoalanine; PR8^{5-EU}= 5-EU labelled PR8 stock; HSV^{AHA}= AHA-labelled HSV. Icon of flask taken from BioRender.com.

Although both PLPs and click chemistry did not prove successful in the timeframe of this project, with further persistence they could yet yield useful tools for influenza research. For example, incorporation of an amplification step in the click chemistry protocol might allow detection of the labelled RNA, while testing multiple sets of primers might identify a PLP approach that eliminates the issue of self-primed background. Similarly, there are many other RNA detection methods that we could have established (Table 4). A key caveat of all three approaches explored here, for example, is the requirement for fixed and permeabilised samples. Ideally, such approaches would be cross-referenced with a live-cell imaging approach. RNA tags that can be detected with their cognate RNA binding protein coupled to fluorescent proteins, such as PP7, have been successfully used to track RNA in live cells, as have RNA aptamers such as Spinach and Mango that bind and promote the fluorescence of small fluorogenic ligands (Dolgosheina et al., 2014; Pichon et al., 2018). To our knowledge, such RNA-labelling approaches are yet to be applied to influenza virus, although the PP7 RNA tag has previously been incorporated into the NA segment of WSN and used to purify vRNPs or cRNPs (York et al., 2013). Live-cell imaging of influenza virus +RNAs has been achieved using molecular beacons: short, oligonucleotide probes with a fluorophore on one end and a quencher on the other, that only fluoresce when bound to target RNA (Pichon et al., 2018; W. Wang et al., 2008). Finally, although not an RNA-labelling technique, one interesting approach that is compatible with live cell imaging and can be used to specifically label incoming genomes is quantum dot (QD)-labelling (S. L. Liu et al., 2012, 2014; Qin et al., 2019). QDs are nanoparticles with useful optical properties, that can be chemically conjugated to particles of interest. They are typically much brighter than traditional fluorescent dyes and have been used for single-particle tracking during live cell imaging (S. L. Liu et al., 2012). Interestingly, a previous study successfully packaged QD-conjugated PA into viral particles and tracked the release of the labelled vRNPs from the endosome (Qin et al., 2019). As QDs can also be electron dense (Giepmans et al., 2005), using QD-labelled viral stocks would allow incoming viral genomes to be specifically detected using EM without requiring CLEM. Furthermore, combining QD-labelled viral stocks with our RNAscope CLEM protocol would allow an additional level of information, with incoming vRNPs distinguished from nascent vRNPs due to the presence of the QD.

2.3.4 Summary and future work

In summary, in this chapter we have established an RNA imaging approach that allows detection of PR8 HA and NA vRNAs and +RNAs. Under conditions in which transcription is inhibited, this approach can also be used to specifically visualise HA and NA cRNAs. Simultaneously, we have also optimised a tagged RT-qPCR approach for quantifying segment-specific vRNA, cRNA and mRNA on the population level. In chapter three, these approaches are applied to understanding the role of the host factor ANP32 in cRNA synthesis. In chapter four, the RT-qPCR approach is used to investigate the effect of host restriction on cRNA synthesis. Unfortunately, time constraints prevented the application of these approaches to the original aim of exploring the subnuclear localisation of FluPol replication. However, it is hoped future work using these approaches will achieve this objective.

Through our collaboration with Dr Lucy Collinson and Dr Alana Burrell at the Crick Institute, we have also established a protocol combining RNAscope staining of FluPol replication products with EM. Although the resolution of the fluorescent staining is low in the preliminary images collected, with future samples we hope to collect super-resolution data using an integrated Light Microscopy-Scanning Electron Microscopy (LM-SEM) system which, alongside increased throughput and quantification, will allow localisation to be confidently described. For optimisation, we chose to use late infection timepoints with abundant viral RNA. Now the protocol is established, work is ongoing to characterise the localisation of viral vRNA and +RNA within the nuclear ultrastructure at earlier timepoints at which we expect first just viral transcription, then transcription and replication, and finally transcription, replication and viral export to be occurring. We also plan to compare the localisation of viral RNA within eHAP cells with the host factor ANP32 ablated. Viral replication is completely inhibited in such cells (see chapter 3), but not transcription. If this work proves successful, it will be interesting to investigate whether any differences in localisation can be observed between host-restricted and host-adapted virus. Although the original rationale for developing this approach was to investigate the movement of IAV RNA through the nuclear ultrastructure during replication, currently very little CLEM has been applied to influenza research and this approach could readily be expanded to investigate other aspects of the IAV life cycle.

CHAPTER THREE: HUMAN ANP32 PROTEINS ENHANCE INFLUENZA A VIRUS cRNA SYNTHESIS

3.1 INTRODUCTION

ANP32 proteins are a family of proviral host factors that are essential for influenza replication (Staller et al., 2019; H. Zhang et al., 2019). Species differences between ANP32 proteins, specifically a 33 aa insertion present in avian ANP32A proteins but absent in human orthologues, underly host restriction of avian polymerase in mammalian cells (Long et al., 2016). Within the literature, there is conflicting data whether ANP32 proteins are required to specifically support vRNA synthesis from a cRNA template, or whether ANP32 proteins support both cRNA and vRNA synthesis. Human ANP32A/B (huANP32A/B) were originally identified as factors present in nuclear extracts which specifically enhance replication from a positive-sense RNA template, but not negative-sense RNA template, in cell-free biochemical assays i.e., as factors with a specific role in supporting vRNA synthesis (Sugiyama et al., 2015). Nevertheless, it was observed that both cRNA and vRNA stabilise the FluPol-ANP32 complex (under conditions in which elongation is blocked) – suggesting ANP32 might bind vRNPs as well as cRNPs during initiation (Baker et al., 2018; Mistry et al., 2020). Moreover, the publication of the structure of an ANP32-stabilised asymmetric FluPol dimer implicated ANP32 in coordinating co-replicative encapsidation, which is presumably required for both cRNPs and vRNPs. Indeed, the structure was resolved with the replicating FluPol bound to a short vRNA template, i.e., poised for cRNA synthesis, and it was shown that mutations that disrupt the FluPol_R:FluPol_E interface of the asymmetric dimer impair cRNP stabilisation (Carrique et al., 2020).

In this chapter we set out to clarify the role of the human ANP32 proteins, huANP32A, huANP32B and huANP32E, in supporting FluPol cRNA synthesis. To do this, we made use of previously generated cell lines in which expression of huANP32 proteins has been ablated by CRISPR-Cas9 gene editing, and compared cRNA accumulation relative to wild-type cells in which ANP32 proteins are expressed at endogenous levels. Initially, we visualised FluPol replication in cells lacking all three ANP32 paralogues, using our *in situ* RNA FISH approach (Section 2.2), and demonstrate that genome replication, but not vRNA nuclear import, is impaired in the absence of huANP32A/B/E. Subsequently, we use a combination of RT-qPCR and *in situ* cRNP stabilisation assays to unambiguously demonstrate that huANP32A/B are essential for efficient cRNA synthesis but are not required for viral transcription, for all strains investigated. We observe strain-to-strain variation in the ability to utilise the third human ANP32 paralogue, huANP32E, to support a low level of cRNA synthesis in the absence of huANP32A/B. Finally, we also characterise the early replication of an H5N1 strain that has adapted to efficiently use the typically weakly-proviral huANP32E and demonstrate that this appears to have been achieved through biasing the FluPol away from transcription and towards a more replicative state.

Generation of the huANP32E-adapted 6:2 Tky05 H5N1 strain (Tky05-Mut) by experimental evolution, and subsequent characterisation of the adaptive mutations, was performed by Dr Carol Sheppard and Dr Daniel Goldhill (Goldhill et al., under revision). Generation of the eHAP knockout cell lines was by Dr Ecco Staller (Staller et al., 2019; Goldhill et al., under revision). Data in Figure 34 and Figure 36 was collected by a master's student, Amalie Rasmussen, under the supervision of this author. The sequence alignment presented in Figure 38 was performed by Dr Carol Sheppard. Figures 33-36, Figure 41, and accompanying text, are adapted from Swann et al., in press. Figure 39 is adapted from Goldhill et al., under revision.

3.2 RESULTS

3.2.1 Visualising FluPol activity in the absence of ANP32 proteins

Human cells express three members of the ANP32 family: huANP32A, huANP32B and huANP32E. Previous work suggested that huANP32A/B are functionally redundant to one another in their proviral activity, whilst huANP32E does not support IAV FluPol activity (Staller et al., 2019; H. Zhang et al., 2019). However, whilst this project was ongoing, it was reported that some strains inefficiently utilise huANP32E to support a very low level of replication (Zhang et al., 2020; Goldhill et al., under revision). Through the course of this project, we therefore made use of two genetically edited cell lines, either eHAP double knockout cells (eHAP dKO) in which expression of the key proviral paralogues huANP32A and huANP32B have been ablated, or eHAP triple knockout cells (eHAP tKO) in which expression of all three huANP32 paralogues (huANP32A/B/E) have been ablated by CRISPR-Cas9 (Staller et al., 2019, Goldhill et al., under revision). eHAP cells are a human immortalised cell line originally based on the chronic myelogenous leukemia cell line KBM-7, that have been previously shown to support influenza polymerase activity and virus replication, and that have been engineered to be fully haploid thus simplifying genetic editing (Essletzbichler et al., 2014).

As an initial insight into the role of ANP32 proteins in FluPol activity, we decided to visualise FluPol replication products during authentic infection, in either eHAP WT cells or eHAP tKO cells using the RNAscope approach established in the previous chapter. We infected cells using the lab-adapted H1N1 strain PR8 at a high MOI (MOI=3), and fixed at 2, 3 and 4hpi (WT and tKO cells) plus 1hpi (WT cells only). Cells were stained with RNAscope probes targeting NA vRNA and +RNA. As the infections were performed under standard infection conditions (not cRNP stabilisation/replication assays) +RNA staining may be attributed to NA cRNA or NA mRNA due to the cross-reactivity of the +RNA probe (Section 2.2.4). Matched samples were also collected for processing for CLEM, which is ongoing at the EM Science Technology Platform at the Crick Institute.

In WT cells, viral genomes were already present in the nucleus and producing +RNA by 1hpi (Figure 32A). This likely represents primary transcription, as cRNA synthesis requires the accumulation of nascent viral proteins within the nucleus, which are unlikely to be produced at significant levels at this time. By 2hpi, significant accumulation of +RNA was observed in the nuclei and cytoplasm of WT cells, which may correspond to a mixture of cRNA and mRNA. Cytoplasmic +RNA staining likely corresponds to exported NA mRNA transcripts, although export of cRNPs to the cytoplasm has been reported at later timepoints (Chaimayo et al., 2017). In some nuclei, >10 magenta NA vRNA puncta could be observed, which may suggest in some cells vRNA synthesis has started by 2hpi. An example is highlighted here with a white arrowhead. Alternatively, as this infection was undertaken at high MOI (MOI=3), these cells may have received a particularly high dose of infecting vRNA genomes. By 3hpi, full replication was initiated in several nuclei, with dense vRNA and +RNA staining in the nucleus, and some early vRNP export to the cytoplasm. In other cells, less dense vRNA/+RNA nuclear staining was observed and no vRNP export, suggesting infection is less advanced. Finally, by 4hpi, in many WT cells

extensive vRNP export could be observed. In others, despite intense NA vRNA staining in the nucleus, vRNP export is yet to start. Again, this highlights the heterogeneity observed in infection when investigated at the single cell level. It is worth noting that this infection occurred more rapidly than those described in the previous chapter (Figure 19 and Figure 23), likely due to the higher MOI.

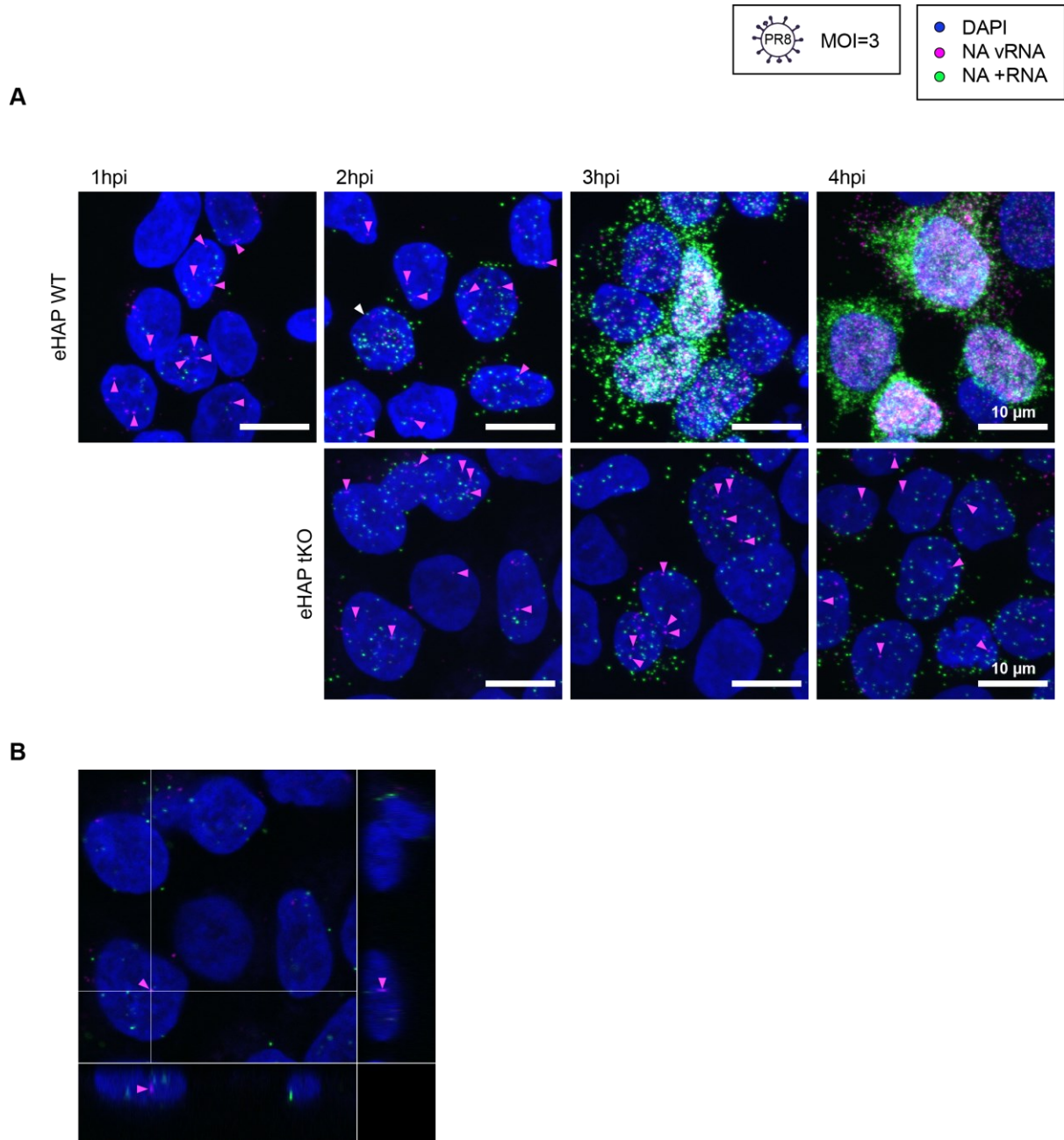


Figure 32: Visualising FluPol replication in the absence of ANP32 proteins.

(A) Representative maximum intensity projections showing infections with PR8 (MOI=3) in human eHAP WT cells 1, 2, 3 and 4hpi, and eHAP tKO cells 2,3 and 4hpi, prior to hybridisation with RNAscope probes targeting NA vRNA and +RNA. Magenta arrowheads highlight a subset of NA vRNA puncta. White arrowhead indicates example nucleus putatively undergoing early replication. All scale bars are 10 μ m. (B) Orthogonal view of the eHAP tKO 2hpi timepoint in (A), illustrating vRNP import into the nucleus in the absence of ANP32A/B/E. Magenta arrowheads highlight NA vRNA staining. Images were visualised using a Leica SP5 inverted confocal microscope and 63x objective. eHAP WT= eHAP wild-type cells, eHAP tKO= eHAP ANP32A/B/E knockout cells, hpi= hours post-infection, MOI= multiplicity of infection.

It has previously been suggested that ANP32 proteins may be essential for nuclear import of viral factors, rather than replication. Our imaging analysis provides evidence to refute this hypothesis for vRNPs at least, as NA vRNA puncta could be directly observed within the nuclei of infected tKO cells (Figure 32B). However, it remains possible that ANP32 plays a role in the nuclear import of nascent nuclear-localising viral proteins, such as NP, the polymerase proteins and NS2/NEP, later in infection. At the earliest timepoint visualised (2hpi), similar +RNA staining could be observed in the tKO cells as in the WT, consistent with previous data demonstrating that primary transcription does not require ANP32 proteins (Figure 32A) (Bi et al., 2019; Nilsson-Payant et al., 2021; Staller et al., 2019; Sugiyama et al., 2015). Unlike WT cells, no tKO nuclei were observed with >10 NA vRNA puncta, 2hpi. This might suggest some WT cells have genuinely started replication at this timepoint, although more data is required to confirm this observation. From 3hpi onwards, a drastic reduction in +RNA staining could be observed in tKO cells compared to the matched WT sample. Moreover, no accumulation of NA vRNA over time was observed. This demonstrates that ANP32 proteins are essential for genome replication. However, as some of the +RNA staining observed in the tKO cells could be due to primary cRNA, this data does not allow the role of ANP32 in cRNA synthesis to be determined.

3.2.2 cRNA synthesis is inhibited in human cells lacking ANP32A/B

To specifically investigate whether ANP32 proteins are required for cRNA synthesis, eHAP WT and eHAP dKO cells were infected with PR8 (MOI=3) and the accumulation of NA vRNA, cRNA and mRNA quantified at two different timepoints using the tagged RT-qPCR approach described in Section 2.2.1. We chose to focus on early timepoints of infection (3hpi and 6hpi) to minimise the indirect effect of reduced vRNP templates on cRNA synthesis. At 6hpi, a significant reduction in all three RNA species was observed in dKO compared to WT cells (Figure 33A,B,C). At 3hpi, an ~10 fold reduction in the accumulation of NA cRNA was already apparent in the dKO cells, however no difference in either NA vRNA or NA mRNA accumulation was yet observed. This suggests that the reduction in cRNA accumulation observed in dKO cells is not solely an indirect effect due to reduced vRNP template, as has previously been suggested (Staller et al., 2019). Moreover, no significant increase in NA cRNA was observed from 3-6hpi in the dKO cells (analysed using an unpaired two-tailed *t* test). In combination, this suggests that huANP32A/B play a direct role in cRNA synthesis.

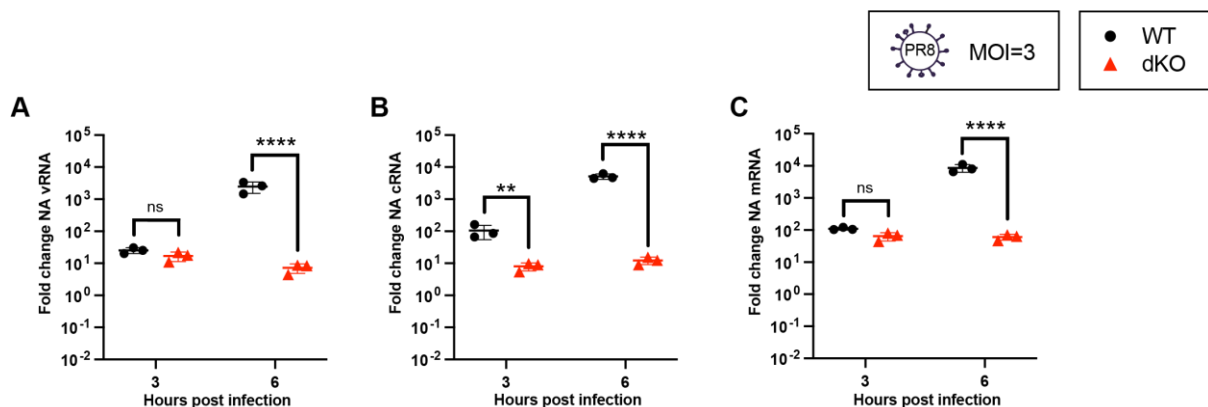


Figure 33: NA cRNA synthesis is inhibited in human cells lacking ANP32A/B.

(A-C) Segment 6 (A) vRNA (B) cRNA or (C) mRNA accumulation over time in eHAP WT vs dKO cells following infection with PR8 (MOI=3). Fold change was calculated over mock infected cells. n=3 biological replicates, plotted as mean $\pm$ s.d.. Significance was assessed using multiple unpaired *t* tests following log transformation, corrected for multiple comparisons using false discovery rate. NA= neuraminidase; WT= eHAP wild-type cells; dKO= eHAP ANP32A/B double knockout cells; MOI= multiplicity of infection; hpi= hours post-infection. ns= not significant; **= $p<0.01$; ****= $p<0.0001$. Figure adapted from Swann et al., in press.

The promoter sequences at the extreme termini of influenza RNA segments are highly conserved, save for at position 4, which can be either a C or a U (Section 1.3.5). C4 and U4 promoters display different replication dynamics. The PR8 strain used in this work bears a C4 promoter, typically associated with the polymerase segments but occasionally present on the NA or M segments, on the NA segment (K.-H. Lee & Seong, 1998). To establish whether ANP32A/B are also required for cRNA synthesis from a segment with the alternative U4 promoter, the experiment was repeated and the accumulation of segment 4 (HA) vRNA, cRNA and mRNA quantified over time using an analogous tagged RT-qPCR approach (Figure 34A,B,C). Following infection there was no increase in either vRNA or cRNA levels over time in the absence of ANP32A/B, confirming ANP32 proteins are essential for replication. Significantly, no increase in cRNA accumulation was observed over input, suggesting ANP32 proteins are essential for primary cRNA synthesis, at least for PR8. In contrast, HA mRNA transcripts increased 50-fold in dKO cells from 0hpi to 2hpi, illustrating that primary transcription does not require ANP32A/B.

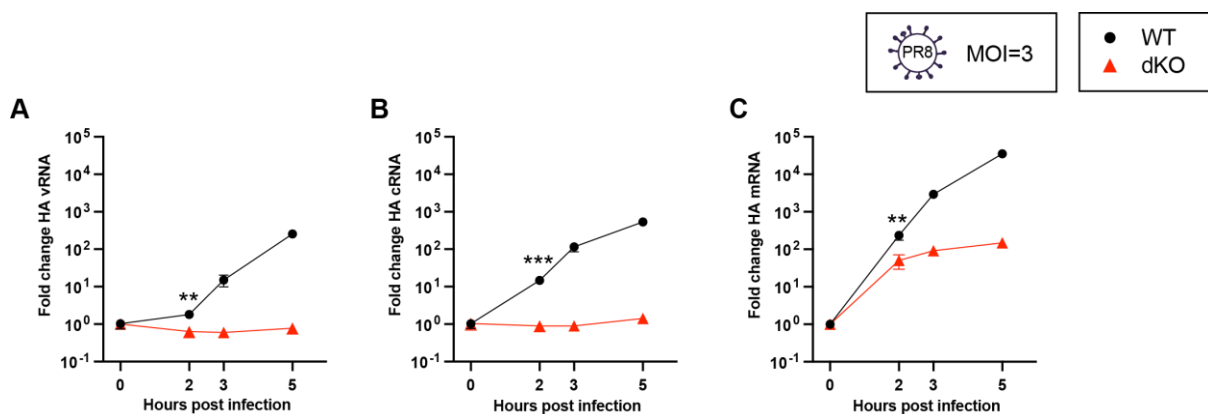


Figure 34: HA cRNA synthesis is inhibited in human cells lacking ANP32A/B.

(A-C) Segment 4 (A) vRNA (B) cRNA or (C) mRNA accumulation over time in eHAP WT vs dKO cells following infection with PR8 (MOI=3). Fold change was calculated over input (0hpi). n=3 biological replicates, plotted as mean $\pm$ s.d.. Significance was assessed at 2hpi using an unpaired *t* test following log transformation. HA= haemagglutinin; WT= eHAP wild-type cells; dKO= eHAP ANP32A/B double knockout cells; MOI= multiplicity of infection; hpi= hours post-infection. ns= not significant; **= $p<0.01$; ***= $p<0.001$; Data collected by Amalie Rasmussen. Figure adapted from Swann et al., in press.

3.2.3 Human ANP32A/B play a direct role in primary cRNA synthesis

To confirm the role of ANP32A/B in supporting PR8 cRNA synthesis is direct, we made use of cRNP stabilisation assays. As previously described (Section 2.2.5), ActD-treated cRNP stabilisation assays manipulate cellular conditions so that only the primary round of cRNA synthesis from incoming viral genomes occurs (Figure 35A) (Vreede et al., 2004, Nilsson et al., 2017). Thus, any reduction in cRNA accumulation in this assay cannot be attributed to changes in vRNA synthesis. Initially, ActD-treated cRNP stabilisation assays were performed in eHAP WT and dKO cells and the accumulation of NA vRNA, cRNA and mRNA analysed by RNAscope and RT-qPCR, 3hpi (Figure 35B,C,D,E). Both incoming NA vRNA (highlighted with magenta arrowheads) and NA +RNA accumulation could clearly be detected in the nuclei of infected WT cells. In contrast, whilst incoming vRNPs could be observed in the nuclei of dKO cells, indicating successful infection, no +RNA staining was observed (Figure 35B). When analysed by RT-qPCR, a highly significant reduction in cRNA was observed in dKO cells as compared to WT cells (Figure 35D). As expected in this assay, no accumulation of NA vRNA or mRNA occurred over background levels (controls lacking transfected PB1, dotted line), in either cell type (Figure 35C,E). Unlike standard infection conditions (Figure 34B), in cRNP stabilisation assays we found some cRNA accumulated above input levels in the dKO cells. This may be an artefact caused by the overexpression of RNP components in this assay, enabling cRNA synthesis and encapsidation in the absence of ANP32A/B, or could suggest a low level of ANP32A/B-independent cRNA synthesis – potentially using ANP32E or through an ANP32-independent mechanism. Equal transfection efficiency of WT and dKO cells was tested by western blot (Figure 35F): slightly less PB2 and NP appeared to be expressed in the WT cells, however we would expect this to reduce the cRNA accumulation in the WT cells, and thus cannot explain the results seen here. In combination, this data corroborates the conclusion that huANP32A/B are required for primary cRNA synthesis, at least for the H1N1 strain PR8.

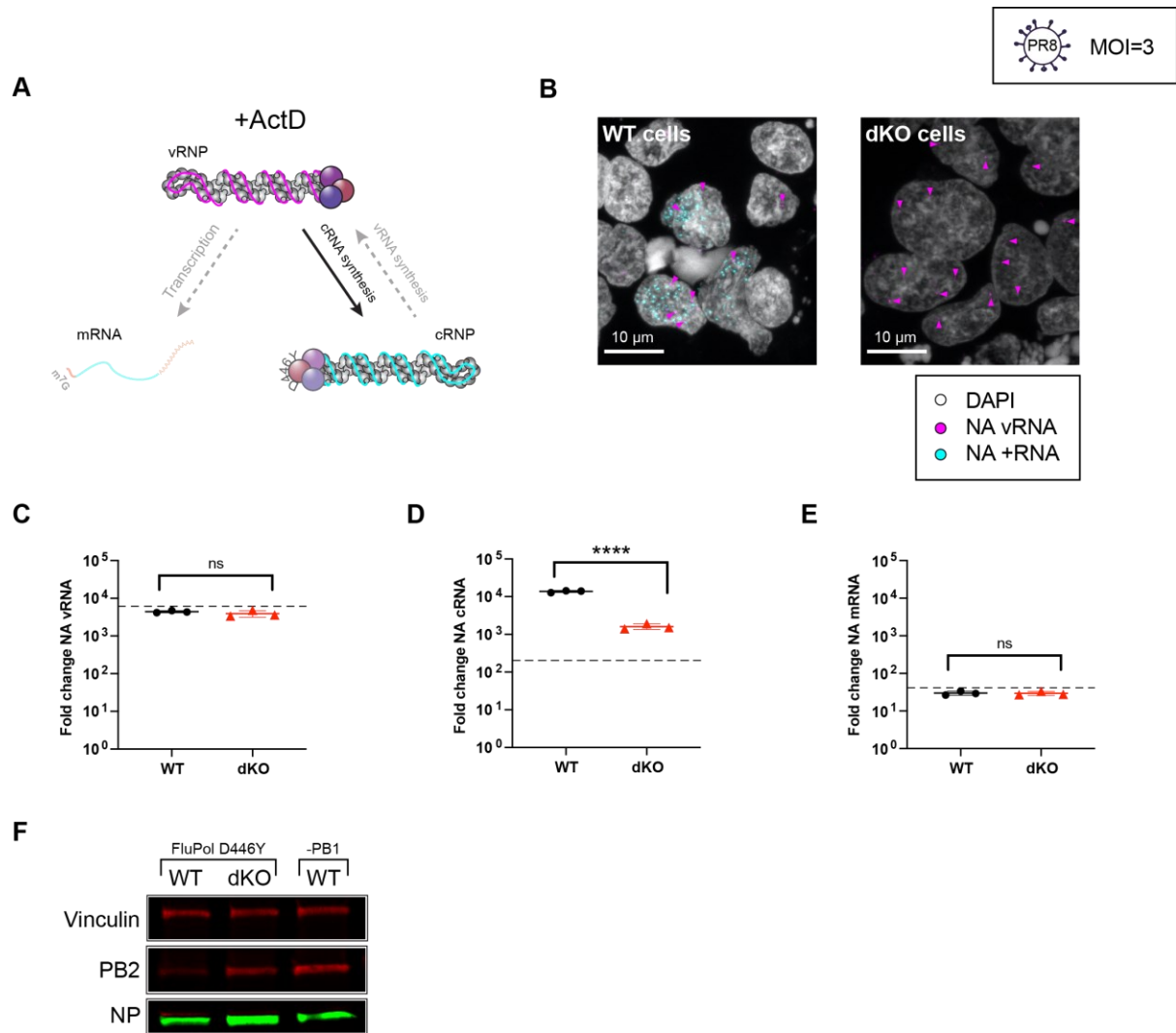


Figure 35: Human ANP32 proteins play a direct role in primary cRNA synthesis.

