

**Study of the interaction between
the influenza A virus polymerase and GTPase Rab11a**



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Abstract

The influenza A virus (IAV) genome that consists of eight viral RNA (vRNA) segments is transcribed and replicated by the viral heterotrimeric RNA-dependent RNA polymerase (RdRP) complex, composed of polymerase acidic (PA), polymerase basic 1 (PB1), and PB2, subunits, in the cell nucleus. Replicated vRNA segments are assembled with RdRP and oligomeric nucleoprotein into viral ribonucleoprotein (vRNP) complexes which are exported from the nucleus and transported across the cytoplasm to be packed into progeny virions. The GTPase Rab11a associated with recycling endosomes is believed to contribute to this process by mediating the cytoplasmic transport of vRNPs. However, how vRNPs interact with Rab11a remains poorly understood. In this D.Phil. project, I have utilised a combination of biochemical and structural approaches to characterise the interaction between the IAV RdRP and Rab11a. Initially, using a GST pull-down assay, I showed that the interaction between the IAV RdRP and Rab11a is direct, and that the 627-domain of PB2 is required for this interaction. Furthermore, my study demonstrated for the first time that Rab11a has a differential specificity for vRNP but not cRNP. Analysis of the Rab11a interactome further revealed that IAV infection reduces Rab11a binding to Rab11-family interacting partners (Rab11-FIPs), thereby altering the stoichiometry of cytoplasmic motor proteins bound by Rab11a. Using mutant Rab11a in a GST pull-down assay, this study provides the first evidence that IAV RdRP binds to the same site on Rab11a as Rab11-FIPs. It was also investigated here whether Rab11a has a role in determining the host specificity of influenza viruses. However, this study could not identify any adaptive mutations in PB2 that may affect the interaction between RdRP and Rab11a. Moreover,

the study also did not identify the exact site of Rab11a binding on the viral RdRP using cryo-electron microscopy. However, performing a small-angle X-ray scattering analysis I showed that C-terminal two thirds of PB2 (PB2-C) and Rab11a form a well-folded protein complex in solution, and confirmed in a controlled pull-down assay that the interaction between PB2-C and Rab11a is direct. Finally, I have also established a workflow to study the interaction between IAV vRNPs and Rab11a in the context of live infected cells using full field cryo-X-ray microscopy. The findings of my D.Phil. work expand our understanding of the cytoplasmic transport of the influenza virus vRNPs. Identifying the exact site of Rab11a binding on the viral RdRP could uncover a novel target site for the development of an influenza antiviral drug.

Declaration

I declare that the contents of this thesis are a product of my own work except for when specifically stated. This work was undertaken between October 2018 and June 2021 under the supervision of Prof Ervin Fodor and Prof Jonathan Grimes at The Sir William Dunn School of Pathology and Diamond Light Source. The length does not exceed the word limit for a D.Phil. thesis submitted to the University of Oxford, Medical Sciences Division.

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Abbreviations

Units

In addition to all SI units of measurement, the following additional units are used:

Å	Ångström (10^{-10} m)
aa	Amino acid
bp	Base pair
Da	Dalton
eV	Electronvolt
<i>g</i>	Standard gravity (9.80665 m s^{-2})
h	Hour
hpi	Hours post infection
hpt	Hours post transfection
kb	Kilobases
L	Protein length
min	Minute
MOI	Multiplicity of infection
nt	Nucleotide
PFU	Plaque-forming unit
pH	$-\log_{10} c$, where c is $[\text{H}^+]$ in mol l^{-1}
ppm	Parts per million
rpm	Revolutions per minute

Other abbreviations

In addition to amino acid one letter abbreviations, the following additional abbreviations are used:

1918	Influenza A/Brevig Mission/1/1918 (H1N1) virus
A _{260/280}	Absorbance at 260 nm and 280 nm
A549	Adenocarcinomic human alveolar basal epithelial cells
AGC	Automatic gain control
Amp ^R	Ampicillin resistance
ANP32	Acidic nuclear phosphoprotein 32
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CaCl ₂	Calcium chloride
Cas	CRISPR-associated protein
CCD	Charge-coupled device
CLEM	Correlative light-electron microscopy
CTD	Carboxyl-terminal domain
CTF	Contrast transfer function
CO ₂	Carbon dioxide
Cpl ^R	Chloramphenicol resistance
CRISPR	Clustered regularly interspaced short palindromic repeats
CRM1	Chromosomal region maintenance 1
cRNA	Complementary ribonucleic acid
cRNP	Complementary ribonucleoprotein
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

dNTP	Deoxynucleoside triphosphate
DPBS	Dulbecco's phosphate-buffered saline
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
ERES	Endoplasmic reticulum exit sites
ERC	Endocytic recycling compartment
FACS	Fluorescent assisted cell sorting
FCS	Fetal bovine serum
FDR	False discovery rate
Fj	Influenza A/duck/Fujian/01/2002 (H5N1) virus
FM	Fluorescence microscopy
GAP	GTPase-activating proteins
GEF	Guanine nucleotide exchange factors
Gen ^R	Gentamicin resistance
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GO	Gene ontology
GTP	Guanosine triphosphate
GTP-γ-S	Guanosine-5'-O-(gamma-thio)triphosphate
GST	Glutathione S-transferase
HA	Hemagglutinin
HCD	Higher-energy collision dissociation
HEK 293T	Human embryonic kidney 293T cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HCl	Hydrochloric acid
HIV-1	Human immunodeficiency virus 1
HRV	Human rhinovirus
IAV	Influenza A virus

IBV	Influenza B virus
ICV	Influenza C virus or irregular coated vesicle
IDV	Influenza D virus
IPTG	Isopropyl β -d-1-thiogalactopyranoside
K ⁺	Potassium ion
LB	Lysogeny broth
LC-MS/MS	Liquid chromatography and tandem mass spectrometry
M1	Matrix protein 1
M2	Matrix protein 2
m ⁷ G	N7-methyl guanosine
MDCK	Madin-Darby canine kidney cells
Mg ²⁺	Magnesium ion
MgCl ₂	Magnesium chloride
mRNA	Messenger ribonucleic acid
MTOC	Microtubule organising centre
NA	Neuraminidase
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NEP	Nuclear export protein
NES	Nuclear export signal
NLS	Nuclear localization signal
NP	Nucleoprotein
NPC	Nuclear pore complex
NS1	Non-structural protein 1
NSAF	Normalized spectral abundance factor
NT60	Influenza A/Northern Territory/60/1968 (H3N2) virus
NTP	Nucleotide triphosphate
OTG	Octylthioglucoside
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1

PB2	Polymerase basic protein 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PEI	Polyethylenimine
PLGEM	Power law global error model
PNK	T4 polynucleotide kinase
PolII	RNA polymerase II
PSM	Peptide-to-spectrum matches
Rab	Ras-related in brain protein
Rab11-FIP	Ras-related in brain protein 11 family interacting protein
Ran	Ras-related nuclear protein
RBD	Rab11 binding domain
RE	Recycling endosome
RdRP	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RNaseA	Ribonuclease A
RNP	Ribonucleoprotein
rRNA	Ribosomal ribonucleic acid
RT	Room temperature (22°C)
SAF	Spectral abundance factor
SAXS	Small-angle X-ray scattering
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
Sf9	<i>Spodoptera frugiperda</i> clone 9
SIM	Structured illumination microscopy
siRNA	Small interfering ribonucleic acid
STN	Signal-to-noise ratio

SXT	Soft X-ray tomography
TBE	Tris-borate-ethylenediaminetetraacetic acid buffer
TEAB	Triethylammonium bicarbonate buffer
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
TEV	Tobacco etch virus
TCEP	Tris(2 carboxyethyl)phosphine buffer
TXM	Transmission X-ray microscope
U2OS	Human bone osteosarcoma epithelial cells
Viet	Influenza A/Viet Nam/1203/2004 (H5N1) virus
vRNA	Viral ribonucleic acid
vRNP	Viral ribonucleoprotein
WCL	Whole cell lysate
WSN	Influenza A/WSN/1933 (H1N1) virus

1 Introduction

1.1 General introduction

Influenza virus infections are serious threats to human health, causing contagious respiratory diseases. Transmission of influenza virus occurs either by a direct inhalation of infectious droplets or aerosols or by indirect contact followed by self-inoculation of the upper respiratory tract or conjunctival mucosa (Kuiken and Taubenberger, 2008). Influenza infection can elicit injury in both the proximal (conducting) airways and distal airways (alveoli and parenchyma) where gas exchange occurs (Bruder *et al.*, 2006). Most human influenza infections are confined to the proximal airways; these are uncomplicated and result in a transient trachea-bronchitis with signs both local (nasal obstruction, cough, sore throat) and systemic (fever, headache, chills, anorexia, myalgia) symptoms (Bruder *et al.*, 2006; Kuiken and Taubenberger, 2008). However, in a small number of cases, most often in young children, the elderly, and the immunocompromised, the infection can extend to the lower respiratory tract. There, often in the presence of a secondary bacterial infection, the infection can cause a fulminant pneumonia, the most common complication of influenza virus infection (Kim *et al.*, 2002). A combination of the direct cytolytic effect caused by influenza virus infection, and the indirect effect of host response results in damage to the alveolar epithelium, which allows fluid from the alveolar capillaries to flood into the alveolar lumina, causing severe, and in some cases fatal, respiratory dysfunction (Kuiken and Taubenberger, 2008).