(A) Schematic illustrating influenza polymerase activity under the conditions of an ActD-treated cRNP stabilisation assay. D446Y= catalytically dead FluPol. (B-E) ActD-treated cRNP stabilisation in eHAP WT vs dKO cells, following infection with PR8 (MOI=3), 3hpi. (B) Accumulation of segment 6 vRNA and +RNA analysed using RNAscope. Magenta arrowheads highlight a subset of NA vRNA-stained puncta. Images are representative maximum intensity projections, visualised using a Leica SP5 inverted confocal microscope and 63x objective. Segment 6 (C) vRNA (D) cRNA or (E) mRNA accumulation analysed by RT-qPCR. Dotted line indicates background RNA present in control samples transfected with a plasmid mix lacking PB1. Fold change was calculated over mock infected cells. n=3 biological replicates, plotted as mean $\pm$ s.d.. Significance was assessed using an unpaired *t* test following log transformation. (F) Matched western blot for (B-E). Figure adapted from Swann et al., in press.

3.2.6 Primary transcription is not dependent on human ANP32 proteins

Our infection data suggests that ANP32 proteins are not required for primary transcription. Nevertheless, at later infection timepoints, reduced mRNA accumulation is observed (Figure 33,34). To confirm this is an indirect effect due to reduced vRNP template, we chose to undertake a variation on the cRNP stabilisation assay using an alternative inhibitor, cycloheximide (CHX), in place of ActD. CHX inhibits cellular translation instead of transcription, and therefore under these conditions both cRNA synthesis and primary transcription occur in the absence of vRNA synthesis (Figure 36A). As both cRNA and mRNA accumulate in this version of the assay, it is not suited to *in situ* analysis (which cannot distinguish these two RNA species). Thus, only RT-qPCR analysis was performed.

CHX-treated cRNP stabilisation assays were performed using PR8 (MOI=3) in eHAP WT, dKO and tKO cells. No accumulation of vRNA over input was observed in any of the cell types over time (Figure 36B), confirming efficient inhibition of vRNA synthesis using CHX. In agreement with ActD-treated cRNP stabilisation assays (Figure 35D), a decrease in cRNA accumulation (>3 fold) was seen in both dKO and tKO cells compared to WT, although significance was only reached at 5hpi (Figure 36C). In contrast, mRNA increased (>200 fold) in all three cell types from 0 to 5hpi, and no significant difference was detected in the presence or absence of ANP32 proteins (Figure 36D). This confirms that primary transcription does not require ANP32 proteins, as has previously been reported (Bi et al., 2019; Nilsson-Payant et al., 2021; Staller et al., 2019; Sugiyama et al., 2015), and that differences observed at later timepoints in full infections are an indirect effect due to reduced genome replication.

In the ActD-treated cRNP stabilisation assay presented in Figure 35, we were intrigued to observe a low level of cRNA accumulation in dKO cells. As previously described, although the third ANP32 orthologue huANP32E was originally thought to be virologically inert, during this project a new study reported that IBV could use huANP32E to support a low level of polymerase activity (Z. Zhang et al., 2020). Moreover, it had been observed that some viral strains could grow to low titres in either human cells lacking huANP32A/B or chicken cells lacking just chicken ANP32A (chANP32A is thought to be the sole strongly proviral ANP32 paralogue in avian cells) (Section 1.4.3, Figure 11) (Long, Idoko-Akoh, et al., 2019; H. Zhang et al., 2019). Thus there was a growing awareness that other ANP32 orthologues may provide a low level of proviral support under certain conditions. In this CHX-treated stabilisation assay, we observed a small but significant difference ($p=0.0018$) in cRNA accumulation in the dKO and tKO cells, again implying huANP32E may support a low level of cRNP stabilisation, at least with PR8. However, slightly less PB2 and NP protein were pre-expressed in the tKO cells compared to the dKO cells when assessed by western blot (Figure 36E). Therefore, we cannot exclude that this underlies the difference in cRNA accumulation observed between the dKO and tKO cells here.

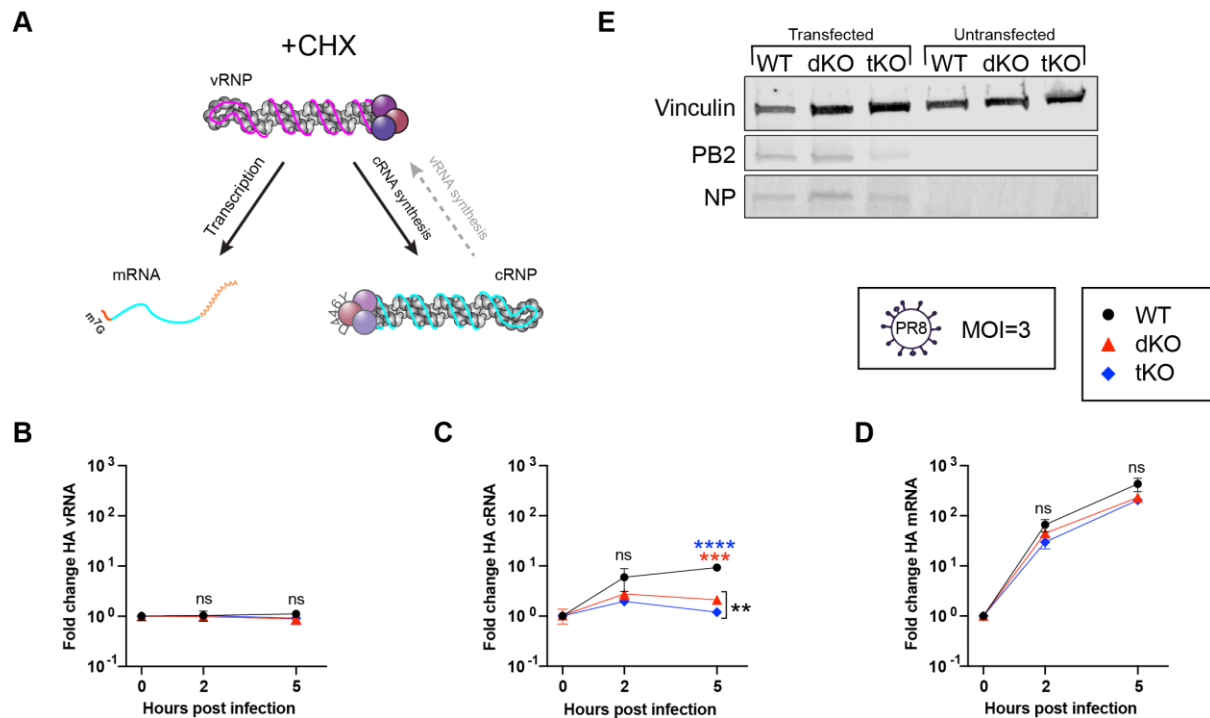


Figure 36: Primary transcription is not dependent on human ANP32 proteins.

(A) Schematic illustrating influenza polymerase activity under the conditions of a cRNP stabilisation assay treated with CHX. (B-E) cRNP stabilisation assay with CHX in eHAP WT, dKO and tKO cells. Segment 4 (B) vRNA (C) cRNA or (D) mRNA accumulation following infection with PR8 (MOI=3). Fold change was calculated over input (0hpi). n=3 biological replicates, plotted as mean $\pm$ s.d.. Significance was assessed using multiple unpaired *t* tests following log transformation, corrected for multiple comparisons using false discovery rate. (E) Matched western blot for (B-D). CHX= cycloheximide; WT= eHAP wild-type cells; dKO= eHAP ANP32A/B double knockout cells; tKO= eHAP ANP32A/B/E triple knockout cells; HA= haemagglutinin; MOI= multiplicity of infection; hpi= hours post-infection. ns= not significant; **= $p < 0.01$; ***= $p < 0.001$; ****= $p < 0.0001$. Data collected by Amalie Rasmussen. Figure adapted from Swann et al., in press.

3.2.4 Tky05 can use human ANP32E to support cRNA synthesis

One strain that has been observed to replicate to low titres in ANP32A/B dKO cells is a 6:2 reassortant consisting of the internal genes from a Eurasian-lineage highly pathogenic avian influenza virus H5N1 strain A/turkey/Turkey/1/2005 (Tky05) with PR8 HA and NA (Goldhill et al., under revision). Unusually for an avian strain, this lineage naturally bears the human-like PB2 627K signature (Long et al., 2013). To investigate the growth phenotype of Tky05 in the absence of huANP32A/B, we undertook infection time-courses in eHAP WT and dKO cells, and quantified the accumulation of NA vRNA, cRNA and mRNA using RT-qPCR (Figure 37A,B,C).

As seen with the H1N1 strain PR8, no accumulation of Tky05 NA vRNA could be observed in the dKO cells by 5hpi (Figure 37A). In contrast, whilst for PR8 no increase in cRNA accumulation was observed by 5hpi during authentic infection in dKO cells either (Figure 33B, 34B), for H5N1 Tky05 a highly significant increase in NA cRNA accumulation over input ($p < 0.0001$, analysed using an unpaired two-tailed *t* test) was observed at this timepoint (Figure 37B). The increase in cRNA in the dKO cells was significantly less (~ 2 log, $p < 0.0001$) than that observed in the eHAP WT cells, suggesting that

ANP32A/B are required, but not completely essential, for full support of Tky05 cRNA synthesis in authentic infection. As previously observed with PR8, mRNA transcripts increased 50-fold in dKO cells from 0hpi to 2hpi, demonstrating that ANP32A/B are not required for Tky05 primary transcription (Figure 37C).

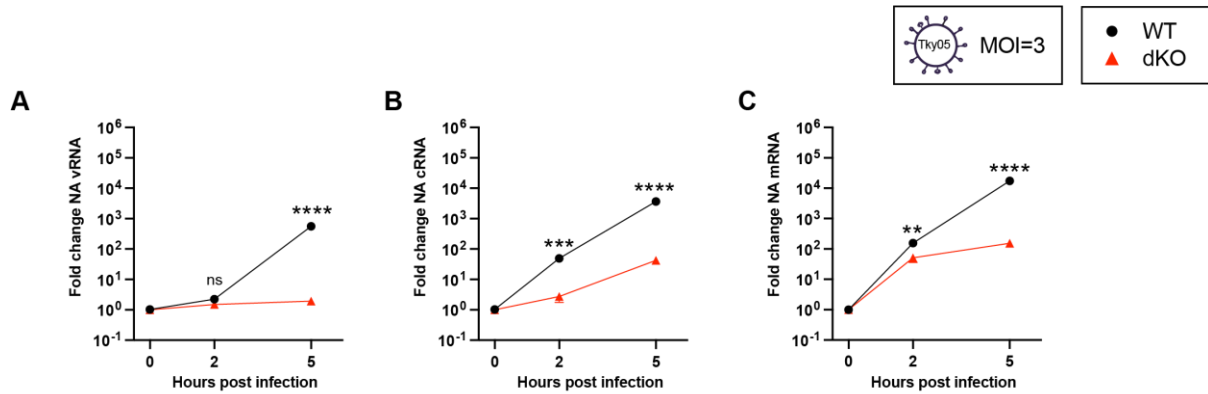


Figure 37: Tky05 can undergo cRNA synthesis in human cells lacking ANP32A/B.

(A-C) Segment 6 (A) vRNA (B) cRNA or (C) mRNA accumulation over time in eHAP WT vs dKO cells following infection with Tky05 (MOI=3). Fold change was calculated over input (0hpi). n=3 biological replicates, plotted as mean $\pm$ s.d. Significance was assessed using multiple unpaired *t* tests following log transformation, corrected for multiple comparisons using false discovery rate. NA= neuraminidase; WT= eHAP wild-type cells; dKO= eHAP ANP32A/B double knockout cells; MOI= multiplicity of infection; hpi= hours post-infection. ns= not significant; **= $p<0.01$; ***= $p<0.001$; ****= $p<0.0001$.

To understand whether the low-level of Tky05 replication in dKO cells could allow the virus to evolve to grow efficiently in the absence of huANP32A/B, experimental evolution was performed using serial passage of the Tky05 strain in dKO cells. Within two passages, this resulted in an adapted Tky05 strain capable of robust replication in the absence of ANP32A/B. This mutant strain was subsequently characterised as having adapted to use the third human ANP32 paralogue, huANP32E, to support efficient replication, through the acquisition of two mutations in the polymerase proteins: PB1 K577E and PA Q556R (Goldhill et al., under revision). A sequence alignment of the three human ANP32 paralogues is depicted in Figure 38.



Figure 38: Sequence alignment of the three human ANP32 paralogues: huANP32A, huANP32B and huANP32E.

Sequence alignment was performed by Dr Carol Sheppard using Clustal Omega (Goujon et al., 2010; Sievers et al., 2011) and visualised using Jalview (Waterhouse et al., 2009). Colour coding of amino acids is according to the Clustal X colour system of amino acid properties.

To investigate whether the initial ability of Tky05 to replicate inefficiently in eHAP dKO cells is also dependent on ANP32E, or whether low level replication is independent and the ability to use ANP32E subsequently evolved to restore efficient replication, we undertook further infection time-courses using the wild-type Tky05 strain (Tky05-WT) in eHAP WT, dKO and tKO cells and quantified the accumulation of NA vRNA, cRNA and mRNA. Simultaneously, we also compared the replication of the experimentally-evolved ANP32A/B-independent Tky05 strain (Tky05-Mut) (Figure 39). Although Tky05-Mut demonstrated slightly delayed vRNA synthesis at the early 2hpi timepoint, overall both strains accumulated equivalent quantities of vRNA in WT cells, suggesting that the mutations acquired by Tky05-Mut are not disadvantageous for vRNA synthesis in the presence of huANP32A/B (Figure 39A). In dKO cells, efficient vRNA synthesis could be observed for Tky05-Mut in the absence of huANP32A/B, albeit over a log lower than in WT cells. As previously observed (Figure 37A), Tky05-WT did not accumulate vRNA in dKO cells. Neither virus accumulated vRNA in tKO cells, consistent with at least one ANP32 protein being essential for IAV genome replication, and with the mechanism of Tky05-Mut replication in dKO cells being adaptation to huANP32E.

cRNA synthesis was slightly delayed for Tky05-Mut in WT cells, with less cRNA accumulating at earlier timepoints compared to Tky05-WT (Figure 39B). However by 8hpi, both viruses accumulated similar levels of cRNA. In dKO cells, both Tky05-WT and Tky05-Mut accumulated similar levels of cRNA at 2hpi and 5hpi. This suggests Tky05-Mut does not stimulate primary cRNA synthesis in dKO cells, and that the mutations acquired specifically increase vRNA synthesis. By 8hpi, significantly more cRNA was present in Tky05-Mut infected dKO cells than in dKO cells infected with Tky05-WT, which may be an indirect effect due to increased vRNP template. ANP32E clearly contributes to the ability of Tky05-WT to undergo cRNA synthesis in dKO cells, as cRNA accumulation was almost completely ablated in tKO cells for both Tky05-WT and Tky05-Mut. Nevertheless, a general upwards trend in cRNA accumulation over time could be observed in the tKO cells for both viruses. In the case of Tky05-WT, the increase from 0hpi to 8hpi reaches significance ($p < 0.0001$, analysed using an unpaired two-tailed t test). This

may suggest a very low level of cRNA synthesis can occur in the absence of any stimulating ANP32 protein, either in a host factor independent manner or through the support of yet further host factors that can weakly substitute for ANP32 proteins. It would be interesting to quantify levels of cRNA at later timepoints to determine whether this trend continues.

Unexpectedly, considering ANP32 is implicated in supporting genome replication and not transcription, adaptation to huANP32E appeared to impair transcription. In both WT and tKO cells, Tky05-Mut accumulated significantly less mRNA than the WT virus at all timepoints (Figure 39C). This suggests that the adaptive mutations function by shifting the FluPol towards a replication mode. In the dKO cells, Tky05-Mut accumulated significantly less mRNA than WT at 2hpi, when only primary transcription can occur. By 8hpi however, Tky05-Mut accumulated significantly more mRNA, likely due to increased vRNP template compared to WT virus. Nevertheless, in all cell types, and for both virus, mRNA transcripts increased by at least 100-fold over the course of infection. This suggests that ANP32E, like ANP32A/B, is not directly required for primary transcription.

Overall, the lower cRNA accumulation by Tky05-WT in tKO cells compared to dKO cells suggests that the low level of activity observed in dKO cells is due to an innate ability to inefficiently use huANP32E to support cRNA synthesis. Presumably, it is this ability that then provides the opportunity for the virus to further adapt to huANP32E to achieve robust replication. Nevertheless, parallel passaging of a virus that does not show a low level of replicative activity in dKO cells, such as PR8, was not performed, and it is possible given the opportunity most viruses will evolve to use huANP32E.

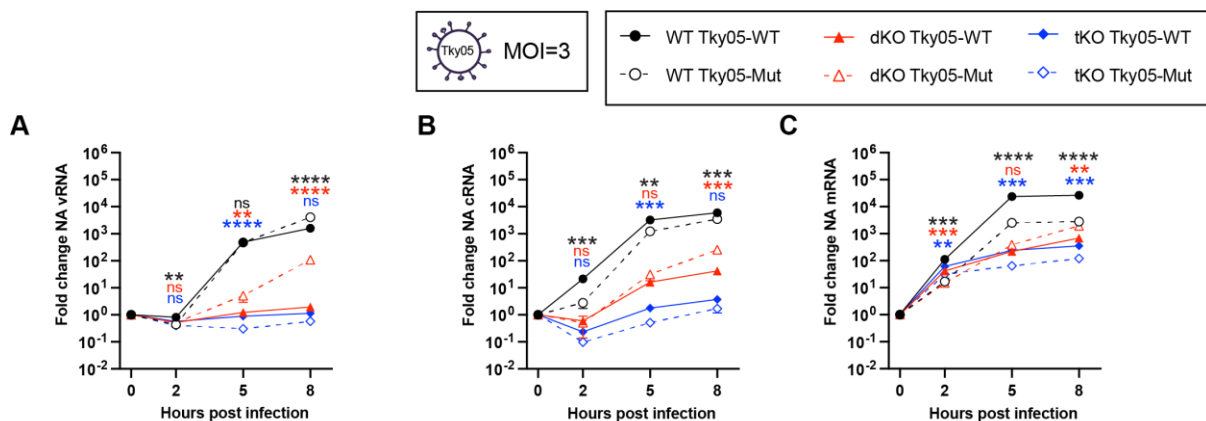


Figure 39: Tky05 can use human ANP32E to support cRNA synthesis.

(A-C) Segment 6 (A) vRNA (B) cRNA or (C) mRNA accumulation over time in eHAP WT, dKO and tKO cells following infection with Tky05-WT or Tky05-Mut (MOI=3). Fold change was calculated over input (0hpi). n=3 biological replicates, plotted as mean $\pm$ s.d.. Statistical significance was assessed using multiple unpaired *t* tests following log transformation, corrected for multiple comparisons using the false discovery rate. Statistical comparisons are between Tky05-WT and Tky05-Mut within the same cell line at each timepoint. Comparisons for WT cells are shown at the top, dKO cells in the middle and tKO cells at the bottom (indicated by matching colour to traces). NA= neuraminidase; WT= eHAP wild-type cells; dKO= eHAP ANP32A/B double knockout cells; tKO= eHAP ANP32A/B/E triple knockout cells. Tky05-WT= wild-type Tky05; Tky05-Mut=Tky05 evolved to replicate efficiently in dKO cells; MOI= multiplicity of infection; hpi= hours post-Infection. ns= not significant; **= p<0.01; ***= p<0.001; ****= p<0.0001. Figure adapted from Goldhill et al., under revision.

The FluPol activity of either Tky05-WT or Tky05-Mut in the three cell types (eHAP WT, dKO, tKO) is summarised in Figure 40. The analysis of the early replication of Tky05-Mut suggests that adaptation to huANP32E involves biasing the FluPol away from transcriptional activity, as evidenced by the reduced transcription observed compared to Tky05-WT when grown in either WT or tKO cells and reduced primary transcription when grown in dKO cells (Figure 39C, 40). Unexpectedly, the adaptive mutations in Tky05-Mut appear to specifically boost vRNA synthesis, rather than increasing replicase activity in general, as Tky05-Mut also shows reduced cRNA synthesis when grown in WT cells (Figure 39B (black lines), Figure 40). Indeed, considering the reduced cRNP template, it is remarkable that vRNA synthesis remains equivalent between the two strains in WT cells, apart from a small delay for Tky05-Mut at 2hpi (Figure 39A (black lines)). This suggests that Tky05-Mut undergoes higher levels of vRNA synthesis in WT cells but this is masked by reduced cRNP template. The equivalent vRNA synthesis from both viral strains in WT cells also demonstrates that adaptation of Tky05 to huANP32E in dKO cells lacking huANP32A/B does not then result in overall impaired FluPol activity when the adapted virus is grown in WT cells in which huANP32A/B are present. Finally, no significant difference is observed in primary cRNA synthesis between the two strains in dKO cells (Figure 39B (red lines, 0-5hpi)). Again, this suggests that the FluPol adaptations in Tky05-Mut specifically enhance vRNA synthesis, rather than replicase activity in general. This implies that the initial low level of cRNA synthesis undertaken by unadapted Tky05-WT in the dKO cells is sufficient to support overall robust replication, despite being significantly lower than that undertaken in WT cells. Therefore, the adaptations present in Tky05-Mut FluPol are specifically acquired to restore vRNA synthesis in the absence of huANP32A/B.

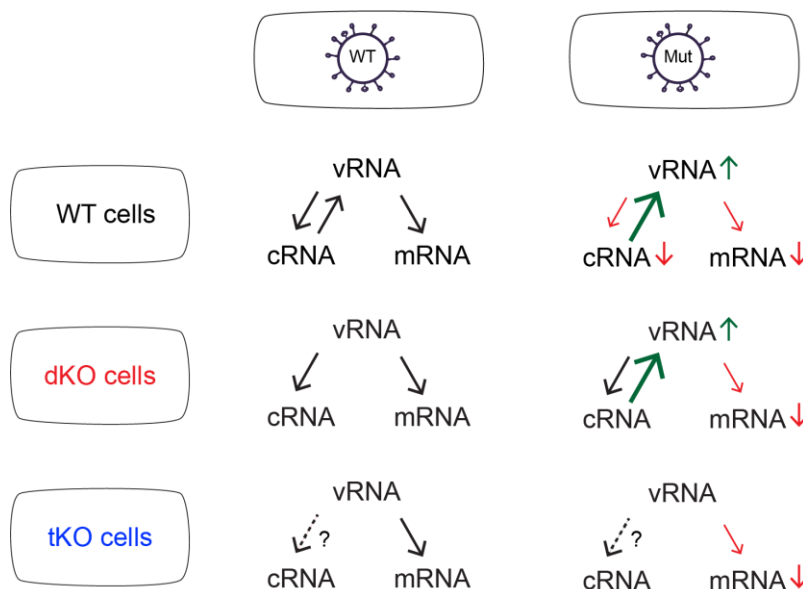


Figure 40: Schematic of the activity of Tky05-WT and Tky05-Mut in eHAP WT, dKO and tKO cells.

Activity of Tky05-Mut is compared to the activity observed in the same cell type by Tky05-WT, i.e., comparisons have been made within cell types but not across cell types. Green arrows indicate increased activity, red arrows indicate reduced activity. WT= Tky05-WT; Mut= Tky05-Mut.

3.2.5 Human ANP32 proteins play a direct role in primary cRNA synthesis of additional human IAV strains

To broaden our conclusions, we decided to investigate whether huANP32 proteins are required for cRNA synthesis in further human IAV strains. Specifically, we quantified vRNA and cRNA accumulation in eHAP WT, dKO and tKO cells following infection with 6:2 reassortant viruses with PR8 external genes (to normalise entry) and internal genes representative of either a pandemic human H1N1 strain, A/Eng/195/2009(H1N1) (H1N1pdm09) or a human H3N2 strain, A/Aichi/2/1968(H3N2) (Figure 41A-D).

Consistent with our investigations using PR8 and Tky05, no vRNA accumulation was observed for either strain in the dKO or tKO cells, demonstrating that huANP32A/B are absolutely essential for vRNA synthesis (Figure 41A,C). Moreover, a significant reduction in cRNA accumulation was already observed in both dKO and tKO cells by 2hpi, strongly suggesting a direct role for ANP32 proteins in cRNA synthesis (Figure 41B,D). In the case of the H3N2 strain, no increase in cRNA was observed in the dKO or tKO cells over input, suggesting huANP32A/B are essential whilst huANP32E is insufficient to support primary cRNA synthesis. In contrast, in the case of the H1N1(pdm09) strain, a small but significant increase in cRNA accumulation over input was observed in the dKO cells by 5hpi ($p=0.03$, analysed using an unpaired two-tailed t test). Furthermore, a small but significant difference in cRNA accumulation was observed between the dKO and tKO cells at 5hpi (Figure 41B). Taken together, this suggests a low level of support by huANP32E and again highlights the strain-to-strain variability in the ability to (inefficiently) utilise huANP32E.

As with Tky05, with the H1N1(pdm09) strain we observed a very small upwards trend in cRNA accumulation in the tKO cells. This increase did not reach statistical significance from 0 to 5hpi, so could represent variation within the assay. Nevertheless, to exclude the possibility that this minimal cRNA accumulation represented primary cRNA synthesis in the absence of further rounds of replication and amplification, i.e., the H1N1(pdm09) strain does not directly require ANP32 proteins for cRNA synthesis, we undertook a CHX-treated cRNP stabilisation assay using the H1N1(pdm09) strain in eHAP WT and tKO cells (Figure 41E,F,G). In this assay, a highly significant reduction in cRNA accumulation was observed in the absence of ANP32 proteins, 5hpi, confirming that ANP32 directly supports cRNA synthesis in this strain. Therefore overall, this data confirms that ANP32 proteins are required to directly support robust cRNA synthesis for a diverse range of IAV strains.

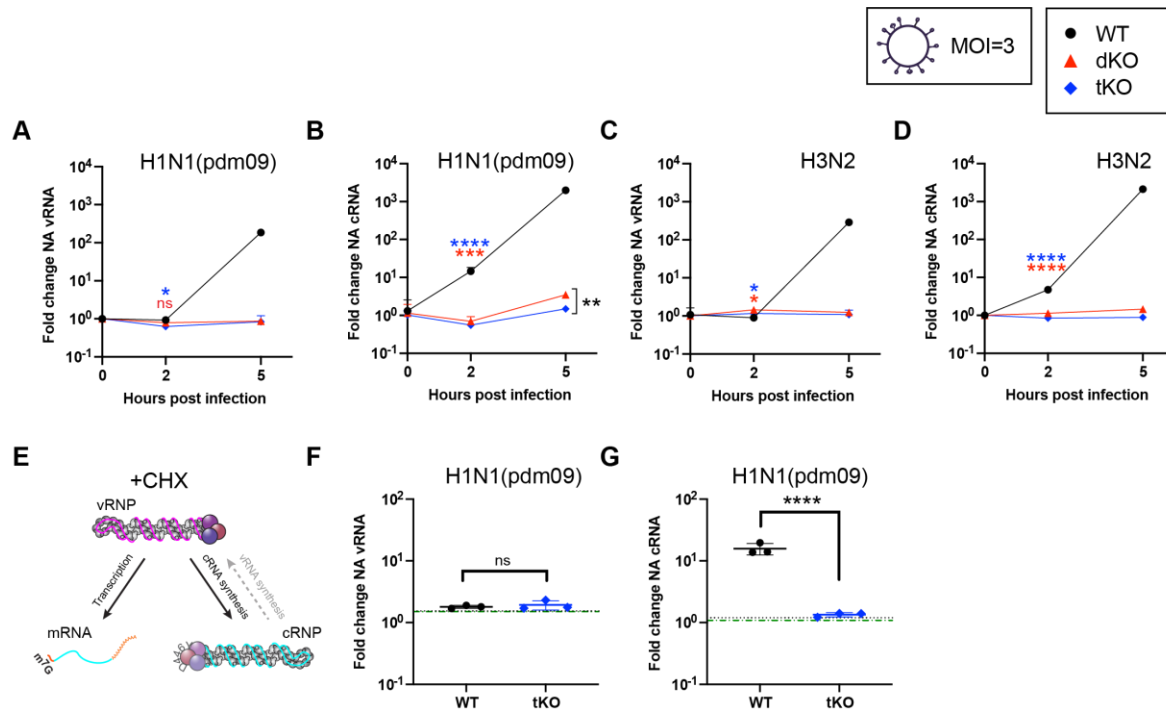


Figure 41: Human ANP32 proteins play a direct role in primary cRNA synthesis of additional human IAV strains.

(A-D) Segment 6 (A,C) vRNA accumulation or (B,D) cRNA accumulation over time in eHAP WT, dKO and tKO cells following infection with (A,B) H1N1(pdm09) (Eng195) or (C,D) H3N2 (Aichi68) (MOI=3). Fold change was calculated over input (0hpi). n=3 biological replicates, plotted as mean $\pm$ s.d.. Significance at 2hpi (WT vs dKO (bottom, red) or WT vs tKO (top, blue)) was assessed using an unpaired *t* test following log transformation. (E) Schematic of CHX-stabilised cRNP stabilisation assay. (F,G) Segment 6 (F) vRNA or (G) cRNA accumulation during a CHX-treated cRNP stabilisation assay in eHAP WT and tKO cells, following infection with Eng195 (MOI=3). Fold change was calculated over input (0hpi). Black dotted line indicates background levels of vRNA/cRNA present in WT cells transfected with a control plasmid mix minus PB1, 5hpi; green dotted line indicates background levels of vRNA/cRNA present in WT cells transfected with a control plasmid mix minus NP, 5hpi. n=3 biological replicates, plotted as mean $\pm$ s.d.. Significance was assessed using an unpaired *t* test following log transformation. hpi= hours post-infection. ns= nor significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$. Figure adapted from Swann et al., in press

3.3 DISCUSSION

In this chapter we investigated the mechanism by which ANP32 proteins support influenza replication. Using previously generated cell lines with expression of either ANP32A/B (dKO) or ANP32A/B/E (tKO) ablated by CRISPR-Cas9, we unambiguously confirm that ANP32 is required to support the viral polymerase in cRNA synthesis, but not viral transcription. Our biological data is therefore compatible with the current interpretation of the ANP32-stabilised asymmetric dimer complex as a replication platform responsible for coordinated encapsidation of nascent cRNA/vRNA into RNPs. Although our data does not agree with previous *in vitro* analysis (Sugiyama et al., 2015), *in vitro* assays frequently do not recapitulate *in vivo* activity. Importantly, cRNA synthesis has been shown to occur from purified vRNP templates in the absence of additional polymerase protein *in vitro* (York et al., 2013), despite nascent encapsidating components proving completely essential *in vivo* (Vreede et al., 2004). As the replicase model implicates ANP32 in loading the encapsidating polymerase onto nascent cRNA, such data might imply that the role of ANP32 would be similarly disposable under *in vitro* conditions. Moreover, ANP32 is known to interact with the PB2-627 domain (Baker et al., 2018; Mistry et al., 2020), which is similarly essential *in vivo* but dispensable *in vitro* (Nilsson et al., 2017).

Although the literature would suggest ANP32 is required for vRNA synthesis (Nilsson-Payant et al., 2021; Sugiyama et al., 2015), we cannot conclude a direct role from this data, as it is possible no vRNA accumulation is observed due to the upstream effect on cRNA synthesis. The lack of vRNA accumulation during Tky05 infections in dKO cells - despite a low level of cRNA accumulation – could be interpreted as a, potentially more stringent, requirement for huANP32A/B for this step (Figure 37A,B). Nevertheless, as vRNA synthesis follows cRNA synthesis, we cannot exclude vRNA accumulation occurs later in infection, and it would be interesting to investigate later timepoints to answer this question. Indeed, we know that a small amount of infectious Tky05 virus can be isolated from dKO cells 72hpi, suggesting that a low level of vRNA synthesis must eventually occur in the absence of huANP32A/B.

Although all strains investigated required ANP32A/B for robust cRNA synthesis, and demonstrated significantly impaired cRNA accumulation in dKO cells, we observed strain-to-strain variation in the ability to use the final ANP32 paralogue, ANP32E, to support a low level of cRNA synthesis at early infection timepoints. Two of the four strains investigated here, Tky05 (H5N1) and Eng195 (H1N1), were capable of inefficient cRNA synthesis in dKO cells (Figure 37B, 41B). In contrast, PR8 and Aichi68 showed no cRNA accumulation from 0-5hpi during authentic infection in dKO cells (Figure 33B, 34B, 40D), although PR8 showed some activity over background in an ActD-treated cRNP stabilisation assay (Figure 35D) and slightly higher activity compared to tKO cells in a CHX-treated cRNP stabilisation assay (Figure 36C). It would be interesting to map the genetic differences between strains that can or cannot utilise huANP32E. Previously, the inability of huANP32E to support robust IBV FluPol replication has been mapped to a single substitution (N129E) (Z. Zhang et al., 2020). This is the same region (residues 129/130) that underlies the inability of chANP32B and msANP32A to support IAV FluPol replication (Long, Idoko-Akoh, et al., 2019; Staller et al., 2019; H. Zhang et al., 2019). Residues 129/130

map to the interface between the ANP32 LRR C-terminus and the encapsidating FluPol in the asymmetric FluPol dimer structure (Figure 42) (Carrique et al., 2020). Specifically, the asparagine at position 129 of ANP32A interacts directly via hydrogen bonding with the lysine at position 391 in the P3 subunit of the ICV FluPol_E. The aspartic acid at position 130 directly interacts with a lysine at position 608 in the P3 subunit (Carrique et al., 2020). Speculatively, variation in the residues present on the encapsidating FluPol interface might allow the N129E substitution that is present in huANP32E, relative to huANP32A, to be tolerated in some strains. The P3 subunit in ICV is equivalent to PA in IAV, and the authors state that P3 K391 is equivalent to PB1 K413 in IAV (Carrique et al., 2020). In all four strains investigated here, the residue at position 413 is a lysine, suggesting variation at this specific residue cannot explain the differences observed here. However, it is possible that other residues in the region may be able to compensate for a suboptimal interaction. However, as there is currently only an ANP32A-ICV structure available for the ANP32-stabilised asymmetric dimer complex, it is difficult to precisely map the interactions between ANP32 proteins and IAV FluPol.

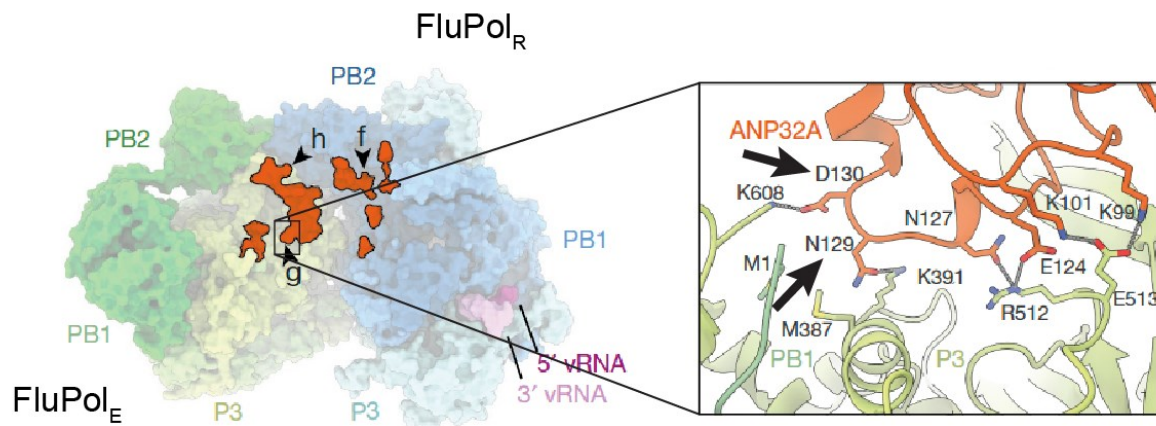


Figure 42: Substitutions at position 129 on ANP32A map to the interaction with the encapsidating FluPol in the ANP32A-stabilised asymmetric ICV FluPol dimer structure.

ANP32A-ICV FluPol interface, with interacting regions highlighted in orange. Figure adapted from Carrique et al., 2020, with permission from Springer Nature. Residues 129 and 130 are highlighted with black arrowheads. N129 interacts with residue K391, whilst D130 interacts with K608, of the P3 subunit of the ICV FluPol.

Alternatively, differences in other interfaces of the complex, for example increased stability at the ANP32 LRR N-terminus with the replicating FluPol (region F in Figure 42) or the FluPol_R:FluPol_E interface, might reduce the dependency on this additional contact point. We noted that Tky05-WT, Tky05-Mut and Eng195 show a small upwards trend in cRNA accumulation over time in the tKO cells. Although this trend only reached significance over input by 8hpi for Tky05-WT infection, we did not measure beyond 5hpi in the case of Eng195. This might suggest the FluPol_R:FluPol_E interface in the asymmetric dimer is more stable in these strains, allowing a low level of ANP32-independent cRNA synthesis. It would be interesting to see whether this trend continues at later timepoints of infection. If this effect is real, it could suggest that some strains could evolve to replicate in the complete absence of ANP32 proteins. However, as ANP32 is also required to recruit NP to the nascent RNP (F. Wang et al., 2022), it is possible that the level of replication is limited by the loss of this function, and it is this

that would need to be rescued with further evolution – potentially through recruiting an alternative host factor with a negatively charged intrinsically disordered domain (IDD). Nevertheless, it is worth noting that these experiments were undertaken at a moderately high MOI (MOI=3) and it is possible that such low level replication would not be seen if we repeated these experiments at a lower MOI, or in the context of a naturally acquired infection.

ANP32 proteins are believed to specifically support influenza genome replication and are not involved in viral transcription. Therefore, we were initially surprised to find that adapting the avian IAV Tky05 to use the weakly proviral ANP32 orthologue (huANP32E) resulted in reduced FluPol transcription relative to the original, WT strain. This result might suggest that the two adaptive mutations, PB1 K577E and PA Q556R, bias the FluPol away from transcription and towards a 'replicase' mode. This might suggest that part of the essential role of ANP32 is in regulating the level of transcription vs replication, potentially through stabilising the replicating polymerase.

Even more surprisingly, the adaptive mutations do not map to any of the interfaces present in the ANP32-stabilised asymmetric FluPol dimer structure (Figure 43A). Instead, PA Q556R and PB1 K577E map to the symmetric FluPol dimer interface (Figure 43B), that is implicated in primer realignment required specifically for vRNA synthesis (Fan et al., 2019). Further characterisation suggests that the adaptive mutations destabilise this dimer (Goldhill et al., under revision). Nevertheless, our characterisation of the early replication of Tky05-Mut suggests PA Q556R and PB1 K577E promote vRNA synthesis (Figure 39, 40). With our current understanding of replication, it is somewhat counter-intuitive that destabilising mutations in the symmetric dimer interface (associated with primer realignment required for vRNA synthesis (Fan et al., 2019)) would boost vRNA synthesis. Plausibly, in the absence of a strongly pro-viral ANP32 protein, increasing the overall concentration of free FluPol protomers in the nucleus through reducing the strength of the symmetric dimer would encourage the weaker ANP32E-stabilised asymmetric dimer to form, and forming this structure is overall more advantageous for vRNA synthesis than having a strong symmetric dimer. In agreement with this mechanism, a recent pre-print has linked ubiquitination of the neighbouring lysine at position 578 on PB1 to regulation of the symmetric dimer and promotion of replication (Günl et al., 2022). Nevertheless, it is difficult to explain why this mechanism does not also increase cRNA synthesis. Overall, the characterisation of Tky05-Mut hints that there is more to understand both about the regulation of FluPol replication, and how ANP32 fits into the picture. This work is also important from a translational perspective, as it highlights opportunities for viral escape should drugs targeting the ANP32-FluPol interaction be developed.

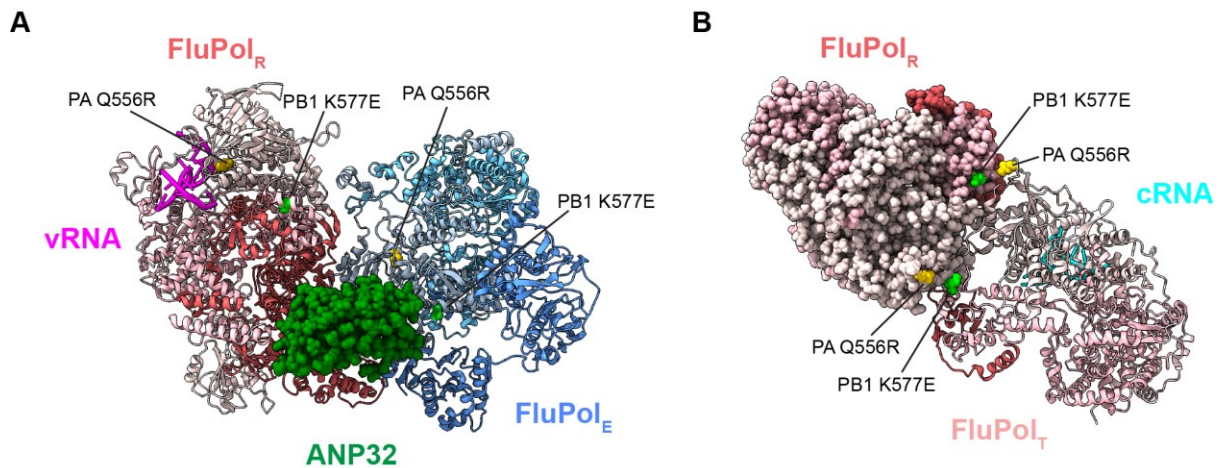


Figure 43: Adaptive mutations that allow Tky05-Mut to utilise human ANP32E mapped to the asymmetric and symmetric FluPol dimer structures.

(A) Cryo-EM structure of the chicken ANP32A-stabilised asymmetric ICV FluPol dimer structure with short 47 nt vRNA fragment (Protein Data Bank (PDB) accession code:6XZR) (Carrique et al., 2020) with adaptive mutations that allow Tky05-Mut to utilise huANP32E highlighted in green and yellow (Goldhill et al., under revision). (B) Cryo-EM structure of the symmetric IAV (H3N2) dimer structure with cRNA promoter (PDB accession code:6QX8) (Fan et al., 2019), with adaptive mutations that allow Tky05-Mut to utilise huANP32E highlighted in green and yellow (Goldhill et al., under revision). FluPol protomers have been arbitrarily labelled FluPol_R and FluPol_T (*trans*-activating FluPol). Structural models were prepared with ChimeraX (Pettersen et al., 2021).

Our preliminary *in situ* characterisation of replication in WT and tKO cells dramatically illustrates the essential role ANP32 proteins play in FluPol replication, as well as the heterogeneity in infection that has been highlighted in previous single-cell studies (Heldt et al., 2015; Russell et al., 2018, 2019; D. Wang & Ma, 2017). As only primary transcription can occur in tKO cells (possibly with the exception of a tiny amount of cRNA synthesis in strains such as Tky05), comparing infections in WT and tKO cells may allow transcribing vs replicating vRNPs to be distinguished. In the future we aim to combine CLEM, in collaboration with the Crick, with co-localisation analysis using standard light microscopy to answer such questions as whether the vRNP template moves away from the euchromatin/host Pol II CTD (the established platform for transcription) in order to undergo genome replication. As a tKO cell can also be used as a model for host restricted FluPol, such comparisons may also shed light on the spatial details of host restriction. A challenge of this approach will be comparing robust infections in WT cells with abundant replication with abortive infections in tKO cells that will host very little influenza RNA.

In summary, we have used our *in situ* RNA FISH approach to directly visualise replication in the absence and presence of huANP32 proteins. Matched CLEM analysis is ongoing. Further investigations are required to utilise this approach to better understand FluPol regulation within the context of the highly organised nucleus. We have also demonstrated that huANP32A/B strongly enhance cRNA synthesis, in support of the structural data implying ANP32 is required to encapsidate nascent cRNPs and vRNPs (Carrique et al., 2020). There appears to be strain-to-strain variation in the ability to use huANP32E to weakly support cRNA synthesis. Such an ability may predispose certain strains to readily escape restriction in the absence of ANP32A/B and may undermine translational work focused on disrupting the ANP32-FluPol interaction.

CHAPTER FOUR: AVIAN INFLUENZA A VIRUS POLYMERASE CAN UTILISE HUMAN ANP32 PROTEINS TO SUPPORT cRNA SYNTHESIS

4.1 INTRODUCTION

The polymerase from avian influenza virus (AIV) replicates poorly in mammalian cells, resulting in a strong selection for mammalian-adaptive mutations (Long, Mistry, et al., 2019). The best-described of these is PB2 E627K, which is sufficient to allow robust replication of an avian origin polymerase in the mammalian nucleus and has been shown to be an adaptation to mammalian paralogues of the essential host factor ANP32 (Long et al., 2016; Subbarao et al., 1993). In the previous chapter, we demonstrate that human ANP32 proteins are required to support the polymerase in cRNA synthesis, as well as vRNA synthesis. Consequently, we would expect unadapted AIV FluPol carrying PB2 627E (FluPol 627E) to be restricted in the production of both cRNA and vRNA during infection of mammalian cells. However, several reports suggest that host restriction specifically occurs at the level of vRNA synthesis (Bi et al., 2019; Mänz et al., 2012; Nilsson et al., 2017).

In this chapter we investigate the point at which host restriction acts using a pair of isogenic viruses based on the highly pathogenic H5N1 avian strain 5092 (A/England/turkey/50-92) that differ only at position 627 on PB2 (5092E (627E) or 5092K (627K)) (Long et al., 2013). First, we confirm that 5092 FluPol requires human ANP32 (huANP32) proteins to directly support cRNA synthesis. Next, we make use of cRNP stabilisation and replication assays using 5092E and 5092K in mammalian cells to investigate at which step of genome replication host restriction is imposed. In accordance with the literature, we find that 5092 FluPol with the avian PB2 627E signature is specifically restricted in vRNA synthesis, and not cRNA synthesis, and can be rescued through exogenous expression of chicken ANP32A. This suggests that avian FluPol is compatible with huANP32 proteins for cRNA synthesis, however incompatible with huANP32 proteins for vRNA synthesis. We suggest that huANP32 proteins play an additional, as yet structurally undefined, role in supporting vRNA synthesis, and it is this activity that is specifically blocked when avian influenza infects mammalian cells.

Finally, to try and map this proposed vRNA-specific activity to a specific domain of ANP32, we investigate whether huANP32B LCAR deletion mutants can recreate the host restricted phenotype (functional cRNA synthesis, inhibited vRNA synthesis), with a mammalian polymerase. Although this proved unsuccessful, this is logical considering the LCAR has since been implicated in RNP encapsidation and therefore cRNA synthesis (F. Wang et al., 2022). Based on our current understanding of ANP32, we therefore propose alternative mutants that might allow the vRNA-specific activity to be mapped to a specific ANP32-domain/motif. We also discuss alternative explanations of our data that could be experimentally explored.

Data presented in Figure 45 was collected by a master's student, Amalie Rasmussen, under the supervision of this author. HuANP32B mutant proteins were generated by Ecco Staller (Staller, 2021; Staller et al., 2021). Data presented in Figures 44, 45 and 47, and the accompanying text, are adapted from Swann et al., in press.

4.2 RESULTS

4.2.1 Human ANP32 proteins play a direct role in primary cRNA synthesis of the H5N1 strain 5092

To investigate whether host restriction of avian FluPol in mammalian cells acts at the level of cRNA synthesis, as well as vRNA synthesis, we made use of a previously generated pair of isogenic viruses based on the avian strain A/turkey/England/50-92/1991 (5092) that differ only in the residue at position 627 on PB2: either the wild-type PB2 627E (5092E) or the 'humanising' mutation PB2 E627K (5092K) that allows efficient use of human ANP32 proteins (Long et al., 2013, 2016). 5092 is a historical pre-Asian lineage H5N1 highly pathogenic AIV, that has been reported to be restricted to *Galliformes* (Long et al., 2013; G. W. Wood et al., 1995). As with other virus strains used in this study, the viruses were rescued with PR8 external genes (in this case HA, NA and M).

We first confirmed that 5092 FluPol is also dependent on ANP32 proteins for cRNA synthesis through comparing the accumulation of NA vRNA, cRNA and mRNA during infection in either wild-type (WT) eHAP cells, or eHAP triple knockout cells that lack expression of huANP32A, huANP32B and huANP32E (Figure 44A,B,C). For this work we chose to use the 'humanised' virus (5092K), which replicates robustly in mammalian cells. As with previous strains, we observed no accumulation of cRNA or vRNA over input in the tKO cells (Figure 44A,B), confirming huANP32 proteins are required for efficient cRNA and vRNA synthesis. Interestingly, 5092 FluPol demonstrated similar dynamics as both the H1N1 Eng195 and H5N1 Tky05 FluPol in tKO cells (chapter three, Figure 39B and Figure 41B), in which cRNA levels initially decrease relative to input, before slowly recovering over time, resulting in an upwards trend in cRNA accumulation (Figure 44B). This may suggest that 5092 can use endogenous huANP32E to undergo a low level of cRNA synthesis in double knockout (dKO) cells, as seen with other strains that demonstrate this kinetic profile in tKO cells, although we did not test this. As with Tky05 and Eng195, it may also suggest that 5092 undergoes a very low level of ANP32-independent cRNA synthesis.

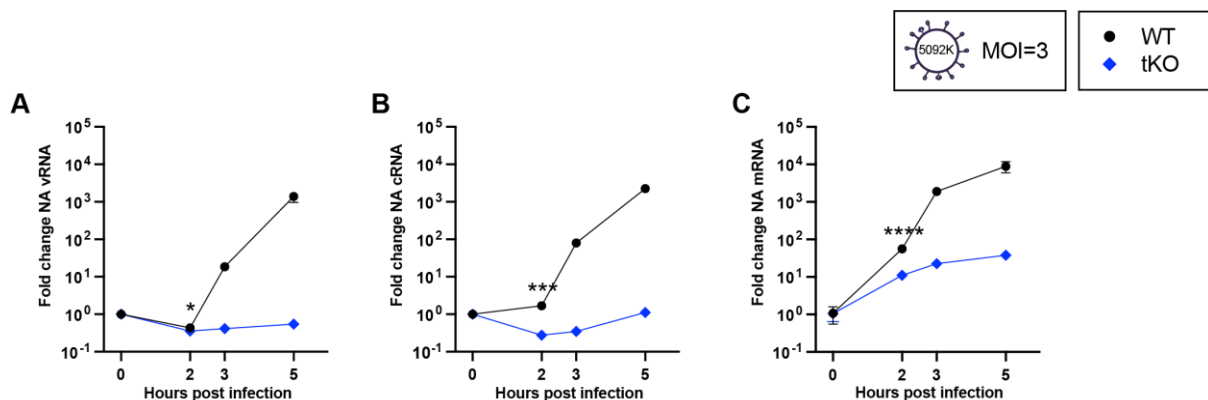


Figure 44: 5092K requires human ANP32 proteins for efficient cRNA synthesis.

(A-C) Segment 6 (A) vRNA (B) cRNA or (C) mRNA accumulation over time in eHAP WT vs tKO cells following infection with 5092K (MOI=3). Fold change was calculated over Input (0hpi). n=3 biological replicates, plotted as mean $\pm$ s.d.. Significance at 2hpi was assessed using an unpaired *t* test following log transformation. WT= eHAP wild-type cells; tKO= eHAP ANP32A/B/E triple knockout cells; NA= neuraminidase; MOI= multiplicity of infection; hpi= hours post-infection. ns= not significant; * = $p < 0.05$; ***= $p < 0.001$; ****= $p < 0.0001$. Figure adapted from Swann et al., in press.

To confirm the role of ANP32 in cRNA synthesis is direct, we also made use of a CHX-treated cRNP stabilisation assay, in which cRNA synthesis and mRNA synthesis can occur, but vRNA synthesis is blocked (Figure 45A,B,C). A small (<1 fold) but significant difference was observed in the accumulation of HA vRNA in the WT and tKO cells by 5hpi, suggesting CHX-treatment did not fully inhibit replication in this experiment (Figure 45A). Nevertheless, we concluded it was unlikely that the larger (>5 fold), significant difference seen in HA cRNA accumulation by 5hpi was solely driven by the small reduction in vRNA synthesis (Figure 45B). Combined with the infection data, we therefore concluded that huANP32 proteins play a direct role in supporting cRNA synthesis during 5092 infection. As with previous strains, no difference in primary transcription was observed, confirming 5092 FluPol does not require ANP32 proteins for transcription (Figure 45C).

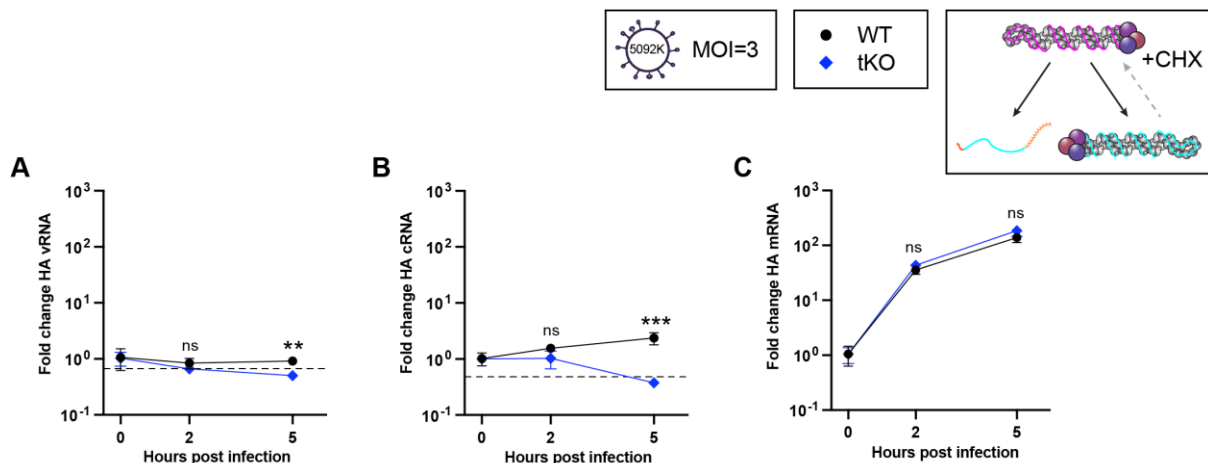


Figure 45: Human ANP32 proteins are required for primary cRNA synthesis of the H5N1 strain 5092.

(A,B,C) CHX-treated cRNP stabilisation assay in eHAP WT and tKO cells. Segment 4 (A) vRNA (B) cRNA or (C) mRNA accumulation following infection with 5092K (MOI=3). Fold change was calculated over input (0hpi). Dotted line indicates background levels of vRNA/cRNA present in WT cells transfected with a control plasmid mix minus PB1, 5hpi. n=3 biological replicates, plotted as mean $\pm$ s.d.. Significance was assessed using multiple unpaired *t* tests following log transformation, corrected for multiple comparisons using false discovery rate. CHX= cycloheximide; WT= eHAP wild-type cells; tKO= eHAP ANP32A/B/E triple knockout cells; HA= haemagglutinin; MOI= multiplicity of infection; hpi= hours post-infection. ns= not significant; **= $p < 0.01$; ***= $p < 0.001$. Data was collected by Amalie Rasmussen. Figure adapted from Swann et al., in press.

4.2.2 Characterisation of 5092E replication in mammalian cells

Next, we characterised the restriction of PB2 627E FluPol activity during replication in mammalian cells. We infected HEK-293T cells with either 5092E or 5092K at either high (MOI=3) or low (MOI=0.1) MOI, and quantified the accumulation of NA vRNA, cRNA and mRNA at 0, 2, 5 and 8hpi. Viral inputs were normalised on genome copies rather than infectious titres as we wished to standardise the input number of vRNPs. As expected, 5092E demonstrated attenuated replication relative to 5092K in mammalian cells at both MOIs investigated. In the high MOI infection, attenuation was particularly apparent at 5hpi, with all three RNA species reduced ~1 log compared to 5092K (Figure 46A,B,C). By 8hpi, this difference had narrowed as 5092K replication peaked earlier than 5092E. In the case of NA mRNA, 5092E accumulated almost equivalent levels as 5092K by 8hpi (Figure 46C). In contrast, attenuation was more pronounced in the low MOI infection, although the maximal difference was still observed at 5hpi (Figure 46D,E,F). It was interesting to observe that at the lower MOI, 5092E FluPol demonstrated delayed NA vRNA synthesis, with no accumulation up to 5hpi, followed by a rapid increase 5-8hpi. In contrast, some cRNA accumulation occurred 2-5hpi. This is reminiscent of our observation in *in situ* replication assays that NA cRNA can be observed in isolation at earlier timepoints (Figure 26) and highlights a degree of differential regulation for the two steps of genome replication. Speculatively, vRNA accumulation may necessitate a higher concentration of nascent FluPol components within the nucleus, due to the requirement of vRNA synthesis for two different FluPol dimers to form (possibly simultaneously as a trimer), as opposed to the single dimer required for cRNA synthesis. Therefore, at earlier timepoints when FluPol concentrations are lower, cRNA may accumulate but produce very little vRNA. It is possible a similar kinetic profile would be observed with 5092K at this MOI, just shifted earlier, however as we did not sample between 2 and 5hpi this detail was missed.

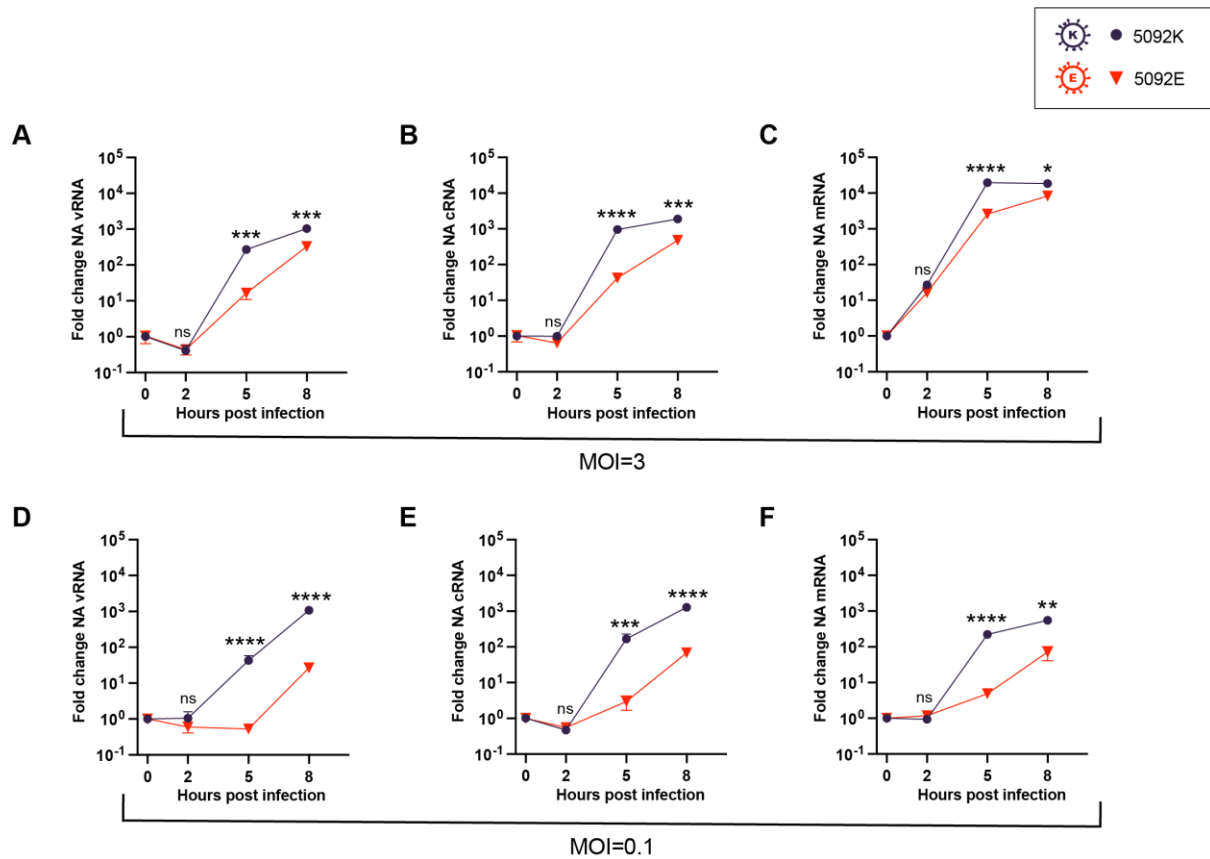


Figure 46: Characterisation of 5092E replication in mammalian cells.