Influenza viruses of genera A and B are responsible for annual epidemics of seasonal influenza that affect 10-30% of the global population and kill 290,000-650,000 people each

year (Krischuns *et al.*, 2021). In addition to seasonal epidemics caused by antigenic drift events, transmission of influenza virus strains from non-human species to humans can lead to occasional pandemics, which can have devastating consequences in terms of mortality and economic costs. Four major influenza pandemics have been recognised for which viral genome sequence data are available (Sorrell *et al.*, 2011). The 1918 “Spanish” influenza pandemic, the deadliest single event in recorded human history, killed an estimated 50 million people worldwide (Taubenberger *et al.*, 2013). Reconstruction of the viral genome from tissues of victims has showed that the pandemic was not caused by a reassortant virus but rather an entirely avian-derived H1N1 virus that adapted to humans (Taubenberger *et al.*, 2005). Remarkably, all three pandemic viruses since 1918 contain gene segments derived from the 1918 virus. Thus, the past 103 years can be considered as a single pandemic era with the founding 1918 pandemic virus leading to emergence of pandemic progeny viruses in 1957, 1968, and 2009 (Taubenberger *et al.*, 2013). In recent decades, pandemic influenza has continued to produce numerous unanticipated events that expose fundamental gaps in scientific knowledge. In the world that we live in, with ever increasing globalisation, rapid population growth, and an increase in travel demand, future influenza pandemics are inevitable. Currently, the only clinically relevant anti-influenza drugs are neuraminidase inhibitors, and we urgently need new drugs, preferably effective against all influenza subtypes (Pflug *et al.*, 2017). Understanding the molecular mechanisms underlying the influenza virus life cycle is, therefore, crucial to the development of next-generation anti-influenza drugs and to subsequently heightening our pandemic preparedness.

1.2 Influenza virus taxonomy and classification

Influenza viruses are members of *Orthomyxoviridae*, a family of negative-sense segmented RNA viruses, which replicate in the nucleus. *Orthomyxoviridae* is divided into seven genera: *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Influenzavirus D*, *Thogotovirus*, *Isavirus*, and *Quaranjavirus*. The four *Influenzavirus* genera differ in host range and pathogenicity, *Influenzavirus A* and *Influenzavirus B* being the only genera of medical importance in humans. Influenza B viruses (IBVs) and influenza C viruses (ICVs) are both isolated almost exclusively from humans, although IBVs have been also isolated from seals and ferrets while ICVs have been also isolated from swine and dogs (Taubenberger *et al.*, 2008). Influenza D viruses (IDVs), which share approximately 50% homology with ICVs, have even narrower range of hosts, as they have been only isolated from swine and cattle (Asha and Kumar, 2019). Influenza A viruses (IAVs), however, have a much broader host range. Namely, they can infect a wide variety of warm-blooded animals, such as humans, swine, horses, bats, seals, whales, poultry, and even aquatic birds (Taubenberger *et al.*, 2008). IAVs are further divided into subtypes by antigenic characterisation of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), the two major viral antigens. Studies on the ecology of IAVs established that aquatic birds serve as the natural reservoir for all known subtypes of IAV, and, moreover, that aquatic birds are the primordial source of all influenza viruses in other species (Wallensten, 2007). Currently, there are 19 HA (H1-H19) and 11 NA (N1-N11) subtypes described.

1.3 The influenza A virus structure

Influenza A virus (IAV) virions are enveloped pleomorphic particles. While the filamentous IAV morphology is typical for clinical isolates of influenza virus with their length as long as several micrometres and diameter of 55 nm, laboratory-passaged influenza strains are spherical varying in shape from true spheres to oblong or even bacillary forms with a diameter ranging from 80 nm to 120 nm (Calder *et al.*, 2010; Harris *et al.*, 2006). Despite IAV pleiomorphy, most virions have three features in common (Harris *et al.*, 2006): 1) the virus-encoded matrix 2 (M2) protein, and glycoproteins HA and NA are embedded in the outer host-derived lipid bilayer membrane, 2) the envelope is supported underneath by the matrix 1 (M1) protein, and 3) the IAV genome consists of eight unique negative-sense segments of single-stranded viral RNA (vRNA) ranging in size from ~0.9 to 2.3 kb with a total genome size of approximately 13.5 kb (Lee *et al.*, 2017). Each vRNA segment is associated with oligomeric nucleoprotein (NP) and contains 13 and 12 conserved nucleotides at the 5' and 3' termini, respectively, which form a 'panhandle' structure that associates with a single heterotrimeric RNA-dependent RNA polymerase (RdRP), composed of polymerase acidic (PA), polymerase basic 1 (PB1) and PB2 subunits (te Velthuis and Fodor, 2016). The vRNA-NP-RdRP complex is referred to as a viral ribonucleoprotein (vRNP) complex, which functions in IAV gene expression and genome replication. In addition to these structural proteins, non-structural protein 1 (NS1) and nuclear export protein (NEP) have been found at low levels within IAV virions (Hutchinson *et al.*, 2014). In addition to these ten major proteins (PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, NEP) IAV encodes accessory proteins, which are not incorporated into virions, and their involvement in the IAV pathogenesis is not fully understood (Vasin *et al.*, 2014). A summary of the IAV genome

segments and their known encoded proteins in given in **Table 1.1**. In addition to viral proteins, over 300 host-encoded proteins were also found to be incorporated in IAV virions (Hutchinson *et al.*, 2014). A schematic diagram and electron micrographs of the IAV virions are presented in **Figure 1.1**.

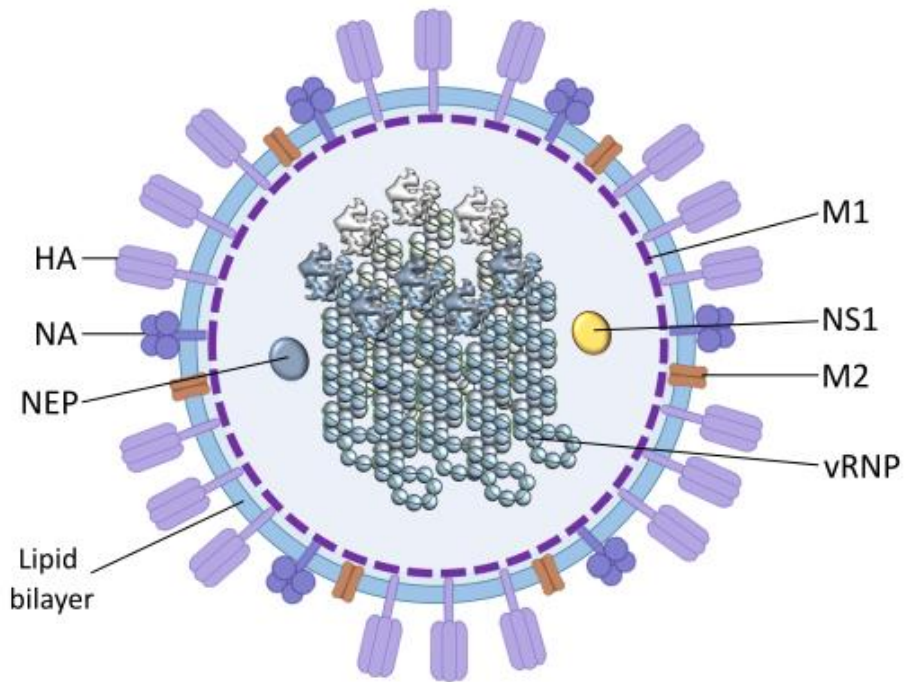
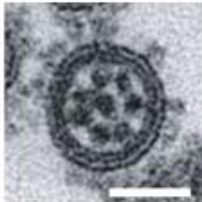
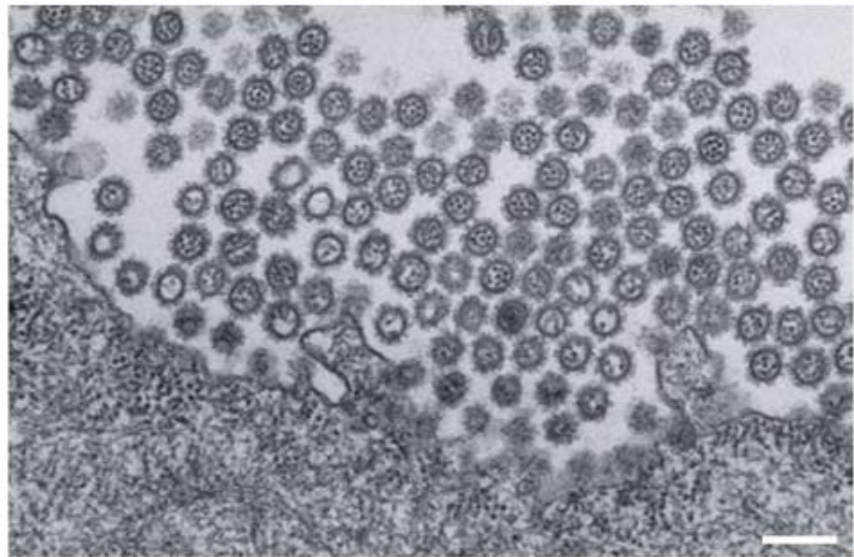
A**B****C****D**

Figure 1.1: The influenza A virion.

(A) Schematic of the influenza A virus virion showing all structural proteins and vRNP complexes. Negative stain electron micrographs of influenza A/WSN/1933 (H1N1) virions in **(B)** vertical and **(C)** horizontal planes and of **(D)** budding virions from Madin-Darby Canine (MDCK) cells. Adapted from Noda *et al.* (2006). Scale bar for **B** and **C** = 50 nm. Scale bar for **D** = 200 nm. Parts of the figure were made using BioRender.

Table 1.1: Influenza A virus genome segments and their encoded proteins.

Genome segment	Length (nt)	Protein	Length (aa)	Reference to the initial publication
1	2341	PB2	759	Fields and Winter, 1982
		PB2-S1	508	Yamayoshi <i>et al.</i> , 2016
2	2341	PB1	757	Winter and Fields, 1982
		PB1-N40	718	Wise <i>et al.</i> , 2009
		PB1-F2	87	Chen <i>et al.</i> , 2001
3	2233	PA	716	Fields and Winter, 1982
		PA-X	252	Wise <i>et al.</i> , 2012
		PA-N155	562	Muramoto <i>et al.</i> , 2013
		PA-N128	535	Muramoto <i>et al.</i> , 2013
4	1778	HA	566	Winter <i>et al.</i> , 1981
5	1565	NP	498	Winter and Fields, 1981
6	1413	NA	454	Fields <i>et al.</i> , 1981
7	1027	M1	252	Winter and Fields, 1980
		M2	97	Winter and Fields, 1980
		M3	9	Lamb <i>et al.</i> , 1981
		M4	54	Shih <i>et al.</i> , 1998
		M42	99	Wise <i>et al.</i> , 2012
8	890	NS1	230	Winter <i>et al.</i> , 1981
		NEP	121	Winter <i>et al.</i> , 1981
		NS3	188	Selman <i>et al.</i> , 2012
		NEG8	216	Clifford <i>et al.</i> , 2009

1.4 The influenza A virus life cycle

IAVs commonly infect and replicate in alveolar type I and type II epithelial cells throughout the respiratory tree, resulting in mostly only acute symptoms due to their relatively fast life cycle (Morens *et al.*, 2008). During their life cycle, IAVs interact with multiple host factors and cell pathways, which can regulate pathogenesis, immune response and host range restriction, making both viral and host factors important determinants of the extent and severity of the infection (Flerlage *et al.*, 2021). A schematic diagram of the IAV life cycle is presented in **Figure 1.2**.

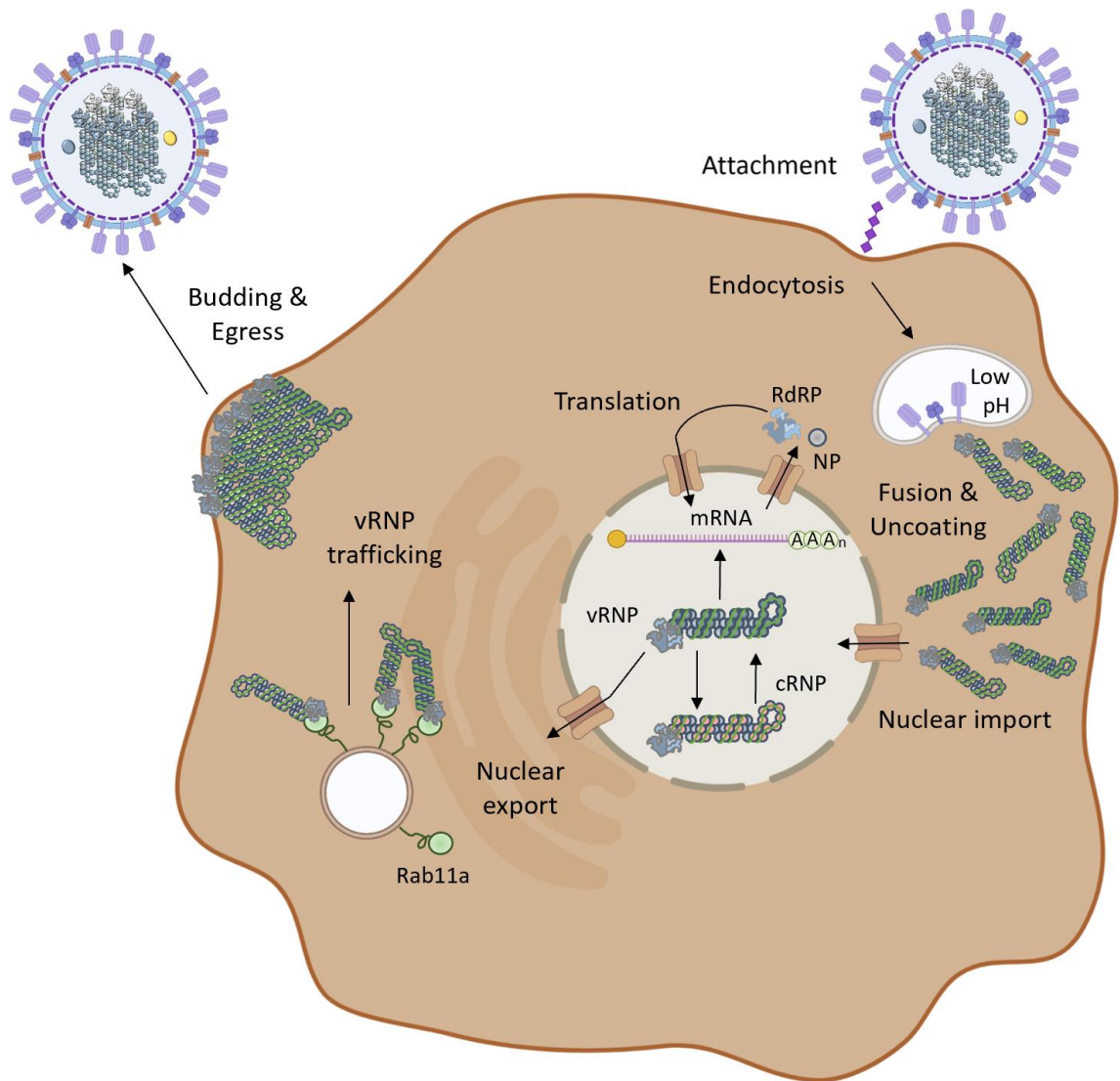


Figure 1.2: Life cycle of the influenza A virus.