(A-F) RT-qPCR quantification of segment 6 RNA in HEK-293T cells following infection with either 5092E or 5092K. Fold change was calculated over Input (0hpi). n=3 biological replicates, plotted as mean $\pm$ s.d.. Significance was assessed using multiple unpaired *t* tests following log transformation, corrected for multiple comparisons using false discovery rate. (A-C) Segment 6 (A) vRNA, (B) cRNA or (C) mRNA accumulation over time following infection with either 5092K or 5092E (MOI=3). (D-F) Segment 6 (D) vRNA, (E) cRNA or (F) mRNA accumulation over time following infection with either 5092K or 5092E (MOI=0.1). NA= neuraminidase; MOI= multiplicity of infection; hpi= hours post-infection. ns= not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

4.2.3 Avian polymerase is not restricted in cRNA synthesis in mammalian cells

Host restriction of FluPol 627E is believed to be caused by incompatibility with human paralogues of ANP32. Having established that 5092 requires ANP32 proteins for cRNA synthesis, we wished to investigate whether host restriction also acts at both steps of genome replication, i.e., the synthesis of cRNA from vRNA and vRNA from cRNA. To do this we undertook cRNP stabilisation and replication assays in parallel in HEK-293T cells (where only huANP32A/B/E proteins, that are incompatible with FluPol 627E, are available). Precisely, we pre-expressed NP with either catalytically dead 5092 FluPol 627E or 627K (cRNP stabilisation assay) or WT 5092 FluPol 627E or 627K (replication assay), followed by infection with either 5092K or 5092E virus, in the presence of ActD.

ANP32 stabilises an asymmetric FluPol dimer consisting of an RNA-bound replicating FluPol (FluPol_R) and an encapsidating FluPol (FluPol_E) that is poised to capture the nascent strand of RNA to form a new RNP (Carrique et al., 2020). In cRNP stabilisation and replication assays, during primary cRNA

synthesis, FluPol_R is provided by the vRNPs from the infecting virus, whilst FluPol_E is provided by the pre-expressed polymerase. Thus by undertaking the assays with different combinations of pre-expressed polymerase (FluPol 627E or FluPol627K) and input virus (5092E or 5092K), the effect of the 627 residue of either FluPol_R or FluPol_E during primary cRNA synthesis can be differentiated (depicted in the first line of Figure 47A, 'incoming vRNPs'). During vRNA synthesis and subsequent secondary rounds of cRNA synthesis (that only occur in the replication assay), the 627 signature of FluPol_E and FluPol_R are both dictated by the genotype of the pre-expressed polymerase (Figure 47A, bottom two lines).

In Figure 47B and 47C, cRNP stabilisation assays are reported on the left-hand side of the graph whilst replication assays are reported on the right-hand side of the graph. As expected, no vRNA accumulated in the cRNP stabilisation assay (Figure 47B). Surprisingly, the PB2 signature of neither FluPol_R nor FluPol_E influenced cRNP stabilisation: all four FluPol combinations accumulated equivalent amounts of cRNA, including the fully avian combination of 5092E virus with pre-expressed FluPol 627E (Figure 47C). This implies that avian-like and human-adapted FluPol can undergo cRNA synthesis equally well in human cells, i.e., avian FluPol is fully compatible with huANP32 proteins for cRNA synthesis and stabilisation. In the replication assay, samples with pre-expressed FluPol 627E were equally impaired in vRNA and cRNA synthesis, regardless of whether the incoming virus was 5092K or 5092E. Similarly, samples with pre-expressed FluPol 627K were not impaired, and accumulated equivalent quantities of vRNA and cRNA regardless of the incoming virus 627 signature (Figure 47B,C). Thus, the PB2 signature of the pre-expressed polymerase dictated host restriction in replication assays. This is logical considering the pre-expressed polymerase dictates the PB2 signature of both FluPol_E and FluPol_R in vRNA synthesis (see the bottom two rows in Figure 47A). The reduced cRNA accumulation observed when PB2 627E is pre-expressed is likely due to the indirect effect of reduced vRNP template on secondary rounds of cRNA synthesis.

Overall, this data suggests that host restriction solely acts at the level of vRNA synthesis. Significantly, the equivalent accumulation of vRNA and cRNA when infecting with either 5092E or 5092K in the presence of pre-expressed PB2 627K (Figure 47Ai,ii,B,C) demonstrates that bearing 627E on FluPol_R does not impact primary cRNA synthesis, and that the resulting cRNPs are functional for further rounds of replication. Exogenous expression of chANP32A was sufficient to rescue replication for all FluPol combinations in replication assays (Figure 47D,E), confirming that host restriction maps to ANP32.

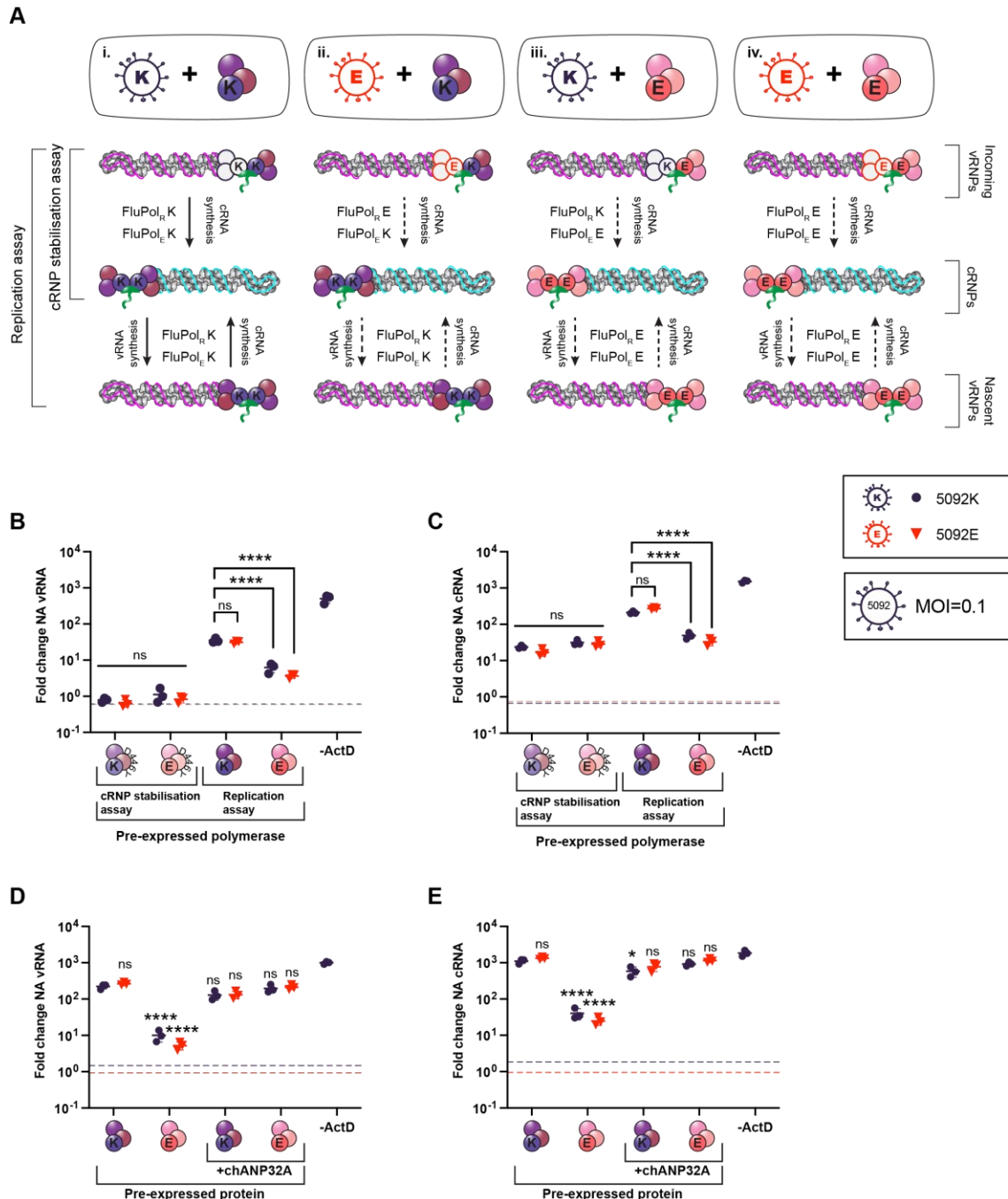


Figure 47: Avian FluPol (PB2 627E) is not restricted in cRNA synthesis in mammalian cells.

(A) Schematic outlining assay set-up and expected polymerase combinations during replication. The infecting virus genotype is indicated by the virus cartoon, whilst pre-expressed polymerase genotype is indicated by the trimeric FluPol cartoon. As indicated on the left, in the cRNP stabilisation assay, only the first layer of activity (primary cRNA synthesis) will occur. Both layers of activity can occur in replication assays. Unknown polymerase activity is indicated by a dashed black arrow. (B,C) Simultaneous cRNP stabilisation and replication assays with ActD, 6 hpi. Segment 6 (B) vRNA and (C) cRNA accumulation following infection with either 5092E or 5092K as indicated (MOI=0.1). Dotted line indicates background levels of vRNA/cRNA present in control replication assays using 5092K (navy) or 5092E (red) with PB2 omitted in the transfection mix. Fold change was calculated over input (0hpi). $n=3$ biological replicates, plotted as mean $\pm$ s.d.. Significance was assessed using one way ANOVA with Dunnett's multiple comparison test, following log transformation. (D,E) Replication assays with ActD and exogenously expressed chANP32A (80 ng plasmid/well), 6 hpi. Segment 6 (D) vRNA and (E) cRNA accumulation following infection with either 5092E or 5092K as indicated (MOI=0.1). Dotted line indicates background levels of vRNA/cRNA present in control replication assays using

5092K (navy) or 5092E (red) with PB2 omitted in the transfection mix. Fold change was calculated over input (0hpi). n=3 biological replicates, plotted as mean $\pm$ s.d.. Significance was assessed using one way ANOVA with Dunnett's multiple comparison test, following log transformation. Pre-expressed polymerase mixes: Stabilisation K= PB2 627K, PB1 D446Y, PA, NP; Stabilisation E= PB2 627E, PB1 D446Y, PA, NP; Replication K= PB2 627K, PB1, PA, NP; Replication E= PB2 627E, PB1, PA, NP. hpi= hours post-infection. ns= not significant; *= p<0.05; ****= p<0.0001. Figure adapted from Swann et al., in press.

4.2.4 cRNA synthesis requires the LCAR tail of huANP32B

Our data demonstrates that ANP32 is required for both steps of genome replication, vRNA to cRNA synthesis and cRNA to vRNA synthesis. Surprisingly however, AIV FluPol can use human ANP32 proteins to support cRNA synthesis without further adaptation, but only avian ANP32 to support vRNA synthesis. This suggests ANP32 may have an additional role in supporting the second step of replication, and it is this activity that is specifically blocked when AIV infects human cells. To explore this proposed vRNA-specific activity further, we attempted to identify a huANP32 mutant that can recreate the host restricted phenotype observed with avian FluPol (5092E), i.e., supports cRNA but not vRNA synthesis, with a mammalian adapted (PB2 627K) polymerase (in this case PR8). Through mapping the vRNA specific activity to a specific domain/motif, such a mutant could elucidate further details as to the mechanism of ANP32-mediated host restriction.

Specifically, we were interested in exploring whether the LCAR was required for cRNA synthesis, as this domain had previously been implicated in host restriction (Camacho-Zarco et al., 2020; Carrique et al., 2020). Moreover, as the LCAR is not resolved in the cryo-EM structure of the ANP32-stabilised asymmetric dimer (Carrique et al., 2020), we hypothesised that it could be dispensable for the formation of the replicase complex, which we expect to be required for both steps of replication. Moreover, LCAR truncation mutants have previously been observed to demonstrate unusual phenotypes where binding to FluPol is eliminated but function only mildly impaired in mini genome assays, which warrants further investigation (Domingues & Hale, 2017; Mistry et al., 2020). This is particularly true for the mutant chANP32A-208, which lacks the entire LCAR domain but retains the intrinsically disordered 33 aa avian insertion, however a milder version of this phenotype is also observed in human ANP32 LCAR truncation mutants (Staller, 2021).

Consequently, we initially investigated two huANP32B mutants: a deletion mutant, huANP32B-delta175, that lacks the majority of the LCAR domain but retains the very C terminal NLS; and the point mutant huANP32B-L128V, which disrupts the binding interface with FluPol_E (Figure 48A). As the replicase complex is believed to be required for both steps of replication, we expected huANP32B-L128V to be impaired in both cRNA and vRNA synthesis, thus this mutant was included as a control. Equivalent expression and nuclear localisation of these mutants has previously been reported (Staller, 2021; Staller et al., 2021). First, we characterised the overall ability of the huANP32B mutants to support the PR8 FluPol constellation in mini genome assays, relative to WT huANP32B, through exogenous expression in eHAP tKO cells. In agreement with previous reports using alternative FluPol constellations (Staller, 2021; Staller et al., 2021), we saw a significant decrease in the ability of both huANP32B-delta175 and huANP32B-L128V to support FluPol activity relative to WT huANP32B (Figure 48B).

huANP32B-delta175 demonstrated the more extreme phenotype, with FluPol activity dropping to around 20% WT activity, whilst huANP32B-L128V dropped to around 30%. Next, we complimented eHAP tKO cells with either WT or mutant huANP32B proteins and undertook ActD-treated cRNP stabilisation assays. cRNA accumulation was quantified using RT-qPCR and normalised to cRNA accumulation in the presence of WT huANP32B. Unexpectedly, we observed some cRNA accumulation over input (compare dotted blue line over dotted black line) and background (samples transfected with mixes lacking either NP or PB2) in the absence of any transfected ANP32 protein. We previously observed a low level of cRNA accumulation for PR8 in cRNP stabilisation assays in dKO cells (chapter three, Figure 35D), however we expected this to be fully ablated in the tKO cells that also lack huANP32E expression. Moreover, all three huANP32B proteins accumulated a significant amount of cRNA over both input and empty plasmid control. Surprisingly, no significant difference was detected in cRNA accumulation between WT huANP32B and ANP32B-L128V, and only a minor, although highly significant, reduction between WT huANP32B and huANP32B-delta175. As it had been speculated that the LCAR domain might load NP onto the nascent RNP (Carrique et al., 2020), in the case of huANP32B-delta175 we wondered whether the minimal phenotype could be explained if NP is not essential under the conditions of a cRNP stabilisation assay. However, no cRNA accumulation was observed in controls lacking pre-expressed NP, confirming NP remains essential in cRNP stabilisation assays. Alternatively, we considered whether it was an artefact caused by overexpression. Consequently, we undertook a preliminary experiment in which the three huANP32B proteins were titrated into the assay in a four-fold dilution series (Figure 48D). Statistical analysis was not performed as the experiment was run in duplicate, however at all dilutions tested all three huANP32B proteins accumulated more cRNA than empty plasmid alone.

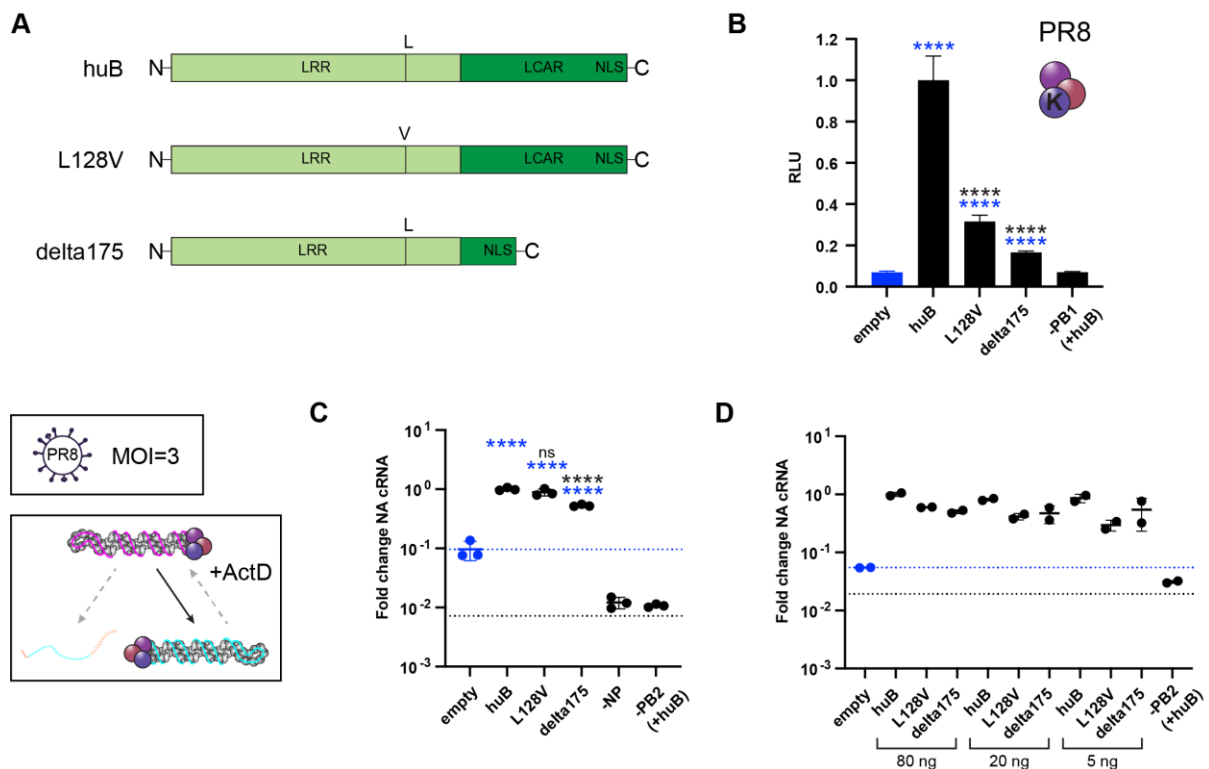


Figure 48: Human ANP32B replicative mutants are not impaired in cRNP stabilisation assays in eHAP tKO cells.

(A) Schematic depicting huANP32B mutants used in this experiment, relative to WT. LRR= leucine-rich repeat; LCAR= low-complexity acidic region; NLS= nuclear localisation signal. (B) Polymerase assay using the PR8 polymerase constellation and transfected huANP32B proteins as annotated (20 ng plasmid/well), in eHAP tKO cells. Data was normalised to huANP32B-WT. Significance compared to no transfected ANP32 (empty) (blue/lower stars) or WT huANP32B (black/upper stars) was assessed using one way ANOVA with Dunnett's multiple comparison test. RLU= relative light units. (C-D) huANP32B-complemented ActD-treated cRNP stabilisation assays in eHAP tKO cells. Segment 6 cRNA accumulation was quantified 6hpi. Fold change was calculated compared to WT huANP32B. Dotted lines indicate input levels of cRNA (black/lower line) and background accumulation of cRNA in the absence of any transfected ANP32 protein (empty plasmid control, blue/upper line). (C) Cells were pre-transfected with 100 ng huANP32B (WT/mutants as annotated) plasmid/well. n=3 biological replicates, plotted as mean $\pm$ s.d.. Significance was assessed relative to no transfected ANP32 (empty) (blue/lower stars) or WT huANP32B (black/upper stars) using one way ANOVA with Dunnett's multiple comparison test, following log transformation. (D) HuANP32B proteins (WT/mutants) were titrated into eHAP tKO cells at the amounts indicated on the x-axis. n=2 biological replicates, plotted as mean $\pm$ s.d.. hpi= hours post-infection; NA= neuraminidase; ActD= actinomycin D; WT= wild-type; huB= wild-type human ANP32B; L128V= human ANP32B-L128V; delta175= human ANP32B-delta175; ns= not significant; ****= $p < 0.0001$.

As eHAP cells have a low transfection efficiency, we wondered whether our complementation data was compromised by variable transfection of the ANP32 expression plasmids. To investigate further, we undertook similar experiments in HEK-293T cells lacking huANP32A/B (293T-dKO) (H. Zhang et al., 2019), a kind gift from the Wang lab, which demonstrate superior transfection efficiency. We also included an additional deletion mutant in these experiments - huANP32B-delta155 - that lacks the final three residues of the C-terminal portion of the LRR in addition to the full LCAR (Figure 49A). Again, we first characterised the mutants using a polymerase assay (Figure 49B). In these cells, huANP32B-L128V mutant showed a relatively modest reduction in polymerase activity of ~20%, relative to WT huANP32B. This contrasts with the earlier data in tKO cells, in which a much greater reduction of ~70% was observed. The huANP32B-delta175 and huANP32B-delta155 mutants showed a more extreme phenotype relative to WT huANP32B, with around 20% and negligible activity, respectively. No activity was observed in the absence of transfected NP or PB1. We next undertook ActD-treated cRNP stabilisation assays in 293T-dKO cells complemented with the different huANP32B proteins. As with the tKO experiments, cRNA accumulated over input in the absence of transfected ANP32 plasmids (empty plasmid control) (Figure 49C). In these cells this is unsurprising, due to the presence of endogenous huANP32E. The dynamic range of the assay in these cells (compare the space between empty plasmid control and WT huANP32B) was narrower than that observed in the eHAP tKO cells, and this could also be due to increased background activity in the empty plasmid control due to support from endogenous huANP32E. In contrast to the eHAP tKO experiments, none of the mutant plasmids showed a significant increase in cRNA accumulation above the level of empty plasmid alone. Nevertheless, although the level of cRNA accumulation did not reach significance, the overall pattern of huANP32B proteins was the same as in tKO cells with WT huANP32B showing the greatest support, huANP32B-L128V showing an intermediate level, and the LCAR deletion mutants showing the lowest level. Similarly, the mutants also showed the same trend as in the polymerase assay. Interestingly, the huANP32B-delta155 mutant accumulated significantly less cRNA than empty plasmid alone, suggesting this mutant may actively disrupt cRNA synthesis in a dominant negative manner as has previously been observed for other ANP32 mutants (Staller et al., 2021).

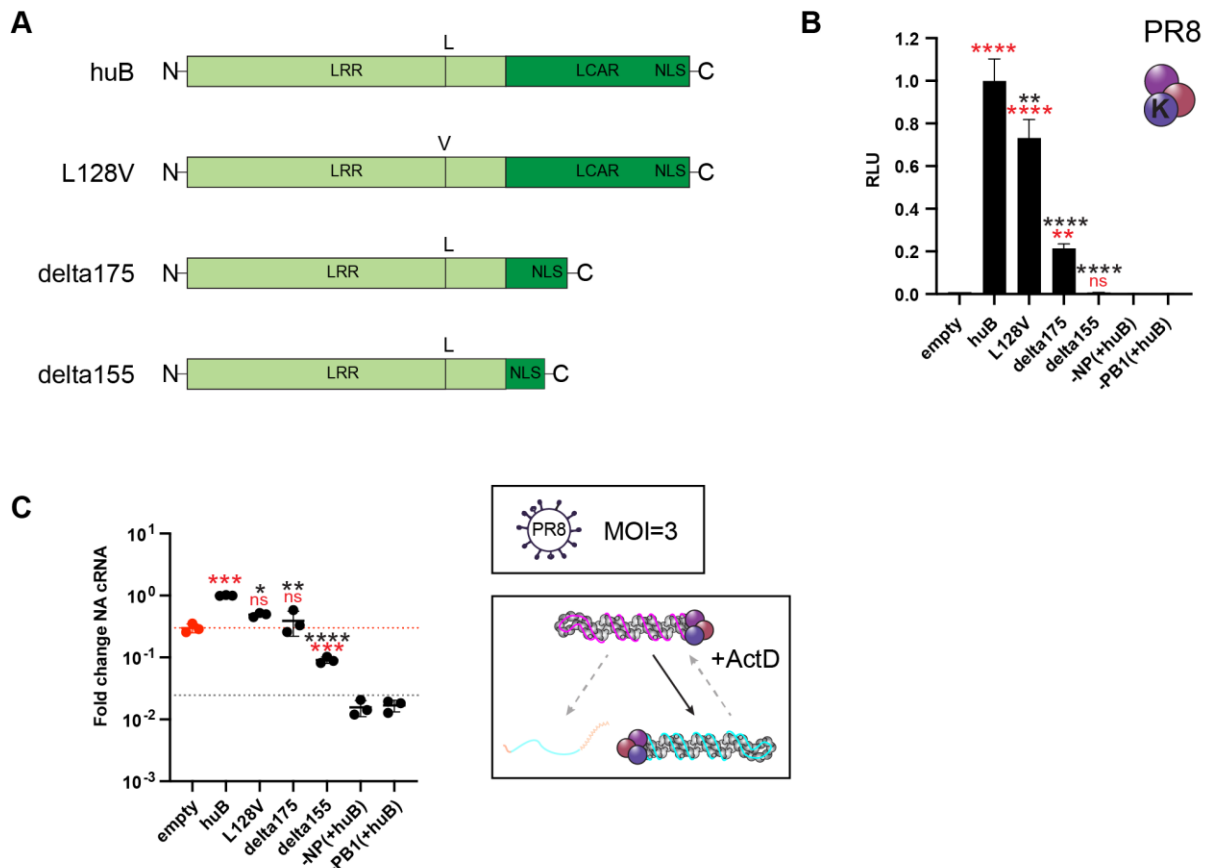


Figure 49: Human ANP32B replicative mutants are impaired in cRNP stabilisation assays in 293T-dKO cells.

(A) Schematic depicting huANP32B mutants used in this experiment relative to WT. LRR= leucine-rich repeat domain; LCAR= low-complexity acidic region; NLS= nuclear localisation signal. (B) Polymerase assay using the PR8 polymerase constellation and transfected huANP32B proteins (20 ng plasmid/well) as annotated, in 293T-dKO cells. Data was normalised to WT huANP32B. Significance compared to no transfected ANP32 (empty) (red/lower stars) or WT huANP32B (black/upper stars) was assessed using one way ANOVA with Dunnett's multiple comparison test. (C) huANP32B-complemented ActD-treated cRNP stabilisation assay in 293T-dKO cells. Segment 6 cRNA accumulation was quantified 6hpi. Fold change was calculated compared to WT huANP32B. Dotted lines indicate input levels of cRNA (black/lower line) and background accumulation of cRNA in the absence of any transfected ANP32 protein (empty plasmid control, red/upper line). Cells were pre-transfected with 20 ng huANP32B (WT/mutants as annotated) plasmid/well. n=3 biological replicates, plotted as mean $\pm$ s.d.. Significance was assessed relative to no transfected ANP32 (empty) (red/lower stars) or WT huANP32B (black/upper stars) using one way ANOVA with Dunnett's multiple comparison test, following Log transformation. hpi= hours post-infection; NA= neuraminidase; ActD= actinomycin D; WT= wild-type; huB= wild-type human ANP32B; L128V= human ANP32B-L128V; delta175= human ANP32B-delta175; delta155= human ANP32B-delta155. ns= not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

In both eHAP tKO and 293T-dKO cells the dynamic range of the complementation assay was small (compare the difference in cRNA accumulation between samples transfected with empty plasmid and WT huANP32B). In this scenario, it can be very difficult to detect intermediate phenotypes such as those we expect from the mutant ANP32B proteins, which may explain why we saw the same trend for the mutants (which also matched the trend in the polymerase assays), but different statistical differences relative to either WT huANP32B or empty plasmid control, in the two cell lines. Potentially this could be improved through increasing statistical power with more replicates per experiment. The small dynamic range observed might be because we are creating an overly supportive environment for

cRNA accumulation, and therefore the FluPol is less dependent on ANP32 support/less inhibited by ANP32 mutants. For example, this could be an artefact of overexpressing FluPol/NP prior to infection. Nevertheless, it is worth noting that we use equivalent quantities of plasmid for the cRNP stabilisation assays as for polymerase assays, in which a good dynamic range is observed. More likely, the small dynamic range is because relatively little cRNA accumulates in these assays even in the presence of WT huANP32B. It is important to note that we've generated conditions in which only vRNA to cRNA synthesis can occur, and so the amplification effect seen in a polymerase assay is not captured. It could be interesting to see if the range could be increased through leaving the assays for longer to allow further cRNA synthesis.

A caveat to these experiments is that we did not run the matched western blots. Including these controls in further experiments will be important to confirm the differences in proviral support are not due to variation in transfection efficiency or expression level. Nevertheless, the overall trend was the same in both cell types, and it was clear that the huANP32B LCAR truncation mutants were impaired in supporting FluPol cRNA synthesis. With our latest understanding of ANP32 this is logical, as it has since been established that the LCAR domain is required to load NP onto both cRNPs and vRNPs (F. Wang et al., 2022). Thus, we were not successful in identifying an ANP32 protein that is specifically impaired in vRNA synthesis.

4.3 DISCUSSION

In this chapter we investigated whether avian polymerase (FluPol 627E) is restricted in both steps of genome replication, i.e. vRNA to cRNA synthesis and cRNA to vRNA synthesis, in mammalian cells. Our data, using the avian H5N1 strain 5092, agrees with earlier reports that FluPol 627E is specifically inhibited in vRNA synthesis (Figure 47) (Bi et al., 2019; Mänz et al., 2012; Nilsson et al., 2017). Nevertheless, this result was unexpected considering we have established that ANP32 proteins are required for cRNA synthesis (chapter three) and host restriction of FluPol PB2 627E is driven by incompatibilities with mammalian ANP32 paralogues (Carrique et al., 2020; Long et al., 2016). Subsequently, our data suggests that human ANP32 proteins are sufficient to support avian polymerase cRNA synthesis, but not vRNA synthesis.

In the replication complex described by Carrique et al., the ANP32A LRR domain bridges the FluPol dimer, forming multiple contacts between FluPol_R and FluPol_E. The flexible LCAR is unresolved, although additional density is present in a groove formed between the two FluPol 627 domains. As the groove is acidic in FluPol 627E, the acidic huANP32 LCAR is likely incompatible with this interaction, whereas the mixture of acidic and basic residues in the 33 amino acid insertion in avian ANP32 proteins is compatible. The authors suggest this additional contact may further stabilise the complex (Carrique et al., 2020). Nevertheless, our data suggests this mismatch can be tolerated for cRNA synthesis. This aligns with the observation that addition of chANP32A to mammalian cells does not stimulate FluPol 627E cRNA synthesis (Bi et al., 2019). The ANP32-FluPol dimer complex is thought to be required to load an encapsidating FluPol onto the promoter of the extruded strand of nascent c/vRNA. Moreover, it has been proposed that the LCAR domain may act as a molecular whip, which recruits NP for loading onto the nascent RNA (Carrique et al., 2020; F. Wang et al., 2022). As our data suggests cRNPs are produced in the absence of a compatible 627-LCAR interaction, it implies host restriction does not act at the level of encapsidation. Instead, the 627-LCAR interaction appears to be required for a further mechanistic role that is specific to vRNA synthesis. For example, this interaction could be of critical importance to recruit the additional *trans*-activating FluPol required for primer realignment, or to provide additional stability that could be essential to form the proposed trimer structure consisting of the asymmetric and symmetric FluPol dimers combined.

Our attempt to map this proposed vRNA-specific activity to mammalian ANP32 proteins through identifying a huANP32 mutant that recreates the host restricted phenotype with a mammalian polymerase, i.e., supports cRNA but not vRNA synthesis, was unsuccessful. We chose to examine LCAR deletion mutants as this domain had been implicated in host restriction but was not resolved in the replicase structure, therefore we hypothesised that it could be dispensable for cRNA synthesis. Nevertheless, considering the LCAR has now been associated with loading NP onto the RNP, it is unsurprising that this domain is required for cRNA synthesis (F. Wang et al., 2022). With more time, it would be interesting to screen a larger panel of ANP32 mutants that plausibly might recreate the host restricted phenotype within the context of our current understanding of the mechanism of ANP32. To do this, we could take two different approaches. Firstly, we could continue trying to recreate the host

restricted phenotype with a FluPol 627K and a mammalian ANP32 protein that is specifically broken in vRNA synthesis. For example, we could try introducing a positive charge at the start of the LCAR, which might recreate the charge clash experienced by FluPol 627E. Interestingly, it was recently reported that chANP32A poorly supports some IBV strains (chANP32A demonstrated a similar level of support as huANP32E), i.e., host restriction reversed (Z. Zhang et al., 2020), and it would be interesting to characterise whether this host restriction also specifically acts at the level of vRNA synthesis. Secondly, it might be possible to generate a chicken ANP32A mutant that recreates the huANP32 phenotype for an avian strain. For example, the hexapeptide motif at the start of the avian specific 33 aa insertion in ANP32A has been strongly implicated in host restriction, and it would be interesting to see whether removing the hexapeptide motif specifically inhibits vRNA synthesis. Nevertheless, as host restriction maps to the LCAR, and the interactions between this intrinsically disordered domain and FluPol have been characterised as non-specific and polyvalent (Camacho-Zarco et al., 2020), destroying such an interaction without disrupting other proviral functions of the protein may prove impossible through genetic approaches. Considering the limitations of the cRNP stabilisation assays for distinguishing ANP32 mutants, such investigations might also require optimisation of this technique, or alternatively a new approach for dissecting effects on vRNA and cRNA synthesis.

It has been reported that avian FluPol is impaired in nuclear import in mammalian cells, due to incompatibilities with the importin- α isoforms present (Gabriel et al., 2008). However, this does not explain the data obtained here, as FluPol 627E proved fully functional for the pioneering round of cRNA synthesis and stabilisation within the nucleus during cRNP stabilisation assays (Figure 47C), whilst the omission of any RNP component (e.g. -PB1, -NP control) eliminates cRNA accumulation in cRNP stabilisation assays (Figure 48C, 49C). Other reports have suggested that host restricted FluPol can generate cRNA in mammalian cells, but that the resulting cRNPs are aberrant (Bi et al., 2019; Mänz et al., 2012; Nilsson-Payant et al., 2021). As our RT-qPCR approach relies on primers that bind to the very 3' end of the cRNA, any signal suggests successful replication of a full-length cRNA segment (although internal deletions upstream of the ~100bp qPCR amplified fragment could be present). This contrasts with primer extension analysis, which typically uses a more 5' proximal primer for reverse transcription (te Velthuis et al., 2018) and is more prone to detecting incomplete cRNA fragments. However, our data cannot exclude the possibility that a full cRNA is replicated but incorrectly packaged and is therefore rendered non-functional. Such a mechanism for restriction fits with the concept of ANP32 as coordinating co-replicative encapsidation. It would be interesting to purify the cRNPs produced by avian FluPol in mammalian cells and examine whether any differences in NP interaction can be detected, or ultrastructural differences observed.

In our data, we also observe that cRNA produced from 5092E is functional for onwards replication so long as the encapsidating FluPol (which will become the replicating FluPol during the subsequent primary round of vRNA synthesis) bears the mammalian-adapted 627K signature (i.e. the scenario depicted in Figure 47Aii) (Figure 47). Thus, cRNA produced from an avian FluPol_R in a mammalian cell is fully functional for onwards replication. It is possible that the presence of pre-expressed FluPol 627K

in this scenario is sufficient to overcome host restriction, either because the 627 signature of FluPol_E dictates host restriction or a single PB2 627K residue present in either FluPol_R or FluPol_E is sufficient to negate the charge clash between the PB2 627 domain and huANP32-LCAR. Nonetheless, we note that in cRNP stabilisation assays equivalent quantities of cRNA accumulate in mammalian cells regardless of whether FluPol_R, FluPol_E or both FluPol molecules in the replication complex bear the avian-like 627E signature, but no cRNA accumulation occurs in the complete absence of ANP32 proteins. This suggests there is at least some functional interaction between huANP32 proteins and FluPol 627E.

In contrast to our findings here, a recent study was published suggesting avian FluPol is restricted in both cRNA and vRNA synthesis in mammalian cells (Nilsson-Payant et al., 2021). In this study, promoter mutants were generated that are specifically blocked in either cRNA or vRNA synthesis and built into positive- or negative-sense viral-like RNAs, respectively. These templates were subsequently used in mini genome assays that could therefore only undergo vRNA or cRNA synthesis, thus allowing the interdependence of the two steps to be unpicked in an alternative manner to the traditional cRNP stabilisation assay. The authors used either the WT PB2 627K polymerase from H1N1 WSN, or an 'avianised' version with engineered PB2 627E, and demonstrated that the avian-like version was restricted in both steps of replication in the promoter mutant backgrounds. This opposing finding could reflect strain-to-strain variation. For example, some viral strains could form weaker interactions between the FluPol_E and FluPol_R interfaces and thus require greater stabilisation by ANP32. Alternatively, it is possible that WSN poorly tolerates the avian-like PB2 627E signature, resulting in a suboptimal polymerase and is therefore generally disrupted in replication – although it is worth noting equivalent replication was observed in chicken DF1 cells. The authors also undertook a simultaneous cRNP stabilisation/replication assay using pre-expressed 627E or 627K polymerase followed by infection with WT WSN bearing PB2 627K, analysed using primer extension analysis. The data from this experiment aligned with the data reported in Figure 47, in that neither infection showed impaired cRNA synthesis, but both showed impaired vRNA synthesis. The authors concluded that this was because host restriction is dictated by FluPol_R, which in their experiment was always 627K. However, as the equivalent infection using WSN 627E was not performed, it is impossible to say whether this is the case, or whether these infections would also accumulate equivalent quantities of cRNA. It would certainly be informative to repeat our experiments in an additional genetic background to see how transferable the conclusions are.

In summary, in this chapter we have established that ANP32 proteins are required to support cRNA synthesis of a further H5N1 avian strain, 5092. Nonetheless, host restriction of 5092E was found to occur specifically at the level of vRNA synthesis. We suggest that huANP32 proteins are sufficient to support avian polymerase in cRNA synthesis, but not vRNA synthesis, implying a fundamental difference in the way ANP32 supports the two steps of replication. However, the mechanistic detail of this additional activity has not been clarified. We also acknowledge that there is a possibility that cRNPs are produced during host restriction but are aberrant for onwards replication. Fundamentally, this is true

for any cRNP containing FluPol 627E, as such a complex cannot undergo vRNA synthesis in a mammalian cell. However, whether there are additional aberrances, for example in RNP structure, that cannot be detected in the assays used here, warrants further investigation.

CHAPTER FIVE: DISCUSSION AND SUMMARY

Influenza A virus is a significant pathogen for multiple reasons. From a human health perspective, IAV is responsible for a large proportion of the annual morbidity and mortality associated with seasonal influenza epidemics and represents a pandemic risk (Paules & Subbarao, 2017). Moreover, avian influenza virus (AIV) impacts both food security and the welfare of livestock and can trigger mass mortality during highly pathogenic AIV outbreaks in wild birds (Blagodatski et al., 2021; Hood et al., 2021; Ramey et al., 2022; Salvesen & Whitelaw, 2021; Verhagen et al., 2021). Combined, these traits make understanding the biology of the virus, and translating this knowledge into impactful solutions, a global priority.

In this work, we focused our research on the influenza RdRp (FluPol) – the single enzyme responsible for driving both viral transcription and genome replication, and an important determinant of host restriction (Naffakh et al., 2008; te Velthuis & Fodor, 2016). The FluPol is an attractive option for the development of novel antiviral drugs due to the multitude of targets it presents including: multiple interaction faces with viral and host factors that could be disrupted using small molecule inhibitors; the endonuclease activity of PA (which has been successfully targeted by the drug baloxavir); and the RdRp catalytic activity (as has been successfully targeted by the nucleotide analogue favipiravir) (Furuta et al., 2017; Hayden et al., 2018, 2022; Keown et al., 2022; Massari et al., 2021; Noshi et al., 2018; Pflug et al., 2017). Moreover, disrupting FluPol interactions with host factors through gene editing has been proposed as a mechanism for generating flu-resistant livestock (Long, Idoko-Akoh, et al., 2019; Salvesen & Whitelaw, 2021). Consequently, increasing our understanding of FluPol, and FluPol-host factor interactions, is likely to have a multitude of downstream translational benefits.

The work presented in this thesis is split into two interlinking projects – firstly the development and validation of new techniques to enable investigations into the spatiotemporal details of FluPol replication (chapter two), and secondly improving our understanding of the mechanism by which the host factor ANP32 supports influenza replication (chapter three) and underlies host restriction of FluPol activity (chapter four).

5.1 The role of host factor ANP32 in FluPol genome replication

At the start of this project, the ANP32-stabilised asymmetric FluPol dimer had not been solved, and the mechanism of both ANP32-mediated proviral support and host restriction remained unclear. Indeed, it was thought that ANP32 was required to specifically support vRNA synthesis (Sugiyama et al., 2015). Nevertheless, some data called this conclusion into question, such as the observation that addition of either vRNA or cRNA can stabilise the interaction between ANP32 and FluPol, suggesting ANP32 binds both vRNPs and cRNPs (Baker et al., 2018; Mistry et al., 2020). Subsequently, the publication of the ANP32-stabilised asymmetric FluPol dimer structure strongly implied the mechanism of proviral support was in coordinating a replicase complex necessary for both vRNA and cRNA synthesis (Carrique et al., 2020). Our data using human ANP32 (huANP32) knockout cell lines, presented in chapter three, provides the final experimental data required to unambiguously associate ANP32 with cRNA synthesis. Importantly, as significantly less cRNA accumulates in the absence of huANP32A/B, even when vRNA synthesis is blocked in cRNP stabilisation assays, we establish that this effect is due to a direct role for ANP32 in enhancing cRNA synthesis and not merely an indirect effect due to reduced vRNP template. Moreover, this phenotype was validated with the polymerase from multiple avian (H5N1, Tky05 and 5092) and human (H1N1, H3N2) strains, and we are yet to observe a strain of IAV that does not rely on ANP32 for cRNA synthesis. This suggests the role for ANP32 in cRNA synthesis is widely applicable.

ANP32 proteins are thought to also be required to support vRNA synthesis, as nascent vRNA also requires encapsidation into vRNPs. Moreover, the original characterisation of huANP32A/B demonstrated that replication from a positive-sense template is stimulated by the addition of huANP32A/B *in vitro* (Sugiyama et al., 2015). Nevertheless, as vRNA synthesis is downstream from cRNA synthesis, our data cannot confirm this *in vivo*, as the effect on vRNA synthesis observed in our assays could be due to reduced cRNP template. A role for ANP32 in vRNA synthesis is strongly implied in our host restriction experiments (Section 4.2.3), in which a virus containing FluPol 627E (5092E) does not accumulate vRNA in mammalian cells despite producing cRNA but can be rescued by the exogenous expression of chANP32A. However, an alternative explanation is that the cRNPs produced in this scenario are aberrant, and that vRNA synthesis is inhibited as it is downstream from this effect. In a report published by Nilsson-Payant et al., the effect of host restriction specifically on vRNA synthesis is investigated in the context of mini genome assays using a positive-sense template (from which the first step of replication will be vRNA synthesis) that carries inhibitory mutations in the cRNA promoter thus can only produce vRNA (Nilsson-Payant et al., 2021). Using this assay, the authors demonstrate that host restricted FluPol PB2 627E is specifically impaired in vRNA synthesis and can be rescued by the addition of chANP32A, thus strongly supporting a direct role for ANP32 in vRNA synthesis. Nevertheless, considering our data shows that host restricted FluPol activity and FluPol activity in the absence of ANP32 proteins do not necessarily recapitulate one another, it would be informative to unambiguously confirm a role for ANP32 in vRNA synthesis through repeating this approach in ANP32 knockout cell lines.

We observed strain-to-strain variation in the absolute requirement for huANP32A/B for cRNA synthesis. Although all IAV strains investigated showed a highly significant reduction in cRNA synthesis in the absence of huANP32A/B, some strains (Tky05, 5092, Eng195) accumulated a low level of cRNA by 5hpi during authentic infections in dKO cells. In part, this ability seemed to map to an inefficient use of the final huANP32 paralogue, huANP32E, as the low level of cRNA accumulation was abolished in triple knockout (tKO) cells that lack expression of huANP32E as well as the main proviral paralogues, huANP32A and huANP32B. This aligns with an increasing awareness that ANP32 proteins that were originally considered completely virologically inert, under certain conditions such as the absence of a preferred paralogue, can provide inefficient support (Zhang et al., 2020; Goldhill et al., under revision). We also noticed that in strains that could inefficiently use huANP32E to support cRNA synthesis, a general upwards trend in cRNA accumulation could be observed in the tKO cells. Although this upwards trend did not reach significance over input by 5hpi, it did reach significance by 8hpi with the one strain (Tky05) that we measured at a later timepoint. This may suggest that other strains would also reach significance if measured later in infection. This trend was not seen for the FluPol from PR8 or Aichi68, that were unable to use huANP32E to support even inefficient replication in authentic infection (although some cRNA accumulation was observed in dKO and tKO cells during cRNP stabilisation assays using PR8). As ANP32 proteins stabilise the asymmetric FluPol dimer structure, we speculate that the strain-to-strain variation we observe in the ability to exploit huANP32E may reflect differences in the stability of the asymmetric FluPol_R:FluPol_E interface, and that stronger dimer interfaces might tolerate weaker ANP32 interactions – or even no ANP32 interaction at all. This could explain why inefficient use of huANP32E and putative activity in tKO cells appear to be linked. It would be interesting to develop assays that could detect differences in the dimer strengths of different strains – although these assays would likely need to be very sensitive, and it might not be possible to differentiate the asymmetric FluPol dimer structure from the alternative symmetric FluPol dimer structure.

If the upwards trend in cRNA accumulation in tKO cells observed with some IAV strains does reflect ANP32-independent cRNA synthesis, the level of activity is nonetheless vanishingly small, and might not be sufficient for productive infection. Nevertheless, considering Tky05 can rapidly adapt to efficiently use huANP32E in the absence of huANP32A/B (Goldhill et al., under revision), we cannot exclude that this low level of activity is sufficient to allow further adaptation. Although there are no further human ANP32 family members to adapt to, other host proteins, for example that contain LCAR or LRR domains, might to be able to substitute for the role(s) of ANP32. For example, one of the major protein constituents of the nucleolus, nucleophosmin (B23), contains an LCAR and is known to interact with vRNPs (Mayer et al., 2007; Reilly et al., 2014). It is also interesting to note that influenza virus is the only negative-sense RNA virus (NSV) currently known to exploit ANP32, however all NSVs replicate within the context of vRNPs and must encapsidate nascent products (te Velthuis et al., 2021). This suggests alternative evolutionary strategies are available to achieve replication and encapsidation, and it is possible these could be co-opted by influenza virus in the absence of ANP32 proteins. Considering the potential translational benefits of disrupting the influenza-ANP32 interaction, understanding the opportunities for the virus to escape is important, as it allows potential negative consequences, such as

increased pathogenicity, to be identified and could inform a multipronged approach to limit such opportunities.

5.2 ANP32 and host restriction

Unexpectedly, although incompatibility with mammalian ANP32 proteins underlies host restriction of AIV FluPol (PB2 627E) (Long et al., 2016), and ANP32 expression is essential for robust cRNA and vRNA synthesis, we found the FluPol from the avian strain 5092 to be specifically restricted in vRNA synthesis, but not cRNA synthesis, in mammalian cells. Moreover, vRNA synthesis in mammalian cells was rescued by exogenous expression of chANP32A, confirming that restriction maps to an inability to use human ANP32 orthologues. These findings are summarised in Figure 50. This suggests that avian FluPol can utilise human ANP32 proteins to support cRNA synthesis without mammalian adaptation, and that either the 33 aa insertion present in avian ANP32A orthologues or acquisition of the mammalian adaptation PB2 E627K are specifically required for efficient vRNA synthesis. We propose that this implies a fundamental difference in the mechanism by which ANP32 supports vRNA to cRNA synthesis and cRNA to vRNA synthesis.

vRNA and cRNA synthesis share some commonalities, as both generate full-length, encapsidated products (te Velthuis & Fodor, 2016). Nevertheless, vRNA and cRNA are produced from different templates with different promoters and are generated using different *de novo* initiation mechanisms (Section 1.3.4) (Deng et al., 2006; te Velthuis & Fodor, 2016). vRNA and cRNA are also found in different quantities in the cell, demonstrate different replication kinetics and are regulated by different complements of host factors (Section 1.3.5) (Hatada et al., 1989; Kawakami et al., 2011; Peacock et al., 2019; Phan et al., 2021). We observe several pieces of evidence that suggest vRNA synthesis has a more stringent, or additional, requirement for ANP32-mediated support. Firstly, in the original biochemical characterisation of huANP32A/B, no increase in FluPol activity was observed from a short negative-sense template in the presence of huANP32A/B (Sugiyama et al., 2015), suggesting that the role of ANP32 in coordinating the replicase complex is not captured in *in vitro* analysis. This aligns with the observation that additional polymerase protein, which is necessary for encapsidation, is also not required for cRNA synthesis from purified vRNPs *in vitro*, despite being essential *in vivo* (Vreede et al., 2004; York et al., 2013). Nevertheless, the addition of ANP32 did increase replication from a short positive-sense template (Sugiyama et al., 2015). Speculatively, this increase could reflect an increased dependency for ANP32 during vRNA synthesis, or a vRNA-specific role. Moreover, we observe several instances where cRNA synthesis can be achieved using a 'suboptimal' ANP32 paralogue for the FluPol under investigation, but vRNA synthesis cannot (AIV FluPol plus huANP32 paralogues, mammalian-adapted FluPol plus huANP32E). As vRNA synthesis is downstream from cRNA synthesis, it is possible this higher stringency reflects the direct effect of a suboptimal host factor and the indirect effect of reduced cRNP template, combined. Nevertheless, the Tky05-Mut virus, that has adapted to efficiently use huANP32E, appears to achieve this adaptation by specifically increasing vRNA synthesis in dKO cells, without increasing cRNA synthesis. Again, this might suggest the key block preventing efficient replication in the unadapted virus is at the vRNA synthesis, and not cRNA synthesis, level.

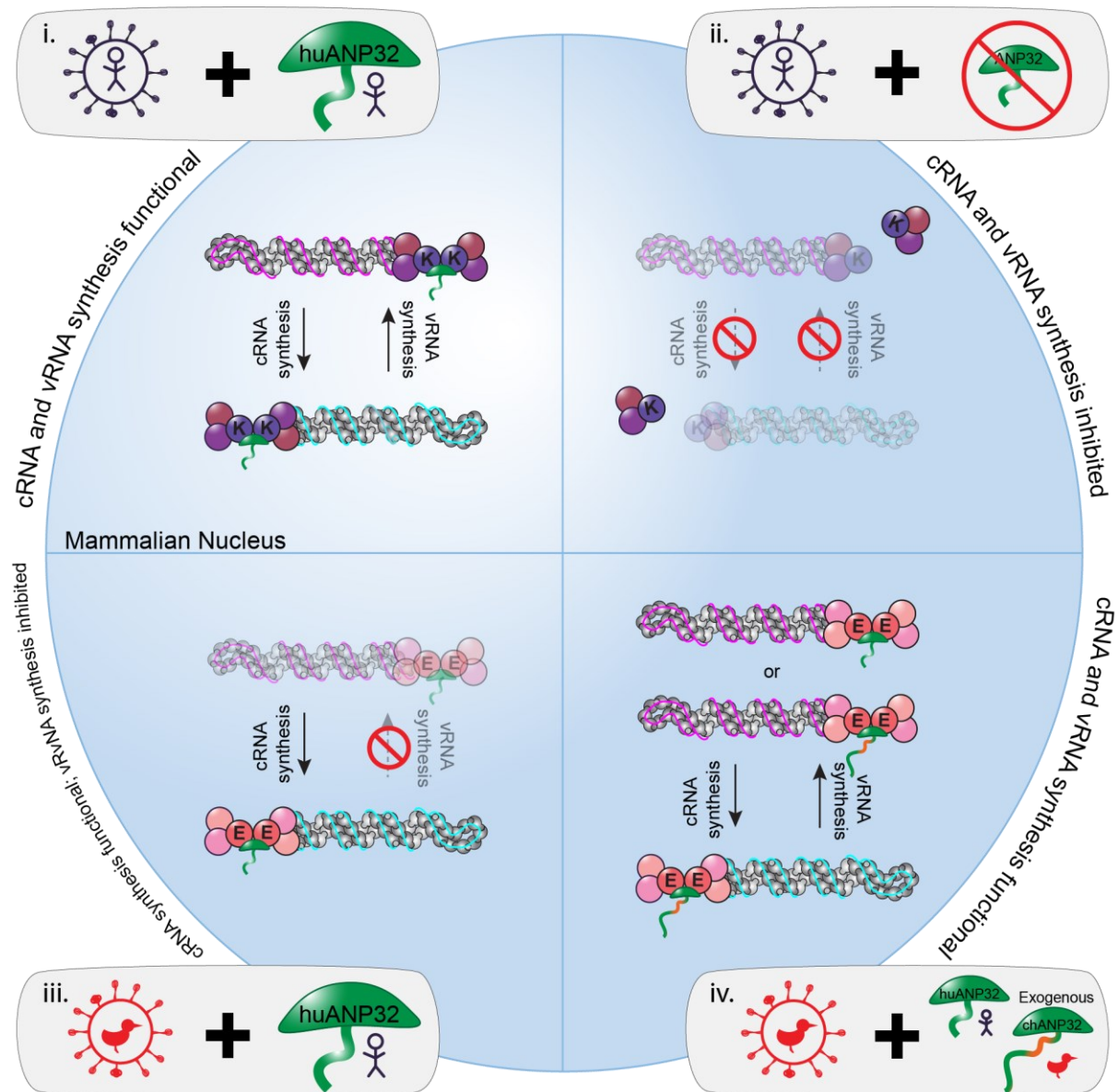


Figure 50: Summarising schematic of the role of ANP32 proteins in genome replication and host restriction.

(i) When mammalian-adapted influenza viruses encoding a lysine at position 627 of the PB2 polymerase protein (PB2 627K) infect wild-type human cells in which only human ANP32 proteins are present, both cRNA and vRNA synthesis can occur. (ii) When influenza viruses (PB2 627K) infect human cells lacking ANP32 protein expression, neither cRNA nor vRNA significantly accumulate, demonstrating that ANP32 is required to support robust cRNA and vRNA synthesis. (iii) When avian influenza viruses encoding a glutamic acid at position 627 of the PB2 polymerase protein (PB2 627E) infect wild-type human cells in which only human ANP32 proteins are present, cRNA synthesis but not vRNA synthesis can occur. As ANP32 proteins are required for cRNA synthesis, this suggests that avian polymerase can functionally interact with human ANP32 proteins to support cRNA synthesis. However, avian polymerase cannot utilise human ANP32 proteins to support vRNA synthesis, thus host restriction is imposed on this step of replication. (iv) In the presence of exogenously expressed avian ANP32A, avian influenza viruses (PB2 627E) can undergo both cRNA and vRNA synthesis, confirming restricted vRNA synthesis in scenario iii maps to aberrant interactions with human ANP32 proteins. huANP32= human ANP32; chANP32= chicken ANP32. Figure adapted from Swann et al., in press.

If host restriction is imposed specifically at the level of vRNA synthesis, this raises the question as to what this block might be. In the context of mini genome assays, host restriction of AIV FluPol in mammalian cells has been shown to be alleviated by the introduction of 3-8 or 3-5-8 mutations in the 3' vRNA promoter (or 5' cRNA promoter) (Section 1.3.6) (Crescenzo-Chaigne et al., 2002; Nilsson-Payant et al., 2021; Paterson et al., 2014). Interestingly, 3-8/3-5-8 mutants appear to function in an ANP32-dependent manner, i.e., they enable AIV FluPol to utilise human orthologues of ANP32 for vRNA synthesis rather than becoming ANP32-independent (Section 1.4.5) (Baker et al., 2018; Mistry et al., 2020). This might suggest that the inability to use huANP32 proteins for vRNA synthesis relates to the promoter. This is intriguing, as one of the key differences between cRNA and vRNA synthesis is the *de novo* initiation strategy: cRNA synthesis uses terminal initiation, whilst vRNA synthesis undergoes internal initiation followed by primer realignment which is dependent on formation of a symmetric dimer with an additional *trans*-activating FluPol. The differences in these strategies may be controlled by differences in the vRNA and cRNA promoter secondary structures and the distance they extend into the FluPol active site (Oymans & te Velhuis, 2018). It would be interesting to investigate whether 3-8/3-5-8 promoter mutants continue to use primer realignment for vRNA synthesis, or whether the promoter mutations switch vRNA synthesis to a cRNA-synthesis-like terminal initiation strategy. The latter could suggest that the host-specific role of ANP32 is in supporting the internal initiation strategy of vRNA synthesis.

A link between the ANP32-stabilised FluPol dimer and the symmetric dimer required for *trans*-activation is implied by the finding that adaptation to huANP32E by Tky05 was (counter-intuitively) achieved through the acquisition of the destabilising mutations PB1 K577R and PA Q566R in the symmetric dimer interface (Goldhill et al., under revision). This could suggest an indirect link – and that maintaining a balance between free 'monomeric' heterotrimeric FluPol complexes and the symmetric and asymmetric dimer complexes is necessary to maintain productive replication (Figure 51A). Alternatively, it has also been proposed that there may be a direct link between the two dimeric complexes, and that the symmetric dimer and asymmetric ANP32-stabilised dimer form at the same time as a trimer of FluPol heterotrimers (Figure 51B). Speculatively, if this is the case, the trimer structure might require greater stabilisation by ANP32 compared to the asymmetric dimer alone. This might explain why cRNA synthesis can tolerate a charge mismatch between the LCAR and 627 domain, whilst a compatible LCAR-627 domain interaction is essential for vRNA synthesis. Nevertheless, there is currently no experimental evidence to support the proposed trimer structure. There is also no structural explanation for ANP32-mediated host restriction, and whilst the proposed model is compelling, it is based on unresolved density in the cryo-EM map. An alternative explanation for the results obtained in this work, for example, could be that the LCAR tail is required to recruit the *trans*-activating FluPol in a comparable manner to its role in recruiting NP, and it is actually the 627 signature of FluPol_T that dictates host restriction. This could explain why the 627 signature does not appear to be important for cRNA synthesis.

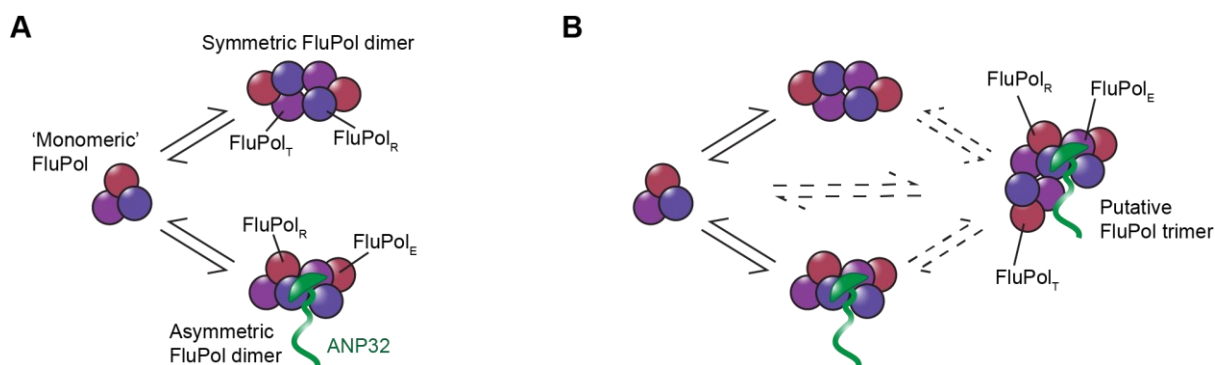


Figure 51: Proposed models for an indirect vs direct link between the host factor ANP32 and the symmetric FluPol dimer.