Influenza A virus infection begins with attachment of the viral HA glycoprotein to host cellular receptor, either α -2,3-linked or α -2,6-linked sialic acid, followed by its entry into the host cell by receptor-mediated endocytosis. After acidification of the endosome, the eight vRNP complexes are released into the cytoplasm and subsequently trafficked into the nucleus of the host cell. In the nucleus, RNA-dependent RNA polymerase (RdRP) replicates the single-stranded negative-sense viral RNA (vRNA) genome segments by copying them into complementary RNAs (cRNAs), which are positive-sense RNAs that form complementary RNPs (cRNPs) and act as templates to produce vRNAs. The RdRP also transcribes vRNA segments into mRNAs, which are 5' capped and 3' polyadenylated. Viral

mRNA is exported into the cytoplasm for translation by cellular mechanism. Newly synthesized viral RdRP subunits (polymerase basic 1 (PB1), PB2 and polymerase acidic (PA)) and nucleoprotein (NP) are imported into the nucleus and bind to vRNA genomic segments and cRNAs to assemble vRNPs and cRNPs, respectively. Newly synthesised vRNPs are exported from the nucleus and transported across the cytoplasm on recycling endosomes in a Rab11a-dependent manner to the cell membrane, where the assembly of progeny virions takes place. Progeny virions are then released by budding. For simplicity only the translation and trafficking of RdRP and NP is shown. Parts of the figure were made using BioRender.

1.4.1 Attachment and entry

Attachment of IAV is initiated upon binding of the viral glycoprotein HA to α 2,3- and α 2,6-linked sialic acid attached to a penultimate galactose of the glycan receptors on the host cell membrane. It is believed that human-adapted HA subtypes primarily bind to α -2,6-linked sialic acids that are mostly found on the upper respiratory epithelial cells of mammals, while the avian-adapted ones prefer to bind to α -2,3-linked sialic acids which are predominantly found on epithelial cells of the bird gut, thereby making the sialic acid linkage a key determinant for host tropism (Dou *et al.*, 2018; Stencel-Baerenwald *et al.*, 2014). Interestingly, it has been shown that it is possible for epithelial cells of pig trachea to produce both α -2,3- and α -2,6-linked sialic acids (Ito *et al.*, 1998). Consequently, pigs can be infected with an avian and mammalian IAV strain simultaneously, therefore, becoming a “mixing vessel” for genetic reassortment and the emergence of new influenza viruses (Ito *et al.*, 1998). Successful binding of the HA glycoprotein to the sialic acid receptor triggers receptor-mediated endocytosis of the IAV virion into the host cell. The internalisation is highly cell-type dependent and mainly occurs in a clathrin-dependent manner, which involves dynamin and the adaptor protein Epsin-1 (Chen and Zhuang, 2008; Roy *et al.*, 2000). However, the IAV can also be internalised by utilising either clathrin- and non-clathrin entry routes in parallel or by macropinocytosis (Rust *et al.*, 2004; Sieczkarski and Whittaker, 2002; de Vries *et al.*, 2011).

1.4.2 Fusion and nuclear import

Once inside the cell, the IAV exploits the transport system via distinct endosomal stages and concurrently occurring pH changes to release vRNPs into the host cell cytoplasm. Irrespective of the internalisation pathway, the IAV virion is trafficked to the endosome, its fusion site. For fusion to occur, HA precursor (HA0) must be first cleaved by host proteases into a receptor binding (HA1) and a fusion (HA2) subunit (White and Whittaker, 2016). Lower pH within the maturing endosome then triggers irreversible structural conformational changes in the viral HA glycoprotein, which exposes its fusion peptide in HA2. Insertion of fusion peptide into the endosomal membrane results in fusion between the endosomal membrane and the viral membrane (Bullough *et al.*, 1994). Simultaneously, lower pH opens the M2 ion channel, which subsequently exposes the viral capsid to elevated levels of protons and K⁺ ions, thereby acidifying the virion and loosening the interactions between M1 and vRNPs (Stauffer *et al.*, 2014). This enables the transfer of vRNPs to the host cytoplasm following HA-mediated fusion. Upon entering the cytoplasm, M1 associates with the nuclear import factor transportin 1, which facilitates removal of vRNP-associated M1 and a subsequent dissociation of the vRNPs from each other (Miyake *et al.*, 2019). M1 removal exposes nuclear localisation signal (NLS) on the NP, thereby enabling it to bind to importin- α , which in turn binds to importin- β (Hutchinson and Fodor, 2013). This allows the vRNP complex to dock at the nuclear pore complex (NPC) and to translocate into the nucleus of the cell.

1.4.3 Viral transcription and replication

Upon successful entry of vRNPs into the host cell nucleus, the RdRP performs distinct processes of transcription of vRNA genome segments and replication of vRNA in the context of vRNPs (te Velthuis and Fodor, 2016). During transcription, the RdRP bound to the vRNA termini in the vRNP (the resident RdRP) synthesises positive-sense viral mRNA with a 5' terminal N7-methyl guanosine (m⁷G) cap and 3' poly adenine (poly(A)) tail (te Velthuis and Fodor, 2016; Walker and Fodor, 2019). The 3' poly(A) tail is added by the RdRP upon its reiterative copying of a short uridine (U) track present near the 5' terminus of all vRNA segments (Poon *et al.*, 1999). Unlike some negative-sense single-stranded RNA viruses that can synthesise an m⁷G cap *de novo* using their own viral mRNA capping machinery (Ortín and Martín-Benito, 2015), the influenza virus RdRP cannot synthesise and methylate a cap structure. Instead, it snatches it from nascent host RNA polymerase II (Pol II) transcripts in a process called 'cap-snatching'. During cap-snatching, the carboxyl-terminal domain (CTD) of the large subunit of Pol II interacts with several binding sites on the RdRP (Engelhardt *et al.*, 2005; Lukarska *et al.*, 2017; Serna Martin *et al.*, 2018). CTD Pol II interaction with the RdRP stabilises the RdRP in a transcription pre-initiation conformation state (**transcriptase**), which is compatible with binding to and cleaving host 5' capped RNA (Serna Martin *et al.*, 2018). After cleavage of the 5' capped RNA, the 3' end of the capped primer enters the polymerase active site where it base pairs with the 3' end of the vRNA template, and primer elongation occurs by template-directed nucleotide addition (Reich *et al.*, 2014). During elongation, the sliding nature of the vRNA to move through active site of the RdRP while the RdRP maintains bound to both vRNA termini in a mechanism called 'processive helical track' (Coloma *et al.*, 2020). The RdRP continues

copying the template while inducing the movement of both vRNP strands until it stutters on an oligo U motif near the 5' end to generate a 3' poly(A) tail (Coloma *et al.*, 2020; Poon *et al.*, 1999). Finally, the mRNA is released and the vRNP goes back to its initial state, allowing a new round of transcription to begin (Coloma *et al.*, 2020).