(A) Maintaining the correct balance between symmetric and asymmetric FluPol dimer complexes and free 'monomeric' heterotrimers may be important for maintaining productive replication. In this model, decreasing the stability of the asymmetric FluPol dimer due to the removal of the preferential paralogues ANP32A and ANP32B disrupts the balance between symmetric and asymmetric dimer forms, and can be counteracted through introducing mutations that decrease the stability of the symmetric FluPol dimer, increasing the concentration of free 'monomeric' polymerase and thus pushing the equilibrium back towards the asymmetric dimer complex. (B) It has been proposed that both the symmetric and asymmetric dimer complexes may form simultaneously as a trimer of FluPol heterotrimers. The formation of this trimer might be dependent on the increased stability provided by an optimal ANP32-FluPol interaction. FluPol_R= replicating FluPol; FluPol_E= encapsidating FluPol; FluPol_T= *trans*-activating FluPol.

As discussed at the end of chapter four, although equivalent quantities of cRNA accumulate from either mammalian adapted (PB2 627K) or avian (PB2 627E) FluPol in mammalian cells, we cannot completely exclude the possibility that the cRNPs produced by the avian FluPol are in some manner aberrant, as has been previously suggested (Bi et al., 2019; Mänz et al., 2012; Nilsson-Payant et al., 2021). Our RT-qPCR analysis suggests that full-length cRNA is produced, as the RT primer binds to the very 3' end of the cRNA. However, in further work we could extract and fully sequence the cRNA produced to check for deletions or differences in error rate. Alternatively, full-length cRNA may be produced but incorrectly complexed with NP. We could investigate this further through purifying tagged cRNPs and looking for differences in NP co-precipitation. Alternatively, aberrant structures have previously been directly visualised using both electron microscopy and high-speed atomic force microscopy (Miyamoto et al., 2022), and it could be informative to apply these techniques here.

In direct contrast to our findings, an alternative study comparing cRNA and vRNA synthesis in mammalian cells using either the WT FluPol from the lab adapted strain WSN, or an 'avianised' version with PB2 K627E, concluded that AIV FluPol is restricted in both cRNA and vRNA synthesis. This study used promoter mutants that are specifically blocked in either cRNA or vRNA synthesis in the context of positive- or negative-sense viral-like RNAs in mini genome assays, thus allowing the interdependence of the two steps to be unpicked in an alternative manner to a traditional cRNP stabilisation assay. Thus, the opposing observations might reflect differences in the assays used. It would be interesting to repeat this alternative approach with the avian strains used in this work to see whether we observe the same effect. Alternatively, the opposing observations could reflect strain-to-strain variation. Intriguingly, a previous study that used WSN PB2 627E/K to investigate host restriction observed that avian FluPol is

specifically restricted in mammalian cells by an inhibitory factor, which is yet to be definitively identified (Mehle & Doudna, 2008). This is the opposite effect to that observed when similar experiments were performed using either 5092 627E/K or a seasonal H3N2 strain (A/Victoria/3/75) bearing either PB2 627E or 627K, for which host restriction was shown to be caused by the absence of a positive factor in mammalian cells (Moncorgé et al., 2010). This positive factor was later identified as chicken ANP32A (Long et al., 2016). Taken together, this may suggest that the mechanism of host restriction for WSN 627E is not the same as for the avian strain 5092. It would be useful to repeat our experiments with additional avian strains, to clarify how widely applicable our findings truly are.

5.3 Visualising FluPol replication

In recent years, there has been an increasing appreciation of the value of single cell approaches that capture biological heterogeneity that is masked at the population level. As imaging approaches inherently capture data at the single cell level, one of the main aims of this work was to develop an approach for visualisation of the RNA products of influenza replication, thus enabling single cell investigations into FluPol activity. Simultaneously, such an approach would also enable investigations into the spatiotemporal details of influenza replication, with the long-term goal of fully understanding the journey of the vRNP through the nucleus. We were successful in establishing RNAscope as a technique for visualising vRNA and +RNA (cRNA/mRNA) and optimising this approach for *in situ* cRNP stabilisation/replication assays to enable specific detection of cRNA as a marker of genome replication. We were also successful in adapting the RNAscope approach for correlative light and electron microscopy (CLEM) through our collaboration with Dr Lucy Collinson and Dr Alana Burrell. However, due to time constraints of this project, our findings using these approaches were limited to preliminary observations.

In future work, key questions we would like to address include:

- ◇ What are the dynamics of early FluPol replication at a single-cell level?
- ◇ Does genome replication occur in the same subnuclear locale as viral transcription (in complex with the host Pol II CTD)? If not, where does genome replication occur?
- ◇ Can we observe spatial differences during infections with host-restricted avian vs mammalian-adapted IAV in mammalian cells?
- ◇ What is the significance of FluPol 'speckles' that have been observed to accumulate when host restricted FluPol PB2 627E is overexpressed, but not mammalian adapted FluPol PB2 627K (Mistry, 2019)?

There are several remaining challenges including how to provide spatial context within the nucleus, where there are no dividing membranes to aid interpretation. It will also be necessary to establish a more high-throughput high-resolution approach if we are to quantify our observations. Nevertheless, although these experiments are challenging, very few imaging studies using influenza have focused on events within the nucleus, leaving much to be discovered. For example, even in our early characterisation of cRNA synthesis in replication assays, we were surprised to observe cRNA

accumulating in the absence of vRNA accumulation at early timepoints (Section 2.2.5). Thus, even the simplest experiment of visualising cRNA and vRNA accumulation over time may provide novel insight into FluPol activity and regulation.

5.4 Summary and outcomes

In summary, in this work we have furthered our understanding of how the essential proviral host factor ANP32 supports influenza replication and underpins host restriction of avian FluPol. Specifically, we have established that huANP32 is required for cRNA synthesis, as well as vRNA synthesis, in human cells. Moreover, host restriction is imposed specifically on vRNA synthesis, suggesting huANP32 paralogues are sufficient for supporting stabilisation of cRNA produced by an avian influenza FluPol, despite being insufficient to support vRNA synthesis. This suggests a difference in the way ANP32 supports vRNA vs cRNA synthesis and suggests mammalian adaptations are acquired to specifically restore efficient vRNA synthesis. We have also successfully developed an approach for visualising influenza replication products *in situ* that is compatible with CLEM and are now poised for single cell investigations into the spatiotemporal details governing influenza replication in the nucleus.

CHAPTER SIX: MATERIALS AND METHODS

6.1 MATERIALS

6.1.1 Cell lines

Cell line	Details	Source	Reference
HEK-293T cells	Human embryonic kidney cells expressing large T antigen of SV40	ATCC	N/A
293T-dKO	293T cells with both human ANP32A and ANP32B ablated by CRISPR-cas9	The Wang lab	(H. Zhang et al., 2019)
MDCK	Madin-Derby Canine Kidney cells	ATCC	N/A
eHAP1 WT	Fully haploid engineered Hap1 cells, derived from the chronic myelogenous leukemia cell line KBM-7. All eHAP1 cells used in this work have been through a CRISPR-Cas9 protocol with a control guide RNA.	Horizon discovery. Control CRISPR-Cas9 performed by Ecco Staller.	(Staller et al., 2019)
eHAP dKO	eHAP1 cells with human ANP32A and ANP32B ablated by CRISPR-Cas9	Barclay Lab	(Staller et al., 2019)
eHAP tKO	eHAP1 cells with human ANP32A, ANP32B and ANP32E ablated by CRISPR-Cas9	Barclay/Fodor Lab	(Goldhill et al., under review)

Table 5: Cell lines used in this study.

6.1.2 Antibodies

Primary Antibody	Extra details	Manufacturer	Catalogue number	Use and dilution
Anti-influenza A virus PB2 protein	Rabbit polyclonal	GeneTex	GTX125926	IF: 1:500 WB: 1:1000
Anti-influenza A virus Nucleoprotein (C43)	Mouse monoclonal	AbCam	ab128193	IF: 1:200-500 WB: 1:500
Anti-RNA polymerase II CTD repeat YSPTSPS (phospho S5) (CTD Ser5P)	Rabbit polyclonal	AbCam	ab5131	IF: 1:1000
Recombinant Anti-vinculin (EPR8185)	Rabbit monoclonal	AbCam	Ab129002	WB: 1:1000
Anti-alpha tubulin (DM1A)	Mouse monoclonal	AbCam	ab7291	WB: 1:5000
Anti-VP5 (HA018)	Mouse monoclonal	Virusys	MAB13576	IF: 1:300

Table 6: Primary antibodies used in this study.

Secondary Antibody	Manufacturer	Catalogue number	Use and dilution
Goat-anti rabbit IgG (H+L) highly cross-adsorbed Alexa-Fluor 647	Invitrogen	A-21244	IF: 1:1000
Goat-anti mouse IgG (H+L) highly cross-adsorbed Alexa-Fluor 568	Invitrogen	A-11036	IF: 1:1000
Goat-anti mouse IgG (H+L) highly cross-adsorbed Alexa-Fluor 647	Invitrogen	A-21235	IF: 1:1000
Goat anti-rabbit IgG H&L (IRDye 680RD) preadsorbed	AbCam	ab216777	WB: 1:10,000
Goat anti-mouse IgG H&L (IRDye 800CW) preadsorbed	AbCam	ab216772	WB: 1:10,000

Table 7: Secondary antibodies used in this study.

6.1.3 Viruses

Name used in study	Details	Full strain name of internal segments	Source
PR8	Full PR8	A/Puerto Rico/8/1934(H1N1) (PR8)	Raul Yusef Sanchez David (Barclay Lab)
5092E	Virus rescued with 5092 internal segments (PB2 WT, PB1, PA, NP, NS) and PR8 external segments (HA, NA and M)	A/turkey/England/50-92/1991(H5N1) (50-92)	Tom Peacock (Barclay Lab)
5092K	Virus rescued with 5092 internal segments with the humanising mutation PB2 E627K (PB2 E627K, PB1, PA, NP, NS) and PR8 external segments (HA, NA and M)	A/turkey/England/50-92/1991(H5N1) (50-92)	Tom Peacock (Barclay Lab)
Tky05-WT	Virus rescued with Tky05 internal segments (PB2, PB1, PA, NP, M, NS) and PR8 external segments (HA, NA)	A/turkey/Turkey/05/2005 (H5N1) (Tky05)	Daniel Goldhill (Barclay lab)
Tky05-Mut	Tky05-WT passaged three times through eHAP dKO cells followed by plaque picking twice	A/turkey/Turkey/05/2005 (H5N1) (Tky05)	Daniel Goldhill (Barclay lab) (Goldhill et al., under revision)
Eng195	Virus rescued with Eng195 internal segments (PB2 WT, PB1, PA, NP, NS) and PR8 external segments (HA, NA and M)	A/England/195/2009 (pH1N1) (Eng195)	Rebecca Penn (Barclay lab)
Aichi68	Virus rescued with Aichi internal segments (PB2, PB1, PA, NP, M, NS) and PR8 external segments (HA, NA)	A/Aichi/2/1968 (H3N2) (Aichi68)	Rescued for this study

Table 8: Virus strains used in this study.

All viruses other than PR8 WT were 6:2 or 5:3 reassortants with PR8 external genes.

6.1.4 Buffers/chemicals

Flu overlay for plaque assays	0.6% agarose (Invitrogen) in minimal essential media (MEM) (Gibco), supplemented with Na ₂ CO ₂ (Invitrogen), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Invitrogen), bovine serum albumin (BSA) fraction V (Invitrogen), Diethylaminoethyl (DEAE) Dextran (Sigma-Aldrich), penicillin-streptomycin (Pen-strep, Gibco), L-glutamine (Gibco) and 0.1% N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) trypsin (Worthington-Biochemical).
Phosphate Buffered Saline-DEPC (PBS-D)	1 L 1x PBS (homemade), treated with 0.1% DEPC (diethyl pyrocarbonate) (Sigma-Aldrich)
Phosphate Buffered Saline-DEPC-Tween (PBS-D-T)	PBSD plus 0.05% tween (Sigma-Aldrich)
Phosphate Buffered Saline-Tween (PBS-T)	1x PBS (Gibco), 0.1% tween
Radioimmunoprecipitation assay (RIPA) lysis buffer	150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (Sodium dodecyl sulfate), 50 mM Tris, pH 7.4, supplemented with an ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail tablet (Roche)
Tris Acetate EDTA (TAE) buffer	40 mM Tris-Acetate pH8, 1 mM EDTA
Tris Glycine SDS (TGS) running buffer	25 mM Tris, 192 mM glycine, 0.1% w/v SDS
Tris Glycine Methanol (TGM) transfer buffer	25 mM Tris, 192 mM glycine, 10% methanol
Tris buffered saline (TBS) wash buffer	20 mM Tris, 137 mM NaCl, pH 7.6
Tris buffered saline-tween (TBS-T) wash buffer	20 mM Tris, 137 mM NaCl, pH 7.6, 0.1% tween

Table 9: Preparation of buffers used in this study.

0.4 M phosphate buffer	Part A stock solution was prepared by dissolving 6.41 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (VWR) in 90 ml of purified H_2O . Part B stock solution was prepared by dissolving 1.38 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (VWR) in 25 ml of purified H_2O . 0.4 M phosphate buffer was prepared by mixing 81 ml of part A stock solution with 19 ml of part B stock solution. The pH was checked using a pH meter (Mettler Toledo) and adjusted to pH 7.4 using drops of 1M NaOH or 1M HCl as needed.
0.2 M phosphate buffer	An aliquot of the 0.4 M phosphate buffer was mixed 1:1 with purified H_2O .
0.1 M phosphate buffer	An aliquot of the 0.2 M phosphate buffer was mixed 1:1 with purified H_2O .
16% formaldehyde	Fifty millilitres of purified H_2O were added to a glass beaker, along with a magnetic stirring bar, and placed within a larger bowl of water heated to 60°C on a stirring hot plate. The 50 ml water level was marked on the side of the beaker and ~10 ml of H_2O was removed. 8 g of paraformaldehyde powder (Agar scientific) were added to the beaker containing ~40 ml H_2O . This was heated at 60°C , whilst stirring. After 15 min, aqueous NaOH was added one drop at a time until the solution became clear. The beaker was allowed to cool to room temperature and topped up with purified H_2O to the 50 ml mark before filtering through a 0.2 μm syringe filter. Formaldehyde solution was used within 1 day following preparation.
8% formaldehyde in 0.2 M phosphate buffer	Equal measures of 16% freshly made formaldehyde and 0.4 M phosphate buffer were mixed. The pH was checked using pH paper and adjusted to ~pH 7.4 using drops of 1M NaOH or 1M HCl as needed.
4% formaldehyde in 0.1 M phosphate buffer	An aliquot of the 8% formaldehyde in 0.2 M phosphate buffer was mixed 1:1 with purified H_2O .
1% formaldehyde in 0.1 M phosphate buffer	An aliquot of the 4% formaldehyde in 0.1 M phosphate buffer was mixed 1:3 with 0.1 M phosphate buffer.
2% uranyl acetate stain	0.2 g of uranyl acetate (Agar Scientific) were dissolved in 10 ml purified H_2O and the solution was filtered through a 0.2 μm syringe filter.
Reynolds' lead citrate stain	0.33 g of lead nitrate (Sigma-Aldrich) and 1.76 g of trisodium citrate (AnalaR) were added to a flask containing 30 ml of pre-boiled purified H_2O which had been allowed to cool to room temperature. The flask was shaken intermittently for 30 min. Carbonate free NaOH was prepared by dissolving 1 g of NaOH (Fischer Chemical) to 25 ml of pre-boiled purified H_2O . After 30 minutes of shaking the lead nitrate and sodium citrate mixture, 5 ml of carbonate free NaOH solution was added. Further NaOH was added in a dropwise manner until the pH of the lead citrate solution reached pH 12.

Table 10: Preparation of buffers used for correlative light and electron microscopy (prepared by Dr Alana Burrell).

Actinomycin D	Actinomycin D (Gibco) was obtained as a powder and dissolved in sterile dimethyl sulfoxide (DMSO, Merck) to a final concentration of 5 mg/mL. Aliquots were stored at -20°C. Working concentrations are 5-10 µg/mL, depending on cell type.
Aminoguanidine	Aminoguanidine (Sigma-Aldrich) was obtained as a powder and dissolved in water to a final concentration of 100 mM. Fresh stock solution was prepared on the day of use. Working concentration is 10 mM.
Azide-fluor 488	Azide-fluor 488 (Sigma-Aldrich) was obtained as a powder and dissolved in DMSO to a final concentration of 500 µM. Aliquots were stored at -20°C. Working concentration is 10 µM.
Copper (II) Sulfate Pentahydrate (CuSO₄)	CuSO ₄ (Sigma-Aldrich) was obtained as a crystalline salt and dissolved in water to a final concentration of 10 mM. Solution was stored at room temperature with parafilm to reduce evaporation. Working concentration is 1 mM.
Baloxavir Acid	Baloxavir acid (MedChemExpress) was obtained as a powder and dissolved in sterile DMSO to a final concentration of 2 mM. Aliquots were stored at -80°C. Working concentrations ranged from 0.05 to 50 nM.
Cycloheximide	Cycloheximide (Sigma-Aldrich) was obtained as a powder and dissolved in sterile DMSO to a final concentration of 100 mg/mL. Aliquots were stored at -20°C. Working concentration is 100 µg/mL.
Sodium ascorbate	Sodium ascorbate (Sigma-Aldrich) was obtained as a salt and dissolved in water to a final concentration of 100 mM. Fresh stock solution was prepared on the day of use. Working concentrations is 10 mM.
THPTA (Tris((1-hydroxypropyl-1H-1,2,3-triazol-4-yl)methyl)amine)	THPTA (Sigma-Aldrich) was obtained as a powder and dissolved in water to a final concentration of 10 mM. Aliquots were stored at -20°C.
5-EU	5-EU (Jena Bioscience) was obtained as a powder and dissolved in sterile ddH ₂ O to a final concentration of 100 mM by heating at 70°C for one minute. Aliquots were stored at -20°C. Working concentrations were 0.5 mM- 4 mM.

Table 11: Preparation of chemical stocks used in this study.

6.2 METHODS

6.2.1 Cell culture

eHAP WT, dKO and tKO cells were maintained in Iscove's modified Dulbecco's medium (IMDM, Gibco) supplemented with 10% fetal bovine serum (FBS, Labtech), 1% penicillin-streptomycin (pen-strep, Gibco) and 1% non-essential amino acids (NEAA, Gibco). MDCK, HEK-293T and 293T-dKO cells were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% FCS, 1% pen-strep and 1% NEAA. When used for infection, HEK-293T cells were seeded on plates coated with poly-L-lysine (Merck) to aid adherence. To coat plates, poly-L-lysine (0.1% w/v) was diluted 1 in 50 in sterile water and incubated in wells for >1 hour followed by three washes in PBS (Gibco). All cells were maintained at 37°C and 5% CO₂.

6.2.2 Cloning

Plasmids

pCAGGS expression plasmids encoding the polymerase subunits (PB2, PB1, PA) and NP from PR8 were subcloned from the reverse genetics system pPoll plasmids (Whiteley et al., 2007). The catalytic mutant PB1 D446Y has been previously described (Biswas & Nayak, 1994), and was introduced into pCAGGS:PB1 (PR8) and pCAGGS:PB1 (Eng195) using overlap extension PCR. pCAGGS expression plasmids encoding 50-92 PB2, PB1, PA and NP (Moncorgé et al., 2010), 50-92 PB1 D446Y and *Renilla* luciferase control (Cauldwell et al., 2013), FLAG-tagged huANP32B and chANP32B constructs (Long et al., 2016) and FLAG-tagged huANP32B-L128V (Staller et al., 2021), as well as the human minigenome reporter plasmid pHOM1-Firefly (Moncorgé et al., 2013) have previously been described. pCAGGS: huANP32B-delta175 and -delta155 were generated and validated by Dr Ecco Staller, and are reported in his thesis (Staller, 2021). Plasmid concentrations were measured using a Nanodrop Lite spectrophotometer (Thermo Fisher), or QIAexpert spectrophotometer (Qiagen). Gel extractions, PCR purifications and mini preps were performed using the respective NEB kit, according to the manufacturer's instructions. Midi and maxi preps were performed using Qiagen hiSpeed plasmid kits, according to the manufacturer's instructions. Sanger sequencing was performed by Eurofins or Source Bioscience.

Heat shock transformation

Transformation into competent NEB 5-α E. coli (NEB) was achieved using heat shock. High efficiency NEB 5-α were used for fresh ligations, whilst subcloning efficiency NEB 5-α were used for retransformations. In brief, approximately 30 ng plasmid, or 2 μL ligated product, were incubated with competent cells on ice for 20 minutes. Bacteria were briefly incubated (1 minute) in a 42°C water bath, prior to a further five-minute incubation on ice. Cells were supplemented with 500 μL Super Optimal broth with Catabolite repression (SOC) media (Invitrogen), and incubated at 37°C for 45 minutes, shaking. Transformed bacteria were plated on Luria-Bertani (LB) agar plates containing 100 μg/mL ampicillin and incubated overnight at 37°C.

Gel electrophoresis

DNA products were separated using 1% gels (1% agarose in TAE buffer) supplemented with Gel Red (Biotium) for DNA visualisation. DNA was mixed with loading dye (NEB) and loaded into gels with 1 kb or 100 bp ladders (NEB) as appropriate. Gel electrophoresis was run for 30-60 minutes at 100 V in 0.5x TAE buffer. Gels were visualised using the gel doc XR+ imaging system (Bio-Rad).

Plasmid digestion

Digested empty pCAGGS plasmid was generated using the appropriate double digest (NotI-HF/XhoI or NotI-HF/MluI-HF (all NEB)). 2 µg empty pCAGGS plasmid was incubated with 1 µL of each enzyme in 1x CutSmart buffer, for 2 hours. Digested plasmid was run out on a 1% agarose gel and the correct sized band gel extracted. To ensure full digestion and limit self-ligation, digested plasmid was subsequently digested again under the same conditions plus the addition of 1 µL Antarctic phosphatase and 1x Antarctic phosphatase buffer (NEB). Twice digested plasmid was then PCR purified and used for subcloning or overlap extension PCR as required.

Ligations

Ligations were performed using T4 DNA ligase (NEB) in 20 µL reactions. 0.3 µL cut and dephosphorylated vector (see above) was incubated with 3 µL digested insert, 1 µL T4 DNA ligase and 1x T4 DNA ligase buffer. Ligations were performed at room temperature for 60 minutes, followed by heat inactivation at 65°C for 10 minutes. 2 µL ligation product was transformed into E. coli using heat shock as described above.

Subcloning

To subclone PR8 RNP components from the respective pPoll plasmids into pCAGGS expression vectors, flanking forward and reverse primers were designed for the protein coding region. Forward primers contained NotI restriction sites and Kozak sequences, whilst reverse primers contained an additional stop codon, to inhibit readthrough and the expression of chimeric proteins, plus a XhoI restriction site. Primers used are in Table 12.

Primer use	Sequence
Cloning PR8 PB2 into pCAGGS F	TATATATAGCGGCCGCGCCACCATGGAAAGAATAAAAGAACTAAG
Cloning PR8 PB2 into pCAGGS R	TTAATTAAC TCGAGTTACTAATTGATGGCCATCCG
Cloning PR8 PB1 into pCAGGS F	TATATATAGCGGCCGCGCCACCATGGATGTCAATCCGACC
Cloning PR8 PB1 into pCAGGS R	TTAATTAAC TCGAGTTACTATTTTTGCCGTCTGAGC
Cloning PR8 PA into pCAGGS F	TATATATAGCGGCCGCGCCACCATGGAAGATTTTGTGCGAC

Cloning PR8 PA into pCAGGS R	TTAATTAAGTCGAGTTACTAACTCAATGCATGTGTAAGG
Cloning PR8 NP into pCAGGS F	TATATATAGCGGCCGCGCCACCATGGCGTCCCAAGGCACC
Cloning PR8 NP into pCAGGS R	TTAATTAAGTCGAGTTATTAATTGTCGTACTCCTC

Table 12: Primers used for subcloning PR8 polymerase components into pCAGGS

Genes of interest were amplified from the pPoll plasmid using KOD Hot Start DNA polymerase (Sigma-Aldrich) in 25 µL reactions according to the manufacturer's instructions. Touchdown PCR was used to avoid the need for PCR optimisation. Thermocycler conditions were as follows:

Step	Temperature (°C)	Time
Hot start – activates KOD polymerase	95	2 min
Touchdown (12 cycles, - 1°C/cycle)	95	20s
	68-57	15s
	70	1 min
Further amplification (23 cycles)	95	20s
	57	15s
	70	1 min
Final extension	70	2 min
Hold	12	-

PCR products were run on 1% agarose gels and the expected size band gel extracted. Gel extracted products were digested using NotI-HF, XhoI and DpnI (NEB) (to digest any contaminating plasmid), then PCR purified. Digested PCR fragments were ligated into digested pCAGGS plasmid using T4 DNA ligase as described above. Ligated plasmids were transformed into supercompetent cells by heatshock. The following day, >2 colonies were picked from each agar plate and set up as 3 mL cultures in LB broth containing 100 µg/mL ampicillin, and incubated at 37°C, shaking, overnight. 500 µL overnight culture was used to generate glycerol stocks. Plasmids were mini prepped from remaining overnight culture and verified by Sanger sequencing. Glycerol stocks from correct plasmids were subsequently used to set up 500 mL overnight cultures in LB plus 100 µg/mL ampicillin, and master stocks of plasmids extracted by maxi prep.

Overlap extension PCR

Generation of the pCAGGS:PB1 D446Y (PR8) and pCAGGS:PB1 D446Y (Eng195) mutants was achieved using overlap extension PCR. For PR8 the same forward and reverse flanking primers were used as for subcloning pCAGGS:PB1 (PR8). To introduce point mutations, forward and reverse internal primers were designed with the desired aspartic acid to tyrosine modification at residue 446 and 15bp complementary flanking regions. Primers used are in Table 13.

Primer use	Sequence
PR8 PB1 forward primer	TATATATAGCGGCCGCGCCACCATGGATGTCAATCCGACC
PR8 PB1 reverse primer	TTAATTAACCTCGAGTTACTATTTTGCCGTCTGAGC
PR8 PB1 D446Y modification forward primer	CTTCAATCCTCTGACTATTTTGCTCTGATTGTG
PR8 PB1 D446Y modification reverse primer	CACAATCAGAGCAAAATAGTCAGAGGATTGAAG
Eng195 PB1 forward primer	TATATATAGCGGCCGCGCCACCATGGATGTCAATCCGACT
Eng195 PB1 reverse primer	TATATATAGCGGCCGCGCCACCATGGATGTCAATCCGACT
Eng195 PB1 D446Y modification forward primer	CTCCAATCATCCGACTATTTTGCTCTCATAGTG
Eng195 PB1 D446Y modification reverse primer	CACTATGAGAGCAAAATAGTCGGATGATTGGAG

Table 13: Primers used to introduce the D446Y mutation into PR8 and Eng195 PB1 using overlap extension PCR.

To introduce the D446Y mutation, initially two separate PCR reactions were set up – one with the forward flanking primer and reverse modification primer, and one with the reverse flanking primer combined with the forward modification primer – that result in the generation of two fragments of the PB1 gene each containing the desired D446Y point mutation. PCR reactions used KOD Hot Start DNA polymerase according to the manufacturer's instructions, 200 ng pCAGGS:PB1 (PR8), and the same touchdown conditions described for subcloning. Resulting products were visualised using gel electrophoresis, gel extracted and eluted in 30 µL molecular grade water (Sigma-Aldrich). To generate the full PB1 mutant fragment, a second PCR was run using the overlapping fragments from the previous PCRs as template DNA, KOD Hot Start DNA Polymerase, and initially omitting forward and reverse primers. Under these conditions, the mutant fragments self-prime and extend, resulting in the generation of the full PB1 fragment with the desired D446Y modification.

Thermocycler conditions were as follows:

Step	Temperature (°C)	Time
Hot start – activates KOD polymerase	95	2 min
Amplification (12 cycles)	95	20s
	55	15s
	70	30s
Final extension	70	1 min
Hold	12	-

Subsequently, 0.75 µL forward and reverse flanking primers were added into the PCR reaction mix and further amplification of the PB1 fragment achieved using the same touchdown thermocycler conditions as for subcloning. PCR product size was checked using gel electrophoresis and the resulting product gel extracted. The PB1 fragment was digested using NotI-HF and XhoI according to the manufacturer's instructions and PCR purified, prior to ligation into NotI-HF/XhoI digested pCAGGS plasmid using T4 DNA ligase as described above.

6.2.3 Mini genome assays

Luminescence read-out

HEK-293T or eHAP cells were cultured in 24 well plates, with each condition in triplicate. Cells were transfected using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions, with pCAGGS expression plasmid mixtures encoding PB2, PB1, PA, NP and *Renilla* luciferase reporter, plus pHOM1 vRNA firefly luciferase reporter (2:2:1:8:2:2, where 1= 20 ng). In experiments including ANP32, 5-100 ng pCAGGS expression vectors encoding FLAG-tagged huANP32B or FLAG-tagged chANP32A were added as indicated in figure legends. Empty pCAGGS vector was added to normalise input DNA quantities as necessary. 20-24 hours post-transfection, cells were lysed with 100 µL passive lysis buffer (Promega) for 15 minutes, rocking. 20 µL of lysate was added to 96 well plates, and bioluminescent signals read using a FLUOstar Omega plate reader (BMG Labtech) using the Dual-Luciferase Reporter assay system (Promega). Firefly signal was divided by *Renilla* to give relative luminescence units (RLU). For assays studying BXA activity, BXA was diluted to the desired concentration in full media and added to cells just before transfection.

RT-qPCR read-out

Assays were performed as for standard bioluminescence read-out, however both the *Renilla* luciferase reporter and pHOM1 vRNA firefly luciferase reporter were omitted from the transfection mix and replaced with 40 ng of the PR8 NA segment under the control of a pPoll reporter (i.e., the PR8 NA

segment from the 12 plasmid rescue system). 20-24 hours post-transfection, cells were lysed with RLT buffer (Qiagen) and stored at -80°C, prior to processing as standard for the tagged RT-qPCR approach (Section 6.2.5) using primer sets targeting segment 6 (NA). For assays studying BXA activity, BXA was diluted to 50 nM in full media, and added to cells just before transfection.

6.2.4 Virology

Viral rescue

Viral rescue for Aichi68 with PR8 externals was achieved using the 8 plasmid reverse genetics system (Hoffmann et al., 2000). HEK-293T cells were seeded in full DMEM in a 12 well plate. The following afternoon, cell media was changed to serum-free OptiMEM (Gibco) and cells transfected with all eight viral segments using Xtreme-gene9 transfection reagent (Roche). In brief, a plasmid mix of 1000 ng of NP and 500 ng of all other viral segments (PB2, PB1, PA, HA, NA, M, NS), expressed in pHW2000 plasmid was prepared, alongside a negative control lacking the PB1 plasmid. 40 µL transfection reagent was added to 460 µL OptiMEM, swirled and allowed to equilibrate at room temperature for 5 minutes. 250 µL transfection mix was then added to each of the prepared plasmid mixes, swirled and incubated at room temperature for 25-30 minutes. Each plasmid mix was subsequently added to a well of HEK-293T cells dropwise. Transfected cells were incubated overnight. The following morning, HEK-293T cells were co-cultured with MDCKs. 12 mL of MDCK cells were trypsinised, washed using centrifugation and resuspended in 20 mL full DMEM. 4 mL of suspended MDCKs was aliquoted into two T25 flasks (8 mL total). Media was removed from the transfected HEK-293T cells and 1 mL of the remaining MDCK suspension added to the well and used to dislodge the HEK-293T cells through pipetting. The resulting MDCK-HEK-293T suspension was subsequently added to the prepared T25 flasks containing 4 mL MDCKs, and cells allowed to settle for 4-5 hours. Once cells had adhered to the flask bottom, full DMEM was removed and cells carefully washed in serum-free DMEM. Media was subsequently replaced with serum-free DMEM supplemented with 1% pen-strep, 1% NEAA and 1 µg/mL TPCK-trypsin. Cells were incubated for 5 days, until cytopathic effect could be clearly observed in the cell sheet, but not in the negative control rescue. The supernatant was then harvested, clarified and stored at -80°C. Titre of the rescue stock was measured by plaque assay, and rescue stock subsequently used to generate a higher titre stock using the protocol described below.

Growing viral stocks

To grow viral stocks, the appropriate viral strain was diluted in serum-free media (MOI = 0.0001). MDCK cells were washed in PBS, and diluted viral inoculum added. Cells were incubated at 37°C for 1 hour with regular tipping. Inoculum was removed and replaced with serum-free media containing 1 µg/mL TPCK-trypsin. 48 hpi supernatant was collected and clarified by centrifugation. Viral stocks were aliquoted and stored at -80°C. Infectious viral titres were calculated by plaque assay.

Plaque assays

IAV infectious viral titres were assayed on MDCK cells. MDCK cells were cultured in 12 well plates to 100% confluency. Viral stocks were serially diluted in a 10-fold dilution to 10⁻⁸ in serum-free media and

100 µL of each dilution added to wells in duplicate. Cells were incubated at 37°C for one hour with occasional shaking. Inoculum was removed, then replaced with 1 mL flu-overlay. Overlay media was allowed to set at room temperature, then plates were incubated upside down at 37°C for 72 hours. Flu-overlay was removed and plaques fixed and stained using crystal violet solution (5% crystal violet (Sigma-Aldrich), 15% EtOH in sterile water) (30 minutes, room temp.). Crystal violet solution was carefully removed. Viral plaques were counted at the lowest dilution at which individual plaques could be resolved, and the titre calculated as the average between the two duplicates.

Viral infections

For infections, virus was diluted in serum-free media. MOIs are indicated in figure legends. To synchronise infection, viral inoculation was performed at 4°C. In brief, cells were pre-incubated at 4°C for 15 minutes. Cells were placed on ice, washed once in PBS, before addition of cold viral inoculum and a further incubation at 4°C for 45 minutes to allow virus adsorption to the surface of cells. Viral inoculum was then replaced with pre-warmed full media and infected plates incubated at 37°C, 5% CO₂. At the appropriate time point, cells were processed for RT-qPCR analysis, imaging analysis or immunoblot as described below.

cRNP stabilisation and replication assays

For replication/cRNP stabilisation assays, cells were transfected using Lipofectamine 3000 with pCAGGS expression plasmid mixtures encoding polymerase components in the ratios 2:2:1:4, PB2:PB1:PA:NP, where 1=20ng (24 well plate), 40ng (12 well plate) or 80ng (6 well plate). For replication assays WT PB1 plasmid was pre-expressed, whilst for cRNP stabilisation assays catalytically dead polymerase (PB1 D446Y) was pre-expressed. For experiments using eHAPs, 20 hours post transfection cells were pre-incubated in either actinomycin D (10 µg/mL), cycloheximide (100 µg/mL), or DMSO control, as indicated, for one hour. Cells were then infected as described above, in the presence of actinomycin D (10 µg/mL), cycloheximide (100 µg/mL), or DMSO control, as indicated. For experiments using HEK-293T cells, pre-incubation was omitted and 20 hours post transfection, cells were infected as described above, with the addition of actinomycin D (5 µg/mL) or DMSO control, as indicated. At the appropriate time point, cells were processed for RT-qPCR analysis, imaging analysis or immunoblot as described below.

6.2.5 RNA extraction and RT-qPCR

Tagged RT-qPCR against vRNA, cRNA and mRNA

For RT-qPCR analysis, HEK-293T or eHAP cells were cultured in 24 well plates, with each condition in triplicate. Following infection/transfection, cells were lysed using buffer RLT or RLT plus (Qiagen), frozen at -80, then total RNA extracted using either the RNeasy RNA extraction kit (Qiagen) with 30-minute on column DNaseI digest, or the QIAasympphony RNA kit (Qiagen). Quantification for haemagglutinin and neuraminidase vRNA, cRNA and mRNA was based on the tagged primer approach developed by Kawakami et al (Kawakami et al., 2011). For each sample, four reverse transcription

reactions were set up using 200 ng RNA/reaction, RevertAid H Minus Reverse Transcriptase (Thermo Fisher) as per the manufacturer's instructions, plus a tagged primer targeting either vRNA or cRNA, a tagged polydT (for viral mRNA) or an untagged polydT (for GAPDH internal control). cDNA was then diluted 1 in 10 and quantified using real-time quantitative PCR using Fast SYBR green master mix (Thermo Fisher) and primer pairs targeting the RT-introduced tag (forward primer) plus a segment-specific reverse primer. qPCR analysis was carried out in duplicate or triplicate on a Viia 7 real-time PCR system (Thermo Fisher). Fold changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method with GAPDH expression as internal control. RT and qPCR primers are presented in Table 14:

Primer use	Sequence
NA vRNA tagged RT primer	<u>GGCCGTCATGGTGGCGAAT</u> GAAACCATAAAAAGTTGGAGGAAG
NA cRNA tagged RT primer	GCTAGCTTCAGCTAGGCATCAGTAGAAACAAGGAGTTT
HA vRNA tagged RT primer	<u>GGCCGTCATGGTGGCGAAT</u> GGAGAGTGCCCCAAATACGT
HA cRNA tagged RT primer	GCTAGCTTCAGCTAGGCATCAGTAGAAACAAGGGTGTT
Tagged poly(dT) RT primer (NA/ HA mRNA)	<u>CCAGATCGTTCGAGTCG</u> TTTTTTTTTTTTTTTTTT
NA vRNA qPCR F	CCTTCCCCTTTTCGATCTTG
NA vRNA qPCR R	GGCCGTCATGGTGGCGAAT
NA cRNA qPCR F	CTTTTGTGGCGTGAATAGTG
NA cRNA qPCR R	GCTAGCTTCAGCTAGGCATC
NA mRNA qPCR F	CTTTTGTGGCGTGAATAGTG
NA mRNA qPCR R	CCAGATCGTTCGAGTCGT
HA vRNA qPCR F	CATACCATCCATCTATCATTCC
HA vRNA qPCR R	GGCCGTCATGGTGGCGAAT
HA cRNA qPCR F	GGGGCAATCAGTTTCTG
HA cRNA qPCR R	GCTAGCTTCAGCTAGGCATC
HA mRNA qPCR F	CTTTTGTGGCGTGAATAGTG
HA mRNA qPCR R	CCAGATCGTTCGAGTCGT
GAPDH qPCR F	AATCCCATCACCATCTTCCA
GAPDH qPCR R	TGGACTCCACGACGTACTCA

Table 14: Primers used for tagged RT-qPCR against PR8 NA vRNA/cRNA/mRNA and HA vRNA/cRNA/mRNA

Quantification of NA vRNA genomes in viral stocks

To calculate the number of input NA vRNA genomes present in a viral stock, RNA was first extracted from 140 μ L viral stock using the QIAamp Viral RNA mini kit (Qiagen) as per the manufacturer's instructions, excluding carrier RNA and eluted into 30 μ L RNase-free sterile water (Qiagen). 12 μ L neat and 12 μ L 1 in 10 dilution of the eluted RNA was subsequently used for cDNA synthesis and RT-qPCR using the tagged primers for NA vRNA as described above. For quantification, a standard curve from 10^9 to 10^0 copies of plasmid DNA containing the NA vRNA fragment/ 10 μ L qPCR reaction was included in each RT-qPCR.

6.2.6 RNAscope

Cells were cultured and infected on glass coverslips (VWR). 16 mm diameter cover slips were placed in 12 well plates and washed in 100% EtOH to sterilise, followed by three washes in PBS. Cover slips were incubated in poly-L-lysine (0.1% w/v) diluted 1 in 50 in sterile water for >1 hour, followed by three washes in PBS. Cells were plated onto coated cover slips and maintained as described above until ~70% confluency. Infections were performed as described above. At the appropriate timepoint, infected cells were washed in PBS and fixed in freshly prepared 4% paraformaldehyde (16% PFA (VWR) diluted in PBS) for 30 minutes, prior to three washes in PBS (without incubation/ rocking) and dehydration in an ethanol gradient (50% EtOH, 5 minutes, 70% EtOH, 5 minutes, 100% EtOH, 5 minutes, fresh 100% EtOH, 10 minutes). Cover slips were stored in fresh 100% ethanol at -20°C until further processing. For staining, incubations in solutions were performed through inverting cover slips onto drops of the appropriate solution on parafilm and incubating in a humidified chamber at the appropriate temperature. To wash, cover slips were returned to 12 well plates and 1 mL of the wash buffer/ PBS added as appropriate. Unless indicated otherwise, all washes were for two minutes, at room temperature, with rocking. Images were collected using either an inverted Zeiss Cell Observer Widefield microscope or a Leica SP5 inverted confocal microscope as indicated in figure legends. All image processing was handled in FIJI (Schindelin et al., 2012; Schneider et al., 2012).

Standard RNAscope staining

RNAscope probes (ACDBio) were designed to target PR8 NA vRNA (channel 1), PR8 NA cRNA/mRNA (+RNA) (channel 2), PR8 HA vRNA (channel 2) and PR8 HA cRNA/mRNA (+RNA) (channel 3). RNAscope staining was performed using an RNAscope fluorescent multiplex kit v1 (ACDBio), according to the manufacturer's instructions. In brief, cells were first rehydrated in an ethanol gradient (70% EtOH, 2 minutes, 50% EtOH, 2 minutes, PBS, 10 minutes). Cells were then treated with protease III diluted 1 in 15 in PBS (10 minutes, room temperature), washed three times in PBS, then incubated in the appropriate probe mixtures (2 hours, 40°C). Cells were washed three times in RNAscope wash buffer, then incubated in the four amplification mixtures (Amp 1-FI, Amp 2-FI, Amp 3-FI, Amp 4-FI) in series, according to the manufacturer's instructions. Following incubation in the final fluorescent probe mixture (Amp 4-FI), cells were kept in the dark. Cells were counterstained using DAPI (10 minutes, room temperature), then mounted onto glass microscope slides using ProLong Gold (Thermo Fisher). Slides were allowed to dry overnight at room temperature then stored at 4°C until visualisation.

RNAscope/indirect immunofluorescence (IIF) co-staining

For RNAscope/immunofluorescence co-staining, RNAscope staining was performed first, followed by IIF. RNAscope staining was performed as described above, up until and including incubation in the final fluorophore mixture (Amp 4-FI). At this point, cover slips were blocked in PBS-T with 2% bovine serum albumin (BSA) (Sigma-Aldrich) for 30 minutes. Cells were incubated in primary antibodies, diluted as appropriate (see Table 6), in PBS-T+2% BSA, then washed three times in PBS. Cells were incubated in secondary antibodies diluted as appropriate (see Table 7), plus DAPI (1:1000), in PBS+2% BSA for 1 hour at room temperature, washed three times in PBS, then mounted onto glass microscope slides using ProLong Gold (Thermo Fisher). Slides were allowed to dry overnight at room temperature then stored at 4°C until visualisation.

6.2.7 Correlative light and electron microscopy (CLEM)

Preparation of samples

For CLEM, cells were cultured and infected as described above for standard RNAscope analysis, however using slightly smaller round glass cover slips (13 mm diameter). At the appropriate timepoint post-infection, 1 mL freshly made 8% formaldehyde in 0.2 M phosphate buffer (warmed to 37°C) was added to cells growing in 1 ml of cell culture medium. After 15 minutes incubation at 37°C, the fixative/medium solution was replaced with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) and cells were incubated for 30 minutes at room temperature. Cells were then stored overnight in 1% formaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C prior to progressive lowering of temperature processing.

Progressive lowering of temperature

Progressive lowering of temperature was carried out using a Leica EM AFS2 freeze substitution and low temperature embedding system. Fixed samples were washed three times in 4°C 0.1 M phosphate buffer (pH 7.4) for 5 minutes each. Samples were added to 4°C 50% ethanol and incubated for 20 minutes; during this time the temperature was gradually reduced to -20°C. Samples were dehydrated by a series of graded ethanol incubations: 70% ethanol for 30 minutes at -20°C; 90% ethanol for 60 minutes at -20°C; and 2x 100% ethanol for 60 minutes at -20°C. Resin was prepared by dissolving 0.3 g benzoin methyl ether (Sigma-Aldrich) in 50 mL pre-catalysed, hard grade, LR White resin (Agar Scientific). Resin infiltration was carried out, in the dark, as follows: 30 minutes incubation in 25% resin 75% (-20°C); 30 minutes incubation in 50% resin 50% ethanol (-20°C); 30 minutes incubation in 75% resin 25% ethanol (-20°C); overnight incubation in 100% resin (-20°C); 30 minutes incubation in 100% resin (4°C). A sheet of aclar film (Agar scientific) was placed on top of the resin to exclude air prior to polymerisation. Polymerisation was achieved over 48 hours, at -20°C, using ultraviolet light emitted from the lamp attachment of the EM AFS2 system (Leica).

Preparation of formvar film

Glass slides (BRAND®) were thoroughly cleaned using a leather chamois and then wiped with Kimwipe™ tissue. Formvar solution (1% formvar in chloroform, TAAB) was added to a custom-made film-casting device along with the pre-cleaned slide and the solution was allowed to drain so that a thin film of formvar was deposited on the surface of the slide. Once dry, the edges of the slide were scraped with a razor blade. After briefly breathing on the slide to create condensation, the formvar film was detached from the slide by lowering vertically into a dish of purified H₂O. A film of the desired thickness gives a silver interference colour. The floating film was scooped up onto a Domino Rack (Electron Microscopy Sciences) so that the film stretched across the holes.

Ultrathin sectioning

Resin-embedded cells were detached from the glass coverslips by briefly immersing in liquid nitrogen. 90 nm ultrathin sections were cut using an ultramicrotome (RMC powertome) and diamond knife (Diatome). Sections were collected in a water droplet using a Perfect Loop (Electron Microscopy Sciences) and transferred to formvar film suspended over holes in a Domino Rack (Electron Microscopy Sciences). Three-hundred mesh gold electron microscopy grids (Agar) were coated with adhesive (Coat-Quick "G", Electron Microscopy Sciences) and placed adhesive side down on top of dried sections adhered to the formvar film. *In situ* hybridisation (RNAscope®) was performed on sections on the day following collection.

RNAscope labelling on ultrathin sections

RNAscope probes were prepared by heating to 40°C for 10 minutes then cooled to room temperature. 2 µl of HA vRNA probe was diluted in 100 µl of NA vRNA probe. RNAscope wash buffer was prepared by heating 50x stock to 40°C for 20 minutes and diluting 1 ml in 49 ml purified H₂O. A double layer of parafilm was placed inside a staining box and an ethanol-cleaned wooden skewer was used to mark small depressions into the Parafilm™ (Bemis) to act as wells for containing the RNAscope® probe and amplification reagents. Kimwipe™ tissue was soaked in purified H₂O and added to the box to maintain humidity during labelling. RNAscope® incubations were performed at 40°C by placing grids, section side down, onto 4 µl droplets within the parafilm wells and washes were performed by dipping grids into 5 microcentrifuge tubes containing buffer. RNAscope labelling was performed as follows:

- Incubation in probe mixture for 1 hr (40°C)
- Five washes in RNAscope wash buffer
- Incubation in Amp1-fl for 15 minutes (40°C)
- Five washes in RNAscope wash buffer
- Incubation in Amp2-fl for 8 minutes (40°C)
- Five washes in RNAscope wash buffer
- Incubation in Amp3-fl for 15 minutes (40°C)
- Five washes in RNAscope wash buffer
- Incubation in Amp4-fl for 8 minutes (40°C)
- Five washes in RNAscope wash buffer

Grids were placed onto drops of DAPI (RNAscope) and incubated for 5 minutes in the dark before washing in purified H₂O.

Light microscopy

For light microscopy imaging, grids were mounted in 50% glycerol in H₂O in the well of a Secure-Seal™ spacer attached to the base of a glass bottom dish (MatTek) and covered by a 13 mm round glass coverslip. Widefield light microscopy imaging was performed using an Axio Observer 7 (Zeiss). High resolution images were captured using 63x oil immersion lens (1.4 na). Signal from the NA genomic probe labelling was captured using excitation and emission settings for Atto 550 (ZEN software Zeiss), signal for the HA genomic probe labelling was captured using excitation and emission settings for Atto 647 (ZEN software Zeiss) and signal for the DAPI labelling was captured using excitation and emission settings for DAPI (ZEN software Zeiss).

Electron microscopy imaging

Following light microscopy imaging, grids were washed by dipping 20 times in purified H₂O. Grids were stained for 10 minutes in 2% aqueous uranyl acetate (in the dark) washed in purified H₂O, stained for 5 minutes in Reynolds' lead citrate (Reynolds, 1963) and washed again in purified H₂O. Electron microscopy imaging was performed using a JEM-1400Flash (JEOL) transmission electron microscope. Montage data was acquired using the Limitless Panorama (JEOL) function.

Alignment of light microscopy and electron microscopy data

Aligned overlays of the light microscopy and electron microscopy images were created using BigWarp plugin (Bogovic et al., 2016) for Fiji (Schindelin et al., 2012). Landmark points were manually placed on distinctive features of the nucleus which were visible in both the light microscopy and electron microscopy data sets and an affine transform was applied to the light microscopy data.

6.2.8 Padlock probes (PLPs)

The protocol for padlock probe staining was adapted from that published by Dou et al (Dou et al., 2017). Cells were cultured on glass cover slips, infected, fixed, dehydrated in ethanol and stored as for RNAscope. Incubations in solutions were performed through inverting cover slips onto drops of the appropriate solution on parafilm and incubating in a humidified chamber at the appropriate temperature. To wash, cover slips were returned to 12 well plates and washed in 1 mL of PBS-D-T. Unless indicated otherwise, all washes were for two minutes in DEPC-treated PBS (PBS-D), at room temperature, with rocking. Images were collected using an inverted Zeiss Cell Observer Widefield microscope. All image processing was handled in FIJI (Schindelin et al., 2012; Schneider et al., 2012).

Padlock probe staining

Prior to staining, cells were rehydrated in an EtOH gradient (70% (2 minutes), 50%, (2 minutes), PBS-D (10 minutes). For *in situ* cDNA synthesis, cells were incubated in 20 uL cDNA synthesis mix (5 μM LNA-

modified cDNA primers (targeting vRNA/cRNA as appropriate) (Merck), 1 mM dNTPs, 1x RevertAid RT buffer, 0.2 µg/µL BSA (NEB), 5 mM DTT (Invitrogen), 0.8U/µL RiboLock RNase inhibitor (Thermo Fisher), 20 U/µL RevertAid-H enzyme (Thermo Fisher), nuclease-free water to 20 µL) (42°C, 3 hours). Cells were washed, nascent cDNA fixed in 4% PFA (10 minutes), then washed again. Prior to annealing, PLP primers (Invitrogen) were phosphorylated using T4 PNK (15 µM PLP primer, 0.75U/ µL T4 PNK (NEB), 1x T4 DNA ligase buffer (NEB), nuclease-free water to 20 µL) (37°C, 30 minutes, 65°C, 20 minutes), followed by PCR purification. For PLP annealing and ligation, cells were incubated in 20 µL ampligase mix (1x ampligase buffer, 0.1 µM phosphorylated PLPs, 20% formamide (Sigma-Aldrich), 0.2 µg/µL BSA, 0.4 U/µL RNaseH (NEB), 0.5 U/µL ampligase (Lucigen), 25 mM KCl, nuclease-free water to 20µL) (37°C, 30 minutes, 45°C, 45 minutes), then washed. For rolling circle amplification, cells were incubated in Phi29 rolling circle polymerase mix (1x Phi29 buffer, 5% glycerol (Thermo Fisher), 0.25 mM dNTPs, 0.2 µg/µL BSA, 1 U/µL Phi29 polymerase (NEB), water to 20µL) (25°C, 16 hours), then washed. Rolling circle products were labelled by incubation in barcode probe mix (2x (30 mM) saline-sodium citrate buffer (pH 7.0) containing 300 mM NaCl (Sigma-Aldrich), 0.1 µM barcode probes (Eurofins), 20% formamide) (37°C, 20 minutes), then washed. Nuclei were counterstained using DAPI (1:1000 in PBS+2%BSA) (10 minutes). Cells were dehydrated in an ethanol gradient (70%, 85%, 99%, 1 minute each), then mounted in ProLong Gold. Cover slips were cured at room temperature overnight, then stored at 4°C until imaged.

6.2.9 Click chemistry

Growing 5-EU labelled IAV stocks

5-EU labelled IAV A/Puerto Rico/8/1934(H1N1) (PR8) stocks were prepared as for normal viral stocks (described earlier) in T25 flasks, however following inoculation were incubated in 5 mL serum-free media containing 1 µg/mL TPCK trypsin and 0.5 mM 5-EU. 24hpi, additional 5-EU was spiked into the serum-free media (25 µL, 100mM). Stocks were harvested at 48hpi.

Click-labelling virus directly on glass coverslips

16mm diameter glass coverslips were placed in a 12-well plate, sterilised in 100% EtOH and washed three times in PBS. Virus stocks (PR8^{5-EU} or HSV^{AHA}) were diluted in PBS to approximately 8x10⁵ pfu/mL and 5µL applied directly to the glass coverslip. Virus was left to adsorb to the glass surface for 15 minutes, then fixed in 4% PFA for 15 minutes. Cover slips were washed in PBS, quenched in 100 mM glycine in PBS (5 minutes), washed in PBS, then permeabilised in PBS with 0.5% Triton-X 100 (Sigma-Aldrich) (15 minutes). Cover slips were washed three times in PBS and incubated in freshly prepared click labelling solution in a humidified chamber in the dark (two hours, room temp.). To prepare click labelling solution, reagents were added in the described order, then mixed at high speed: ddH₂O, 1x PBS, 10 µM azide-fluor 488 (Sigma-Aldrich), 1 mM CuSO₄ (Sigma-Aldrich), 10 mM Sodium Ascorbate, 10 mM aminoguanidine (Sigma-Aldrich), 1mM THPTA (Sigma-Aldrich). Cover slips were washed three times and incubated in primary antibodies (see Table 6 for dilutions) in PBS with 2% BSA plus 0.1% tween, in a humidified chamber in the dark (one hour, room temperature). Cover slips were then washed three times and incubated in secondary antibodies (see Table 7 for dilutions) and DAPI (1:1000), in

PBS with 2% BSA in a humidified chamber in the dark (one hour, room temperature). Cover slips were washed three times in PBS, then mounted onto microscope slides in ProLong Gold. ProLong Gold was allowed to set at room temperature overnight, and slides stored at 4°C until imaging. Images were collected using a Zeiss Axiovert 135 TV. All image processing was handled in FIJI (Schindelin et al., 2012; Schneider et al., 2012).

Click chemistry pulldown followed by M gene analysis

Viral RNA was extracted from 140 µL 5-EU treated and untreated control PR8 viral stocks using the QIAamp Viral RNA mini kit (Qiagen) as per the manufacturer's instructions, excluding carrier RNA and eluted into 30 µL RNase-free sterile water (Qiagen). 100 ng RNA was clicked to azide-biotin and precipitated using streptavidin beads using the click-iT nascent RNA capture kit, according to the manufacturer's instructions (Thermo Fisher). Viral RNA pre- and post- pulldown in either 5-EU treated or untreated stocks was quantified for viral M gene using the WHO Real-time RT-PCR protocol for the detection of Avian Influenza A(H7N9) virus. For quantification, a standard curve from 10⁸ to 10⁰ copies of plasmid DNA containing the M gene vRNA fragment/ 10µL qPCR reaction was included in each RT-qPCR. Primers used are included in Table 15:

Primer	Sequence
IAV M gene F	GACCRATCCTGTACCTCTGAC
IAV M gene R	AGGGCATTYTGACAAKCGTCTA
IAV M gene probe	FAM-TGCAGTCCTCGCTCACTGGGCACG-BHQ1

Table 15: Primers used for M gene quantification

6.2.10 Western blot analysis

To confirm equivalent protein expression during replication/cRNP stabilisation assays, cells transfected in parallel were lysed with homemade RIPA buffer. In brief, cells were trypsinised, centrifuged (500rcf, 5 minutes) and supernatant removed. Cell pellet was resuspended in 150 µL ice cold RIPA buffer and incubated on ice for 30 minutes. Lysates were subsequently clarified through centrifugation at max. speed, for 30 minutes at 4°C. 50 µL Laemmli buffer (BioRad) supplemented with 10% β-mercaptoethanol (Sigma-Aldrich) was added to each lysate. Lysates were stored at -20°C until further processing.

For Western blot analysis, lysates were boiled at 80°C for 10 minutes. Samples were subsequently resolved, alongside a Novex sharp prestained protein standard (Thermo), by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Mini Protean TGX 4-20% gradient pre-cast gels (BioRad) with TGS running buffer, run for 20 minutes at 100 V followed by ~30 minutes at 150-200 V. Subsequently the gel was equilibrated in TGM transfer buffer for 20 minutes then transferred to nitrocellulose membrane (Amersham Protran Premium 0.2 µm NC, GE healthcare) by semi-dry transfer at 10 V for 30 minutes using a BioRad Trans-Blot SD semidry transfer cell (BioRad). Once transferred,

nitrocellulose membranes were placed into falcon tubes and blocked with 5mL TBS wash buffer +5% milk powder (Sigma-Aldrich), rolling at room temperature for one hour. Membranes were briefly washed in TBS, then probed with the appropriate primary antibodies diluted in 5 mL TBS-T+2% milk powder (see Table 6 for dilutions), rolling at 4°C overnight. Membranes were then washed three times in TBS-T (10 minutes, room temperature). Membranes were subsequently probed with secondary near-infrared antibodies diluted in 5 mL TBS-T+2% milk powder (see Table 7 for dilutions), rolling at room temperature for 45 minutes. Membranes were subsequently washed three times in TBS-T (10 minutes, room temperature), then a final wash in TBS (10 minutes, room temperature), before visualisation by fluorescence using a Licor Odyssey Imager.

6.2.11 Statistical analysis

All statistical analysis was performed using Prism 9 software (GraphPad). Statistical tests used are specified in figure legends.

6.2.13 Safety/Biosecurity

All work with influenza virus was performed at Biosafety level 2 facilities, as approved by the Health and Safety Executive of the United Kingdom and in accordance with local rules at St Mary's Hospital, Imperial College London.

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APPENDIX A

	btANP32A	dgANP32A	hrANP32A	huANP32A	msANP32A	pgANP32A	seANP32A
btANP32A		97.6%	98.4%	95.2%	89.2%	97.2%	97.6%
dgANP32A	97.6%		99.2%	95.2%	90.0%	97.6%	99.6%
hrANP32A	98.4%	99.2%		96.0%	89.6%	98.0%	99.2%
huANP32A	95.2%	95.2%	96.0%		88.8%	95.6%	95.2%
msANP32A	89.2%	90.0%	89.6%	88.8%		89.6%	90.0%
pgANP32A	97.2%	97.6%	98.0%	95.6%	89.6%		97.6%
seANP32A	97.6%	99.6%	99.2%	95.2%	90.0%	97.6%	

Sequence alignment was performed using Geneious 11.1.5 (<https://www.geneious.com>) and visualised using Jalview (Waterhouse et al., 2009). Colour coding of amino acids is according to the Clustal X colour system of amino acid properties. batANP32A= bat ANP32A; dgANP32A= dog ANP32A; hrANP32A= horse ANP32A; huANP32A= human ANP32A; msANP32A= mouse ANP32A; pgANP32A= pig ANP32A; seANP32A= seal ANP32A. Sequences used were common vampire bat (*Desmodus rotundus*) ANP32A (GenPept. Accession no. XP_024423449.1), dog (*Canis lupis familiaris*) ANP32A (GenPept. Accession no. NP_001003013.2), horse (*Equus caballus*) ANP32A (GenPept. Accession no. XP_001495860.2), human (*Homo sapiens*) ANP32A (GenPept. Accession no. NP_006296.1), mouse (*Mus musculus*) (GenPept. Accession no. NP_033802.2), pig (*Sus scrofa*) ANP32A (GenPept. Accession no. XP_003121807.3), and monk seal (*Neomonachus schauinslandi*) ANP32A (GenPept. Accession no. XP_021549451.1). Species and isoforms were chosen based on those reported in previous publications (Long et al., 2016; Peacock, Swann, et al., 2020; Staller et al., 2019).