Unlike transcription, replication of the vRNA segments by the RdRP is a two-step process; the vRNA template is first copied into a cRNA, which then acts as template for vRNA synthesis. The replication initiates *de novo* at the 3' terminus of the vRNA template to generate a pppApG dinucleotide following 3' vRNA entry into the RdRP active site in a process called 'terminal initiation' (Fodor and te Velhuis, 2019; te Velhuis *et al.*, 2021). Terminal initiation depends on support by the priming loop, which is extruded from the RdRP active site during steady-state elongation to open the template exit channel (Kouba *et al.*, 2019; te Velhuis *et al.*, 2016). As the nascent cRNA emerges from the RdRP, the cRNA assembles into a complementary RNP (cRNP) structure, a vRNP-like complex that serves as a replicative intermediate. In contrast to terminal initiation on the vRNA template, the vRNA synthesis from an antigenome template initiates internally upon the entry of 3' cRNA terminus into the RdRP active site. As a result of internal initiation, the pppApG dinucleotide must be realigned back to the 3' terminus of the cRNA where it can act as a primer for terminal initiation. The antigenome backtracking is mediated by a conformational change in the priming loop following the binding of a second RdRP (regulatory RdRP) which forms a symmetric dimer with the resident cRNA-associated RdRP (Fan *et al.*, 2019; Oymans and te Velhuis, 2018). In this symmetric dimer, the resident cRNA-associated RdRP acts as a replicating polymerase (**replicase**) while the other acts as a regulatory polymerase, which functions in a trans-activating manner. During replication,

the newly synthesised 5' terminus emerges from the product exit channel of the replicase and is encapsidated by another RdRP (**encapsidase**), which forms an asymmetric dimer with the replicase (Carrique *et al.*, 2020). Currently, it remains unclear at which point the symmetric and asymmetric RdRP dimers form and dissociate (te Velthuis *et al.*, 2021). However, it has been shown that the asymmetric dimer is stabilised by a host factor ANP32A which likely acts as a molecular whip to recruit the NP to the body of RNA, thereby initiating the assembly of RNPs (Long *et al.*, 2016; Reilly *et al.*, 2014).

1.4.4 Nuclear export of progeny vRNP

While cRNA-containing RNPs appear to remain in the nucleus (Tchatalbachev *et al.*, 2001), newly synthesised vRNA-containing RNPs are exported from the nucleus via cellular CRM1-dependent nuclear export. Multiple studies have implicated that the viral M1 and NEP proteins are essential for this process (Paterson and Fodor, 2012). While M1 has been shown to associate with vRNP complexes (Bui *et al.*, 2000; Huang *et al.*, 2001), on its own it is insufficient for nuclear export. Current data support a daisy chain model of nuclear vRNP export in which NEP acts as an adaptor protein linking vRNP-M1 complexes and the importin- β protein CRM1 (Neumann *et al.*, 2000; O'Neill *et al.*, 1998). In this model, the C-terminal domain of M1 binds to the vRNP (Baudin *et al.*, 2001) while the N-terminal NLS of the M1 associates with the C-terminal hairpin of NEP (Akarsu *et al.*, 2003; Shimizu *et al.*, 2011). In turn, CRM1 recognises the nuclear export signal (NES) on the N-terminus of NEP following its activation by Ran-GTP in the nucleus, thereby facilitating the nuclear export of vRNP-M1-NEP complex via the NPC (Eisfeld *et al.*, 2015).

In revised CRM1-dependent nuclear export model, NEP interacts with CRM1 as before through its NLS; however, its C-terminus interacts with both M1 and the viral RdRP, thereby establishing a connection between them (Brunotte *et al.*, 2014). This model could explain the involvement of the NEP in a differential nuclear export of vRNPs but not cRNPs. An earlier study has demonstrated that differential nuclear export is a result of differences in the vRNA and cRNA promoter sequences (Tchatalbachev *et al.*, 2001), which could affect the RdRP conformation, therefore allowing for a model where NEP can interact with vRNP-associated but not cRNP-associated RdRP.

Although data in support of the CRM1-dependent nuclear export models described above have been presented, several studies have called into question the role of NEP in this process. Thus, an NEP- and M1-independent nuclear export has been proposed which involves a direct interaction between NP and the CRM1 export pathway (Digard *et al.*, 2001). Additionally, further studies have found that M1 contains an NES which is capable of mediating CRM1-independent nuclear export (Cao *et al.*, 2012). Taken together, these observations suggest some redundancy in nuclear export of the influenza virus progeny vRNPs and imply that multiple mechanisms might operate simultaneously.

1.4.5 vRNP trafficking to the cell membrane

Soon after the nuclear export, newly synthesised vRNPs accumulate around the microtubule organising centre (MTOC), from where they are transported to the plasma membrane on the surface of the endocytic recycling compartment (ERC) in a Rab11a-dependent manner (Amorim *et al.*, 2011; Bruce *et al.*, 2010; de Castro Martin *et al.*, 2017;

Chou *et al.*, 2013; Eisfeld *et al.*, 2011; Momose *et al.*, 2011). This will be described in more detail in section 1.6.2.

1.4.6 Assembly and budding

As the infection progresses, the apical plasma membrane becomes enriched with viral proteins, which together coordinate the budding around the vRNP complexes. The viral transmembrane proteins HA, NA, and M2 are translocated from the sites of protein synthesis at the endoplasmic reticulum via the Golgi apparatus to the apical plasma membrane. The cytoplasmic tails of HA and NA interact with M1, thereby recruiting it to the sites of virion budding where it polymerises to form the interior matrix (Ali *et al.*, 2000; Noton *et al.*, 2007; Zhang *et al.*, 2000). The M1 matrix then acts as a scaffold for the incorporation of the eight vRNP complexes which have been shown to have an organised 7 + 1 arrangement in a budding virion (Noda *et al.*, 2006). This shed light on the existence of segment-specific interactions between the eight vRNPs. In corroboration, several studies demonstrated connections between the vRNPs, suggesting that the eight vRNPs form a supramolecular genomic complex within the virion (Dadonaite *et al.*, 2019; Fournier *et al.*, 2012; Noda and Kawaoka, 2012). Considering these findings, it is crucial to understand the cellular locations and the mechanisms whereby this genome complex is formed. Currently accepted models link genome transport to genome assembly (Amorim, 2019; Dou *et al.*, 2018; Eisfeld *et al.*, 2015; Gerber *et al.*, 2014; Hutchinson and Fodor, 2013; Lakdawala *et al.*, 2016; Pohl *et al.*, 2016). The first model (dispersed model) predicts that cytosolic Rab11a-vesicles carrying the vRNPs collide transiently, allowing the establishment of RNA-

RNA interactions (Amorim *et al.*, 2011; Avilov *et al.*, 2012a; Chou *et al.*, 2013; Han *et al.*, 2021; Lakdawala *et al.*, 2014). Unlike the dispersed model, another model (compartmentalised model) considers that vRNP binding to Rab11a vesicles impairs and remodels the Rab11a cycle, thereby creating designated sites for genome assembly (Alenquer *et al.*, 2019; de Castro Martin *et al.*, 2017; Kawaguchi *et al.*, 2015; Vale-Costa and Amorim, 2016). As both models are coupled with IAV genome transport and involve cellular GTPase Rab11a, this will be described in more detail in section 1.6.3.

1.5 The influenza virus transcription and replication machinery

Influenza viruses must express a full complement of their genome segments to be productively infectious. Upon successful uncoating, influenza virus vRNPs are released into the cytoplasm and transported into the nucleus of the infected cell where viral transcription takes place (Walker and Fodor, 2019). The RdRP of influenza virus transcribes the vRNA into positive-stranded capped and polyadenylated mRNA for translation of viral proteins in the context of vRNPs. The expression of viral proteins is crucial for replication of the viral RNA genome by the RdRP which proceeds through a positive-sense cRNA replicative intermediate. Recent functional and structural studies of the influenza virus transcription and replication machinery have provided new insights into the molecular mechanisms of action by which influenza viruses transcribe and replicate their RNA genome (Pflug *et al.*, 2017; te Velhuis and Fodor, 2016).

1.5.1 The influenza virus ribonucleoprotein

vRNP complex is composed of a single-stranded vRNA segment, a copy of the RdRP bound to the partially complementary 5' and 3' vRNA termini, and multiple copies of NP. Within each vRNP complex, NP binds to the internal vRNA stoichiometrically such that there is approximately one NP molecule per 24 nucleotides of vRNA (Ortega *et al.*, 2000); however, it remains unknown how many nucleotides are in direct contact with the NP. The result of NP binding is a formation of a flexible helical filament that is closed by a loop at the opposite pole to where the RdRP binds, thereby creating three regions of the vRNP: polymerase end, the central helical filament, and the loop end. The central helical filament is a dihedral helix with two opposite-polarity NP strands that coil to form minor and major grooves (Arranz *et al.*, 2012; Coloma *et al.*, 2020; Moeller *et al.*, 2012). A schematic representation of the vRNP structure is shown in **Figure 1.3**.

NP is a crescent-shaped molecule that is composed of a head and body domain and a 28 amino acid-long tail loop (Ye *et al.*, 2006). In between the head and body domain is a basic groove which forms the RNA-binding surface. Ribonuclease digestion studies show that the RNA in vRNP is susceptible to hydrolytic cleavage suggesting that vRNA is bound on the outside surface of NP (Kingsbury and Webster, 1969; Pons *et al.*, 1969). During the RNP assembly in the nucleus, NP monomers are added to the growing end of the assembling vRNP complex. In this ordered assembly process, the incoming NP polymerises with a resident RNA-bound NP by inserting its tail loop into the insertion groove of the adjacent NP (Turrell *et al.*, 2013; Ye *et al.*, 2006). Phosphorylation and dephosphorylation of NP by cellular kinases and phosphatases, respectively, affect NP oligomerisation and viral replication (Hutchinson *et al.*, 2012; Mondal *et al.*, 2015; Turrell *et al.*, 2015). Interestingly,

NP does not associate with vRNA evenly as evident from chemical probing of the vRNA in vRNPs which revealed the presence of vRNA secondary structures in vRNPs isolated from the virions (Dadonaite *et al.*, 2019). This suggests that the NP helix in vRNPs provides a scaffold for the formation of secondary RNA structures which can facilitate intersegments RNA-RNA interactions and selective packaging of the vRNPs into virions.

Recently, a series of vRNP structures of a full-length native vRNP isolated from virions was described using cryo-EM (Coloma *et al.*, 2020). In these structures, NP monomers can adopt different relative positions in the strands, as evident from docking the NP atomic structure into the different reconstructed vRNP structures (**Figure 1.4A**). Moreover, the structures show that the central helical filament is highly flexible and can adopt different conformations, implying that there is a sliding movement between two strands of the vRNP. This shed light on a potential role of a sliding movement during transcription in the context of vRNPs, which was demonstrated by *in vitro* transcription assay using purified vRNPs from a recombinant virus and imaging their transcription by EM (Coloma *et al.*, 2020) (**Figure 1.4B**). Imaging vRNPs during transcription revealed that the central helical filament remained intact along the vRNP. Intriguingly, NP terminal loops were observed at both helical ends, suggesting that the RdRP moved to the central helical filament from which a putative mRNA thread emerged (Coloma *et al.*, 2020). Importantly, this observation suggests a potential stabilising role of NP in keeping the vRNP in an accessible conformation while maintaining its double helical structure during transcription. This is in agreement with previous studies which showed that NP is required for transcription and replication of vRNP templates longer or equal to 101 nucleotides (Turrell *et al.*, 2015).

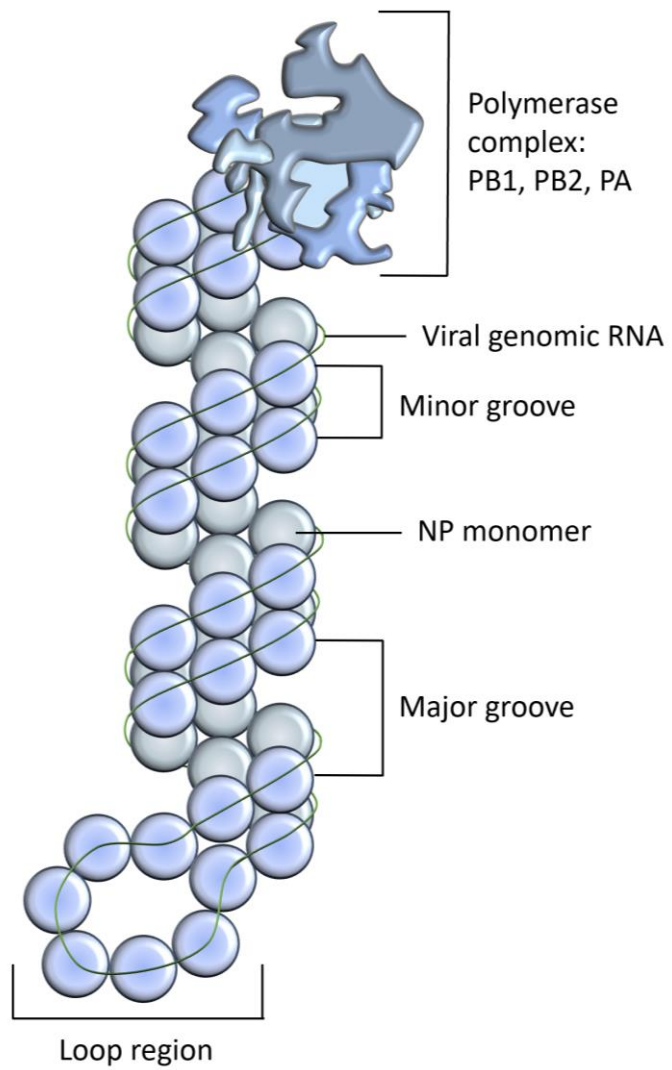


Figure 1.3: Influenza virus ribonucleoprotein complex.

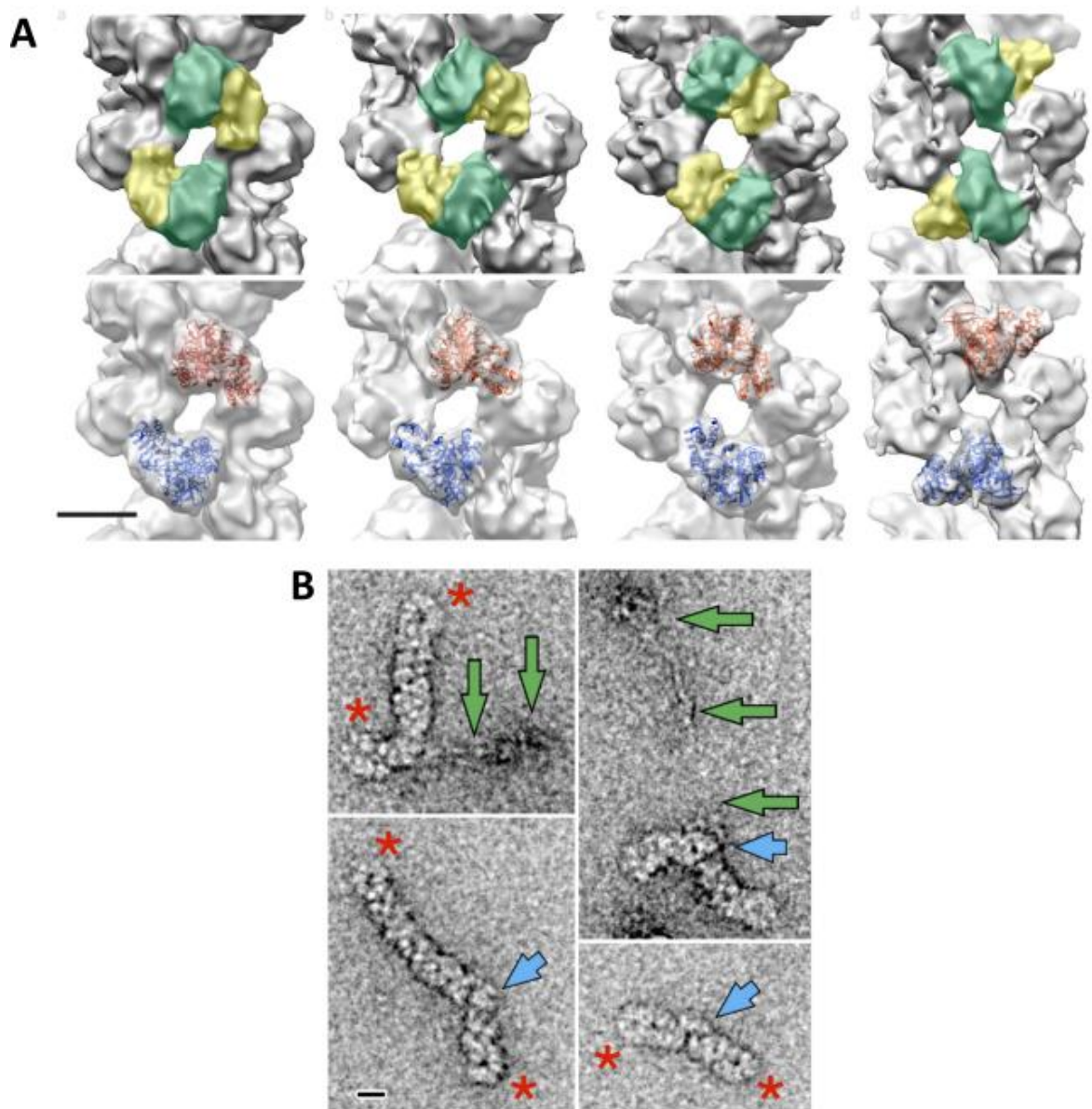


Figure 1.4: Structure of a full-length native vRNP.