Sequence alignment of ANP32B proteins from various species. The alignment shows conserved regions across the sequences, with color coding for amino acid properties. The sequences are aligned in blocks of 70 residues, with some gaps indicated by dashes.

btANP32B/1-259 1 MDMKRR I H L E L R N R T P A A V R E L V L D N C K S N D G K I E G L T A E F V N L E F L S L I N V G L I S V S N L P K L P K L K K L E 70
dgANP32B/1-249 1 MDMDKR I H L E L R N R T P S D V K E L V L D N C R S I E G K I E G L T D E F E E L E F L S T I N V G L T S V A N L P K L N K L K K L E 70
hrANP32B/1-259 1 MDMKRR I H L E L R N R T P A A V R E L V L D N C K S N D G K I E G L T A E F V N L E F L S L I N V G L I S V S N L P K L P K L K K L E 70
huANP32B/1-251 1 MDMKRR I H L E L R N R T P A A V R E L V L D N C K S N D G K I E G L T A E F V N L E F L S L I N V G L I S V S N L P K L P K L K K L E 70
msANP32B/1-272 1 MDMKRR I H L E L R N R T P A A V R E L V L D N C K A M D G K I E G L T D E F V N L E F L S L I S V G L F S V S D L P K L P K L K K L E 70
pgANP32B/1-261 1 MDMKRR I H L E L R N R T P A A V R E L V L D N C K S S D G K I E G L T A E F V N L E F L S L I N V G L I S V S N L P K L P K L K K L E 70
seANP32B/1-259 1 MDMKRR I H L E L R N R T P A A V R E L V L D N C K S N D G K I E G L T A E F V N L E F L S L I N V G L I S V S N L P K L P K L K K L E 70

btANP32B/1-259 71 L S D N R I C G S L D M L A E K L P N L T H L N L S G N K L K D I S T L E P L K K L E C L K S L D L F N C E V T N L N D Y R D S V F K L L P 140
dgANP32B/1-249 71 L S D N R I S G G L E V L A E K C P N L T H L N L S G N K I K D L S T I E P L K K L E N L K S L D L F N C E V T N L N D Y R E N V F K L L P 140
hrANP32B/1-259 71 L S D N R I F G G L D M L A E K L P N L T H L N L S G N K L K D I S T L E P L K K L D C L K S L D L F N C E V T N L N D Y R E S V F K L L P 140
huANP32B/1-251 71 L S E N R I F G G L D M L A E K L P N L T H L N L S G N K L K D I S T L E P L K K L E C L K S L D L F N C E V T N L N D Y R E S V F K L L P 140
msANP32B/1-272 71 L S E N R I F G G L D R L A E E L P S L T H L N L S G N N L K D I S T L E P L K R L D C L K S L D L F G C E V T N R S D Y R E T V F R L L P 140
pgANP32B/1-261 71 L S D N R I Y G G L D M L A E K L P N L T H L N L S G N K L K D I S T L E P L K K L E C L K S L D L F N C E V T N L N D Y R E S V F K L L P 140
seANP32B/1-259 71 L S D N R I F G G L D M L A E K L P N L T H L N L S G N K L K D I S T L E P L K K L E Y L K S L D L F N C E V T N L N D Y R E S V F K L L P 140

btANP32B/1-259 141 Q L T Y L D G Y D R E D R E A - - - - - P D S D A E - - V D G V D E E E D E E E G E D E E D E E D G E E E F D D E E D 195
dgANP32B/1-249 141 Q L T Y L D G Y D R D D K E A - - - - - P D S D A E G Y V E G L D D D E E D E D - E E E Y D E D A V V E D E E D E E E E 196
hrANP32B/1-259 141 Q L T Y L D G Y D R E D Q E A - - - - - P D S D A E - - V D G V D E E E D E E E G E D E E D E E D G E E E F D D E E D 195
huANP32B/1-251 141 Q L T Y L D G Y D R E D Q E A P - - - - - D S D A E - - V D G V D E E E D E E E G E D E E D E E D G E E E F D E E D D 195
msANP32B/1-272 141 Q L S Y L D G Y D R E D Q E A P D S D V E V D S V E E A P D S D G E - - V D G V D K E E E E D E E G E D E E E E D G E E E F D E E D 208
pgANP32B/1-261 141 Q L S Y L D G Y D R D D G E A - - - - - P D S D A E - - V D G V D E E E D E E E G E D E K E E E E D G E E E F D D E E D 195
seANP32B/1-259 141 Q L T Y L D G Y D R E D R E A P - - - - - D S D A E - - V D G V D E E E D E E E G E D E E D E E D G E E E F D D E E D 195

btANP32B/1-259 196 E D E D E D V E G E E D E D V S G E E E E F G H D G E V D E D E D - - D E D E D E D E E E E E S G K G E K R K R E T D D E G E D D - 259
dgANP32B/1-249 197 E G E E D V S G E E E E D E - - - - - E G Y N D G E V D - - - - - D E E D E E D V G E E E - - R G Q K R K R E P E D E G E D D D 249
hrANP32B/1-259 196 E D E D E D G E E D E D V S G E E E E F G H D G E V D D D E D - - D E D E D E E E E E S G K G E K R K R E T D D E G E D D - 259
huANP32B/1-251 196 - - E D E D V E G E D D D E V S E E E E F G L D E E - - - - - D E D E D E D E E E E E G K G E K R K R E T D D E G E D D - 251
msANP32B/1-272 209 E D E D E D V E G E E D E D V S G E E E E F G H D G E V D E D E E - - D E D E D E D E E E E E S G K G E K R K R E T D D E G E D D - 272
pgANP32B/1-261 196 E D E D E D V E G E E D E D V S G E E E E F G H D G E V D E E E D E D E D E D E E E E E E K G E K R K R E T D D E G E D D - 261

	btANP32B	dgANP32B	hrANP32B	huANP32B	msANP32B	pgANP32B	seANP32B
btANP32B		69.8%	96.9%	90.0%	83.5%	93.5%	96.9%
dgANP32B	69.8%		70.2%	69.8%	63.1%	70.7%	71.0%
hrANP32B	96.9%	70.2%		91.2%	84.9%	92.7%	96.9%
huANP32B	90.0%	69.8%	91.2%		80.1%	87.4%	92.3%
msANP32B	83.5%	63.1%	84.9%	80.1%		82.1%	84.9%
pgANP32B	93.5%	70.7%	92.7%	87.4%	82.1%		93.5%
seANP32B	96.9%	71.0%	96.9%	92.3%	84.9%	93.5%	

Sequence alignment and shared sequence identity for mammalian ANP32B proteins.

Sequence alignment was performed using Geneious 11.1.5 (<https://www.geneious.com>) and visualised using Jalview (Waterhouse et al., 2009). Colour coding of amino acids is according to the Clustal X colour system of amino acid properties. btANP32B= bat ANP32B; dgANP32B= dog ANP32B; hrANP32B= horse ANP32B; huANP32B= human ANP32B; msANP32B= mouse ANP32B; pgANP32B= pig ANP32B; seANP32B= seal ANP32B. Sequences used were common vampire bat (*Desmodus rotundus*) ANP32B (GenPept. Accession no. XP_024415874.1), dingo (*Canis lupis dingo*) ANP32B (GenPept. Accession no. XP_025328134.1), horse (*Equus caballus*) ANP32B (GenPept. Accession no. XP_023485491.1), human (*Homo sapiens*) ANP32B (GenPept. Accession no. NP_006392.1), mouse (*Mus musculus*) ANP32B (GenPept. Accession no. NP_570959.1), pig (*Sus scrofa*) ANP32B (GenPept. Accession no. XP_020922136.1), and monk seal (*Neomonachus schauinslandi*) ANP32B (GenPept. Accession no. XP_XP_024415874.1). Species and isoforms were chosen based on those reported in previous publications (Long et al., 2016; Peacock, Swann, et al., 2020; Staller et al., 2019).

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btANP32E/1-265 1 MEMKKRIINLELRNRAP E E V T Q L V L D N C H C V N G E I E G L N D T F K K L E F L S M V N V E L S S L A R L P N L S K L R K K L E 70
dgANP32E/1-264 1 MEMKKRIINLELRNRAP E E V T L V L D N C L C V N G E I E G L N D T F K K L E F L S M A N V E L S S L A R L P S L N K L R K K L E 70
hrANP32E/1-265 1 MEMKKRIINLELRNRAP E E V T L V L D N C L C V N G E I E G L N D A F K K L E F L S M A N V E L S S L A R L P S L N K L R K K L E 70
huANP32E/1-268 1 MEMKKRIINLELRNR S P E E V T L V L D N C L C V N G E I E G L N D T F K K L E F L S M A N V E L S S L A R L P S L N K L R K K L E 70
msANP32E/1-260 1 MEMKKRIINMELKNRAP E E V T L V L D N C L C V N G E I E G L N D T F K K L E F L S M A N V E L S S L A R L P S L N K L R K K L E 70
pgANP32E/1-264 1 MEMKKRIINLELRDRAP E E V T L V L D N C L C V N G E I E G L N D T F K K L E F L S M A N V E L S S L A R L P S L N K L R K K L E 70
seANP32E/1-265 1 MEMKKRIINLELRNRAP E E V T L V L D N C L C V N G E I E G L N D T F K K L E F L S M A N V E L S S L A R L P S L N K L R K K L E 70

btANP32E/1-265 71 L S N N R I S G G L E V L A E K C P N L T Y L N L S G N K I K D I S A L E A L Q N L K N L K S L D L F N C E I T T L E D Y R E N V F E M L Q 140
dgANP32E/1-264 71 L S D N I I S G G L E V L A E K C P N L T Y L N L S G N K I K D L S T V E A L Q N L K N L K S L D L F N C E I T N L E D Y R E S I F E L L Q 140
hrANP32E/1-265 71 L S D N I I S G G L E V L A E K C P N L T Y L N L S G N K I K D L S T V A L Q N L K N L K S L D L F N C E I T N L E D Y R E S I F E L L Q 140
huANP32E/1-268 71 L S D N I I S G G L E V L A E K C P N L T Y L N L S G N K I K D L S T V E A L Q N L K N L K S L D L F N C E I T N L E D Y R E S I F E L L Q 140
msANP32E/1-260 71 L S D N I I S G G L E V L A E K C P N L T Y L N L S G N K I K D L S T V E A L Q N L K N L K S L D L F N C E I T N L E D Y R E S I F E L L Q 140
pgANP32E/1-264 71 L S D N I I S G G L E V L A E K C P N L T Y L N L S G N K I K D L S T V E A L Q N L K N L K S L D L F N C E I T N L E D Y R E S I F E L L Q 140
seANP32E/1-265 71 L S D N I I S G G L E V L A E K C P N L T Y L N L S G N K I K D L S T V E A L Q N L K N L K S L D L F N C E I T N L E D Y R E S I F E L L Q 140

btANP32E/1-265 141 Q I T Y L D G F D Q E D N E A P D S G A E - - D D E D G D E D D E - - E E D E A G P T E G Y E E E E E E E E E - - - - D E D E D E A G S E 201
dgANP32E/1-264 141 Q I T Y L D G F D Q E D N E A P D S E E E - - D D E D G D E D D E - E E E D E A G P P E E Y E E E E E E E E E - - - D E D E D E D E A G S E 204
hrANP32E/1-265 141 Q I T Y L D G F D Q E D N E A P D S E E E - - D D E D G D E D D E E E E D E A G P P E G Y E E E E E E E E E - - - D E D E D E D E A G S E 205
huANP32E/1-268 141 Q I T Y L D G F D Q E D N E A P D S E E E - - D D E D G D E D D E E E E E A G P P E G Y E E E E E E E E E E D E D E D E D E A G S E 208
msANP32E/1-260 141 Q I T Y L D G F D Q E D N E A P D S E E E D D D D E D G D E D E D E D E D E A G P P E G Y E E E E - - - - - - - - D D E D E A G S E 200
pgANP32E/1-264 141 Q I T Y L D G F D Q E D N E A P D S E E E - - D D E D G D E D D E E E E D E A G P P E G Y E E E E E E E E E - - - D D E D E D E A G S E 206
seANP32E/1-265 141 Q I T Y L D G F D Q E D N E A P D S E E E - - D D E D G D E D D E E E E E D E A G P P E E Y E E E E E E E E E - - - D E D E D E D E A G S E 205

btANP32E/1-265 202 L G E G E E E V G L S Y L M K E E I Q D E E D D D D Y V E E G E E E E E E E E E E E G L R G E K R K R D A E D D G E E E D D 265
dgANP32E/1-264 205 L G E G E E E V G L S Y L M K E E I Q D E E D D D D Y V E E G E E - - - E E E E E E E G L R G E K R K R D A E D D G E E E D D 264
hrANP32E/1-265 206 L G E G E E E V G L S Y L M K E E I Q D E E D D D D Y V E E G E E - - - E E E E E E E G L R G E K R K R D A E D D G E E E D D 265
huANP32E/1-268 209 L G E G E E E V G L S Y L M K E E I Q D E E D D D D Y V E E G E E - - - E E E E E E E G L R G E K R K R D A E D D G E E E D D 268
msANP32E/1-260 201 V G E G E E E V G L S Y L M K D E I Q D E E D D D D Y V D E G E E - - - E E E E E E E G L R G E K R K R D A E D D G E E E D D 260
pgANP32E/1-264 207 L G E G E E E V G L S Y L M K E E I Q D E E D D D D Y V E E E - - - - E E E E E E E G L R G E K R K R D A E D D G E E E D D 264

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	btANP32E	dgANP32E	hrANP32E	huANP32E	msANP32E	pgANP32E	seANP32E
btANP32E		89.9%	90.0%	87.5%	84.4%	87.8%	89.6%
dgANP32E	89.9%		98.5%	95.9%	91.4%	95.9%	99.6%
hrANP32E	90.0%	98.5%		96.3%	92.1%	96.6%	98.5%
huANP32E	87.5%	95.9%	96.3%		90.7%	94.4%	96.3%
msANP32E	84.4%	91.4%	92.1%	90.7%		90.3%	91.4%
pgANP32E	87.8%	95.9%	96.6%	94.4%	90.3%		95.9%
seANP32E	89.6%	99.6%	98.5%	96.3%	91.4%	95.9%	

Sequence alignment and shared sequence identity for mammalian ANP32E proteins.

Sequence alignment was performed using Geneious 11.1.5 (<https://www.geneious.com>) and visualised using Jalview (Waterhouse et al., 2009). Colour coding of amino acids is according to the Clustal X colour system of amino acid properties. btANP32E= bat ANP32E; dgANP32E= dog ANP32E; hrANP32E= horse ANP32E; huANP32E= human ANP32E; msANP32E= mouse ANP32E; pgANP32E= pig ANP32E; seANP32E= seal ANP32E. Sequences used were common vampire bat (*Desmodus rotundus*) ANP32E (GenPept. Accession no. XP_024425904.2), dog (*Canis lupis familiaris*) ANP32E (GenPept. Accession no. XP_003639669.1), horse (*Equus caballus*) ANP32E (GenPept. Accession no. XP_001917270.1), human (*Homo sapiens*) ANP32E (GenPept. Accession no. NP_112182.1), mouse (*Mus musculus*) ANP32E (GenPept. Accession no. NP_075699.3), pig (*Sus scrofa*) ANP32E (GenPept. Accession no. XP_020945578.1), and monk seal (*Neomonachus schauinslandi*) ANP32E (GenPept. Accession no. XP_XP_021543732.1). Species and isoforms were chosen based on those reported in previous publications (Z. Zhang et al., 2020), apart from bat and seal, which were chosen based on shared sequence identity with human ANP32E.

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chANP32A/1-281 1 ---MDMKKRIHLELRNRTPSDVKELVLDNCRSYEGKIEGLTDFEELFELSTINVGLASVANLPKLNKLLKLE 70
dkANP32A/1-281 1 ---MDMKKRIHLELRNRTPSDVKELVLDNCRSYEGKIEGLTDFEELFELSTINVGLTSVANLPKLNKLLKLE 70
osANP32A/1-251 1MEFTSLSSQIPDTWRPVDGEVVKELVLDNCRSCGKIEGLTDFEELFELSTINVGLASVANLPKLNKLLKLE 73
zfANP32A/1-282 1 ---MEMKRRIHLELRNRTPSDVKELVLDNCRSYEGKIVGLTDFEELFELSTINVGLTSVANLPKLNKLLKLE 70

chANP32A/1-281 71 LSDNRVSGGLEVLAEKCPNLTHLNLSGNKIKDLGTIEPLKKLENLKS LDFNCEVTNLNDYRENVFKLLPQLT 143
dkANP32A/1-281 71 LSDNRISGGLEVLAEKCPNLTHLNLSGNKIKDLGTIEPLKKLENLKS LDFNCEVTNLNDYRENVFKLLPQLT 143
osANP32A/1-251 74 LSDNRISGGLEVLAEKCPNLTHLNLSGNKIKDLGTIEPLKKLENLKS LDFNCEVTNLNDYRENVFKLLPQLT 146
zfANP32A/1-282 71 LGDNRISSGGLEVLAEKCPNLTHLNLSGNKIKDLGTIEPLKKLENLKS LDFNCEVTNLNDYRENVFKLLPQLT 143

chANP32A/1-281 144 YLDGYDRDDKEAPDSDAEGYVEGLDDEEDEDVLSLVKDRDDKEAPDSDAEGYVEGLDDEEDEDDEEYDDDA 216
dkANP32A/1-281 144 YLDGYDRDDKEAPDSDAEGYVEGLDDEEDEDVLSLVKDRDDKEAPDSDAEGYVEGLDDEEDEDDEEYDDDA 216
osANP32A/1-251 147 YLDGY-----DRDDKEAPDSDAEGYVEGLDDEEDEDDEEYDDDA 186
zfANP32A/1-282 144 YLDGYDRDDKEAPDSDAEGYVEGLDDEEDEDVLSLVKDRDDKEAPDSDAEGYVEGLDDEEDEDDEEYDDDA 216

chANP32A/1-281 217 QVVEDEEDEFEEEEEEDVSGEEEEEDEEGYNDGVDDEDEEDEFDEERGGKKRREPDEGEDDD 281
dkANP32A/1-281 217 QVVEDEEDEFEEEEEEDVSGEEEEEDEEGYNDGVDDEDEEDEFDEERGGKKRREPDEGEDDD 281
osANP32A/1-251 187 QVVEDEEDEFEEEEEEDVSGEEEEEDEEGYNDGVDDEDEEDEFDEERGGKKRREPDEGEDDD 251
zfANP32A/1-282 217 QVVEDEEDEFEEEEEEDVSGEEEEEDEEGYNDGVDDEDEEDEFDEERGGKKRREPDEGEDDD 282

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	chANP32A	dkANP32A	osANP32A	zfANP32A
chANP32A		98.9%	80.8%	95.0%
dkANP32A	98.9%		81.1%	96.1%
osANP32A	80.8%	81.1%		78.7%
zfANP32A	95.0%	96.1%	78.7%	

Sequence alignment and shared sequence identity for avian ANP32A proteins.

Sequence alignment was performed using Geneious 11.1.5 (<https://www.geneious.com>) and visualised using Jalview (Waterhouse et al., 2009). Colour coding of amino acids is according to the Clustal X colour system of amino acid properties. chANP32A= chicken ANP32A; dkANP32A= duck ANP32A; osANP32A= ostrich ANP32A; zfANP32A= zebra finch ANP32A. Sequences used were full-length (unspliced) chicken (*Gallus gallus*) ANP32A (GenPept. Accession no. XP_413932.3), full-length (unspliced) duck (*Anas platyrhynchos*) ANP32A (GenPept. Accession no. XP_038040783.1), ostrich (*Struthio camelus australis*) ANP32A (GenPept. Accession no. XP_009665579.1, and zebra finch (*Taeniopygia guttata*) ANP32A (GenPept. Accession no. XP_012424064.1. Sequences were chosen based on those reported in previous publications (Long et al., 2016).

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chANP32B/1-262 1 MEMKKRLTLELRNKKPGVKKELVLDNCRSDDGKIVGLSSDFENLEFLSMINVNLLSISNLPKLNKLRKLE 70
dkANP32B/1-262 1 MEMKKRLTLELRNKKPGVKKELVLDNCRSDDGKIVGLSSDFENLEFLSMININLLSVSNLPKLNKLRKLE 70
zfANP32B/1-262 1 MEMEKRLTLELRNKKPSVKKELVLDNCRTEGKVVGLSSDFENLEFLSMININLLSVSNLPKLNKLRKLE 70

chANP32B/1-262 71 LSDNRISGGLEVLAERTPNLTHLNLSGNKIKDINTLEPLKKLPNLHSLDLFNC EVTMLINYRESVFTLLP 140
dkANP32B/1-262 71 LSDNRISGGLEVLAERTPNLTHLNLSGNKIKDINTLEPLKKLPNLHSLDLFNC EVTMLINYRESVFTLLP 140
zfANP32B/1-262 71 LSDNRISGGLEVLAERTPNLTHLNLSGNKIKDINTLEPLKKLPNLHSLDLFNC EVTMLINYRESVFTLLP 140

chANP32B/1-262 141 QLTLYLDGFDADDEQEA PDS DPEADGDGLEDEYENGEGEEEEDDDEEDDLDEEVIDEEDDEDDDLGEEEEED 210
dkANP32B/1-262 141 QLTLYLDGFDADDQEA PDS DPEADGDGLEDEYENGEGEEEEDDDEEDDLDEEVIDEEDDEDDDLGEEEEED 210
zfANP32B/1-262 141 QLTLYLDGFDADDEQEA PDS DPEADGDGLEDEYENGEGEEEEDDDEEDDLDEEVIDEEDDEDDDLGEEEEED 210

chANP32B/1-262 211 GVDDEEEDEEEEDGEEDEEDDEADDDLP RGEKRRKNLEDEGEEDPEDEEDDEDD 262
dkANP32B/1-262 211 GVDDEEEDEEEEDGEEDEEDDEADDDLP RGEKRRKNLEDEGEEDPEDEEDDEDD 262
zfANP32B/1-262 211 GVDDEEEDEEEEDGEEDEEDDEADDDLP RGEKRRKNLEDEGEEDPEDEEDDEDD 262

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	chANP32B	dkANP32B	zfANP32B
chANP32B		98.1%	90.8%
dkANP32B	98.1%		90.5%
zfANP32B	90.8%	90.5%	

Sequence alignment and shared sequence identity for avian ANP32B proteins.

Sequence alignment was performed using Geneious 11.1.5 (<https://www.geneious.com>) and visualised using Jalview (Waterhouse et al., 2009). Colour coding of amino acids is according to the Clustal X colour system of amino acid properties. chANP32B= chicken ANP32B; dkANP32B= duck ANP32B; zfANP32B= zebra finch ANP32B. Sequences used were chicken (*Gallus gallus*) ANP32B (GenPept. Accession no. NP_001026105.1), duck (*Anas platyrhynchos*) ANP32B (GenPept. Accession no. XP_027327839.1) and zebra finch (*Taeniopygia guttata*) ANP32B (GenPept. Accession no. XP_030112761.1). chANP32B was chosen based on the isoform reported in previous publications (H. Zhang et al., 2019). Duck and zebra finch isoforms were chosen based on shared sequence identity with chANP32B. Ostrich ANP32B was excluded as there is no sequence available on NCBI.

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chANP32E/1-256 1 MEMKKRINLELRNQAPEEVTLELVLDNCKSSNGEIEGLNDSFKLEFLSMANVQLTSLAKLPTLSKLRKLE 70
dkANP32E/1-258 1 MEMKKRINLELRNQAPEEVTLELVLDNCRSSNGEIEGLNDSFKLEFLSMANVQLTSLAKLPTLSKLRKLE 70
osANP32E/1-209 1 -----MANVLTSLAKLPTLSKLRKLE 22
zfANP32E/1-254 1 MEMKKRINLELRNQAPEEVTLELVLDNCRSSNGEIEGLNDSFKLEQLFLSMANVLTSLAKLPTLSKLRKLE 70

chANP32E/1-256 71 LSDNIIISGGLVLAERCPNLTLYLNLGKNIKDLGTVEALQNLKNLKSLLDFNCEITNLEDYRDSIFDLLQ 140
dkANP32E/1-258 71 LSDNIIISGGLVLAERCPNLTLYLNLGKNIKDLGTVEALQNLKNLKSLLDFSCEITNLEDYRDSIFDLLQ 140
osANP32E/1-209 23 LSDNIIISGGLVLAERCPNLTLYLNLGKNIKDLGTVEALQNLKNLKSLLDFNCEITNLEDYRDSIFELLQ 92
zfANP32E/1-254 71 LSDNVIISGGLVLAERCPNLTLYLNLGKNIKDLGTVEALQNLKNLKSLLDFNCEITNLEDYRDSIFELLQ 140

chANP32E/1-256 141 QITYLDGFDDQEDNEAPDSED-DDDEGGDEDDNDEDEDEAGPPGEYEEDEDDDDGGSDLGEG-EEEEEVGLS 208
dkANP32E/1-258 141 QITYLDGFDDQEDNEAPDSEDDDEEGGDEDDNDEDEDEAGPPGEYEEDEDDDDGGSDLGEG-EEEEEVGLS 210
osANP32E/1-209 93 QITYLDGFDDQEDNEAPDSEDDDEEGGDEDDNDEDEDEAGPPGEYEEDEDDDDGGSDLGEG-EEEEEVGLS 161
zfANP32E/1-254 141 QITYLDGFDDQEDNEAPDSED-DDDEGGDEDDN- DENEAGPPGEYEEDEDDDDGASDLGEG-EEEEEVGLS 207

chANP32E/1-256 209 YLMKEEIQDEDDDDDDYVEEGGDEEEAEGRGEEKRRDPDEEGEEEDD 256
dkANP32E/1-258 211 YLMKEEIQDEDDDDDDYVEEGGDEEEAEGRGEEKRRDPDEEGEEEDD 258
osANP32E/1-209 162 YLMKEEIQDEDDDDDDYVEEGGDEEEAEGRGEEKRRDPDEEGEEEDD 209
zfANP32E/1-254 208 YLMKEEIQDEDDDDDDYV-EGGDEEEAEGVRGEEKRRREPDEEGEEEDD 254

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	chANP32E	dkANP32E	osANP32E	zfANP32E
chANP32E		98.1%	97.1%	93.8%
dkANP32E	98.1%		96.7%	92.6%
osANP32E	97.1%	96.7%		94.7%
zfANP32E	93.8%	92.6%	94.7%	

Sequence alignment and shared sequence identity for avian ANP32E proteins.

Sequence alignment was performed using Geneious 11.1.5 (<https://www.geneious.com>) and visualised using Jalview (Waterhouse et al., 2009). Colour coding of amino acids is according to the Clustal X colour system of amino acid properties. chANP32E= chicken ANP32E; dkANP32E= duck ANP32E; osANP32E= ostrich ANP32E; zfANP32E= zebra finch ANP32E. Sequences used were chicken (*Gallus gallus*) ANP32E (GenPept. Accession no. NP_001006564.2), duck (*Anas platyrhynchos*) ANP32E (GenPept. Accession no. XP_027300275.1) ostrich (*Struthio camelus australis*) ANP32E (GenPept. Accession no. XP_009687883.1) and zebra finch (*Taeniopygia guttata*) ANP32E (GenPept. Accession no. XP_030147225.1). Species and isoforms were chosen based on isoforms reported in previous publications (Z. Zhang et al., 2020), apart from ostrich, for which only one predicted isoform is available on NCBI.

APPENDIX B

Type of work	Legend	Source	Copyright holder	Permission requested	Permission obtained	Licence number
Figure	Figure 1: The complex ecology of influenza A virus (IAV).	Long, Mistry, et al., 2019	© 2018, Springer Nature Limited	03.12.22	Yes	5461301085530
Figure	Figure 2: The unpredictable origins of IAV pandemics.	Long, Mistry, et al., 2019	© 2018, Springer Nature Limited	03.12.22	Yes	5461301085530
Tomogram	Figure 3A: Structure of IAV.	Vijayakrishnan et al., 2013	Licensed with a Creative Commons Attribution 4.0 International (CC BY) license – free to reuse when correctly cited.	Not required	N/A	N/A
Transmission electron micrograph	Figure 3C: Structure of IAV.	Noda et al., 2006	© 2006, Nature Publishing Group	03.12.22	Yes	5461320670346
Figure	Figure 4: Overview of the influenza A virus life cycle.	te Velthuis and Fodor, 2016	© 2006, Nature Publishing Group, a division of Macmillan Publishers Limited.	03.12.22	Yes	5461330366636
Figure	Figure 5: Key FluPol structures.	Pflug et al., 2017	Licensed with a Creative Commons Attribution 4.0 International (CC BY) license – free to reuse when correctly cited.	Not required	N/A	N/A

Figure	Figure 6: The structural basis of transcription.	te Velthuis, Grimes and Fodor., 2021	© 2021, Springer Nature Limited	03.12.22	Yes	5461331481236
Figure	Figure 8: The structural basis of replication.	te Velthuis, Grimes and Fodor., 2021	© 2021, Springer Nature Limited	03.12.22	Yes	5461331481236
Figure	Figure 9: Ultrastructural perturbations visualised in influenza infected cells.	Josset et al., 2008	© 2008, Elsevier B.V.	03.12.22	Yes	5461340124173
Figure	Figure 42	Carrique et al., 2020	© 2020, The Author(s), under exclusive licence to Springer Nature Limited	03.12.22	Yes	5461340518588