(A) 3D reconstructions of different conformations of the central helical filament of the vRNPs with corresponding docking of the NP atomic structure in red and blue (PDB: 2IQH). Adapted from Coloma *et al.* (2020). Scale bar = 50 Å. **(B)** Cryo-EM images of transcribing vRNPs showing a loop at each end of the vRNP (red asterisks), a central helical region (blue arrows), and a putative nascent mRNA thread emerging from the RdRP (green arrows). Adapted from Coloma *et al.* (2020). Scale bar = 150 Å.

1.5.2 The influenza virus RNA-dependent RNA polymerase

The IAV RNA-dependent RNA polymerase (RdRP) is a ~270 kDa heterotrimeric complex composed of three proteins: polymerase acidic (PA or P3 in influenza C and D viruses), polymerase basic 1 (PB1) and PB2 subunit of 713, 756 and 760 residues, respectively (Fodor and te Velthuis, 2019; Pflug *et al.*, 2017) (**Figure 1.5A**). X-ray crystallography and cryo-EM studies showed that the RdRP consists of a core domain (**Figure 1.5B**) and several peripheral domains, which are connected to the core domain through highly flexible linkers (**Figure 1.5C**) (Chang *et al.*, 2015; Fan *et al.*, 2019; Hengrung *et al.*, 2015; Peng *et al.*, 2019; Pflug *et al.*, 2014; Reich *et al.*, 2014; Thierry *et al.*, 2016). The PB1 subunit contributes most to the core domain and contains the RdRP domain with the catalytic residues for RNA synthesis (Fan *et al.*, 2019; Hengrung *et al.*, 2015; Peng *et al.*, 2019; Reich *et al.*, 2014).

The catalytic core of PB1 has the canonical right-hand-like fold and is divided into three subdomains, which are common in all other RdRPs with sequence and/or structural conservations: the fingers (PB1^{fingers}) (including fingertips, PB1^{fingertips}), palm (PB1^{palm}), and thumb (PB1^{thumb}) (Fodor and te Velthuis, 2019; te Velthuis, 2014; te Velthuis *et al.*, 2021). The three subdomains are mainly formed by conserved RdRP motifs, i.e., pre-A motif (motif F) and catalytic motifs A-E, which work together to facilitate viral RNA template binding, nucleotide channelling and binding, and to coordinate two divalent metal ions and to promote catalysis (Kouba *et al.*, 2019; Pflug *et al.*, 2014). Apart from the three conserved subdomains, PB1 has also another feature, which is the priming loop (residues 641-657). The priming loop is an anti-parallel β -hairpin structure that protrudes from the PB1^{thumb} into the polymerase active site, a typical characteristic of the RdRPs that can initiate RNA synthesis *de novo* (Reich *et al.*, 2014). Cartoon models of the IAV RdRP with labelled PB1

subdomains, and PB1 motifs and priming loop are shown in **Figure 1.6A** and **Figure 1.6B**, respectively. The PA subunit is composed of the N-terminal endonuclease domain (PA-N, residues 1-195) and a large PA C-terminal domain (PA-C, residues 258-714), which are connected by a flexible PA-linker (residues 196-257) that consists of three short helices packed on the surface of the PB1 subunit (**Figure 1.6C**). In contrast to PA-N, the PA-C is rather tightly integrated into the RdRP core, specifically PB1^{thumb}. The N-terminal third of PB2 (PB2-N, residues 1-247), which includes the lid domain (PB2^{lid}) and a series of linked modules (PB2^{N-ter}, PB2^{N1}, and PB2^{N2}), is also part of the RdRP core, but the C-terminal two-thirds (PB2-C, residues 248-760) form multiple discrete flexible domains. These flexible regions include the mid domain (PB2^{mid}, residues 247-318), cap-binding domain (PB2^{cap}, residues 319-480), cap-627 linker domain (PB2^{627-linker}, residues 481-537), 627-domain (PB2⁶²⁷, residues 538-679), and the C-terminal nuclear-localisation signal domain (PB2^{NLS}, residues 680-760). The PB2^{mid} and PB2^{627-linker} domains form one unit that is commonly referred to as the mid-link domain (Thierry *et al.*, 2016). Cartoon model of the IAV RdRP with labelled PB2-N and PB2-C domains is shown in **Figure 1.6D**. Overall, the RdRP can be viewed as a complex that is made up of a catalytic core (PB1, PA-C, PB2-N) and flexible peripheral appendices (PA-N and PB2-C). All three RdRP subunits, however, contribute to the formation of the channels that facilitate template RNA entry and exit, nucleotide triphosphate (NTP) entry, and product RNA exit (Fodor and te Velthuis, 2019).

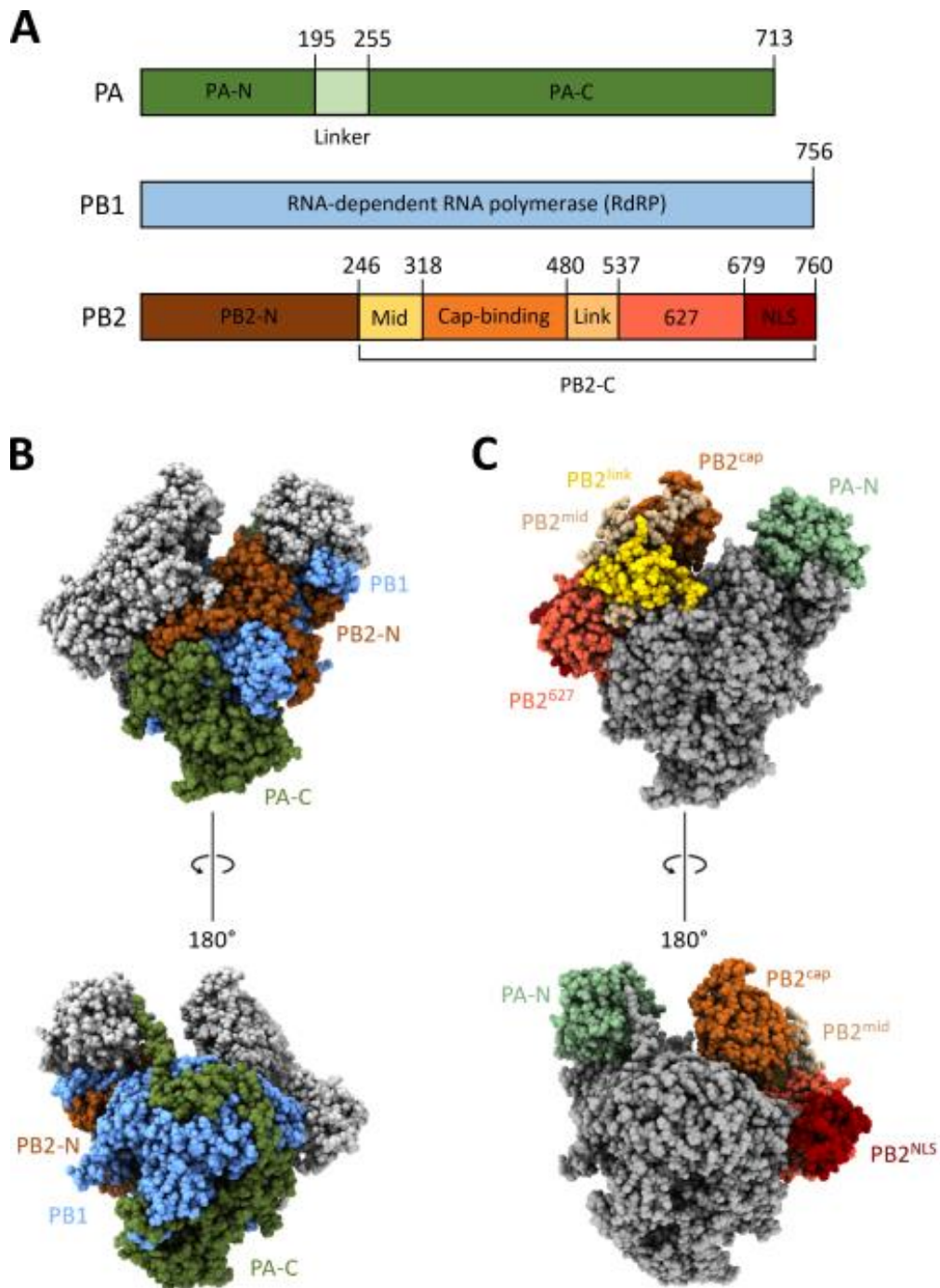


Figure 1.5: Structure of the influenza A virus polymerase.

(A) The position of each domain within the subunit sequence together with the colour code. Structure of the influenza A virus polymerase with coloured **(B)** core globular domain and **(C)** flexible linked peripheral domains (PDB: 6RR7).

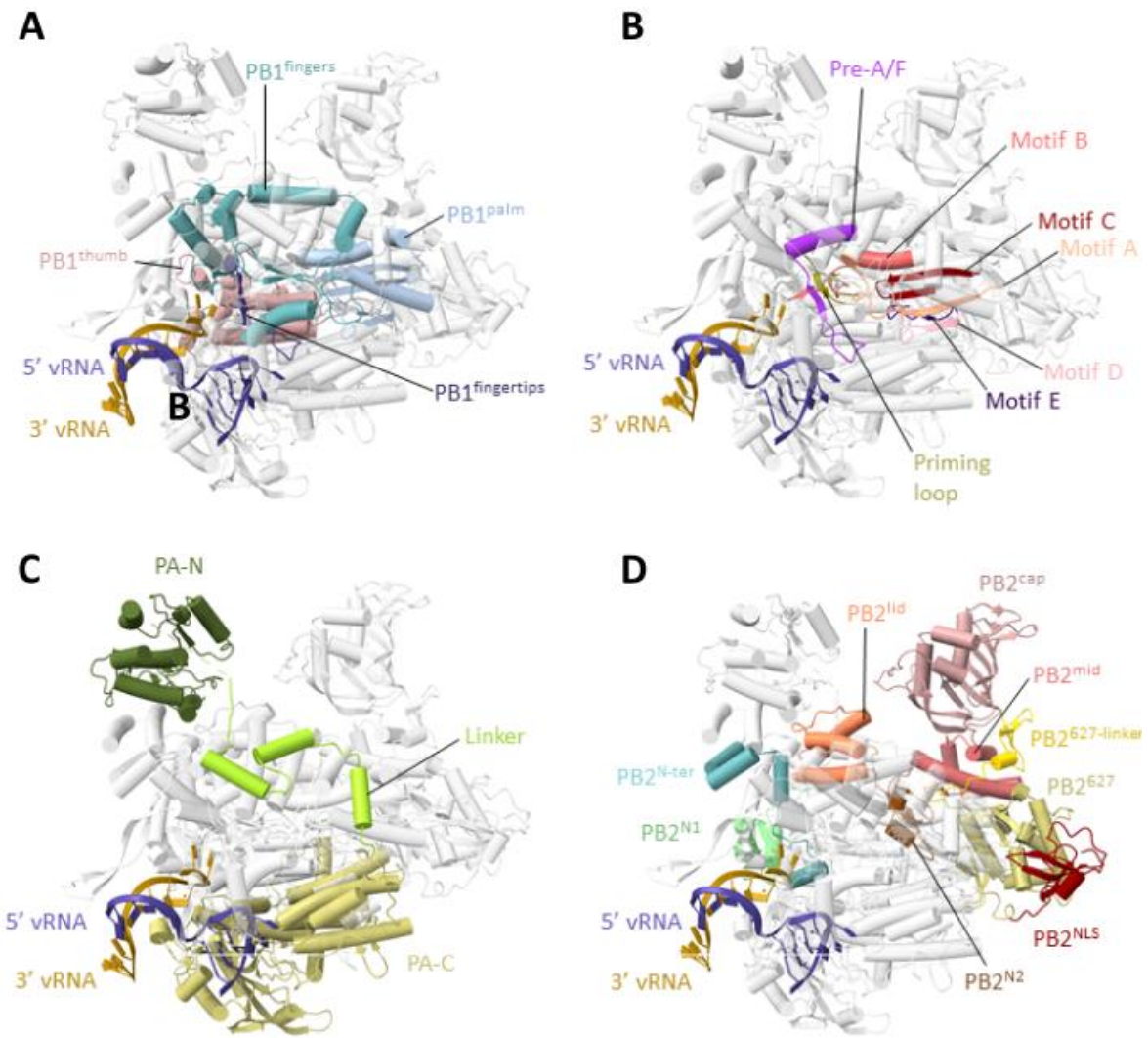


Figure 1.6: Architecture of the influenza A virus polymerase.

Cartoon models of the influenza A virus polymerase bound to a negative-sense viral RNA (vRNA) promoter (PDB: 4WSB) showing the arrangement of the **(A)** PB1 subdomains and the fingertips, **(B)** PB1 pre-A/F motif, motifs A-E, and priming loop, **(C)** PA subunit domains, and **(D)** PB2 subunit domains.

1.5.3 Promoter binding

The RdRP needs to bind the 5' end of the vRNA template to bind to and cleave host capped RNA, and to initiate transcription at the 3' end of the vRNA template (Cianci *et al.*, 1995; Fodor *et al.*, 1994, Fodor *et al.*, 1995; Rao *et al.*, 2003; Tiley *et al.*, 1994). Accordingly, the 5' and 3' termini of the vRNA (vRNA promoter) associate with the RdRP through interactions with highly specific promoter binding sites. Additionally, replication of the vRNA proceeds through a complementary RNA (cRNA) replicative intermediate for which a similar RNA secondary structure to the vRNA promoter was identified (cRNA promoter) (Crow *et al.*, 2004; Pritlove *et al.*, 1995).

The 5' termini of both RNA promoters (nucleotides 1–10) form an intramolecular compact stem-loop structure (the 'hook') and bind near the template entry channel by a pocket formed by the PA and PB1 subunits (Fan *et al.*, 2019; Peng *et al.*, 2019; Pflug *et al.*, 2014; Reich *et al.*, 2014; Robb *et al.*, 2016). Although the cRNA promoter forms a similar 5' hook-like structure and is bound at the same site of the RdRP as the 5' vRNA promoter, its sequence is different from that of the vRNA promoter. In contrast to the 5' vRNA promoter, the 3' vRNA promoter is more flexible and was shown to associate with multiple binding sites depending on the conformation of the RdRP (Robb *et al.*, 2019). Namely, it can either bind in a groove formed near the PB1^{fingers} subdomain (A site) (Reich *et al.*, 2014) or at a site formed by residues of the PB1 and PA subunits (B site) (Peng *et al.*, 2019; Pflug *et al.*, 2014; Wandzik *et al.*, 2020). Interestingly though, the 3' end of the cRNA promoter has been shown to associate with the B site only (Fan *et al.*, 2019). A schematic model of the IAV RdRP with labelled 3' and 5' end binding sites is shown in **Figure 1.7**.

The structures of the RNA-free (apo) influenza A and influenza C virus RdRPs show a highly unstable PB1^{fingers} subdomain and priming loop (Fan *et al.*, 2019; Hengrung *et al.*, 2015) while the same regions are ordered in the influenza A and influenza B RdRPs bound to the 5' vRNA promoter (Pflug *et al.*, 2014; Reich *et al.*, 2014). This suggests that the 5' vRNA promoter facilitates a structural rearrangement of the polymerase active site, thereby stabilising the RdRP catalytic core and acting as a polymerase activator. In contrast, the functional significance of different 3' vRNA binding sites remains undetermined, but they likely correspond to different stages of RNA synthesis, e.g. they may represent a preinitiation site from which the 3' vRNA (A site) and 3' cRNA (B site) enter the RdRP active site (Fodor and te Velhuis, 2019).

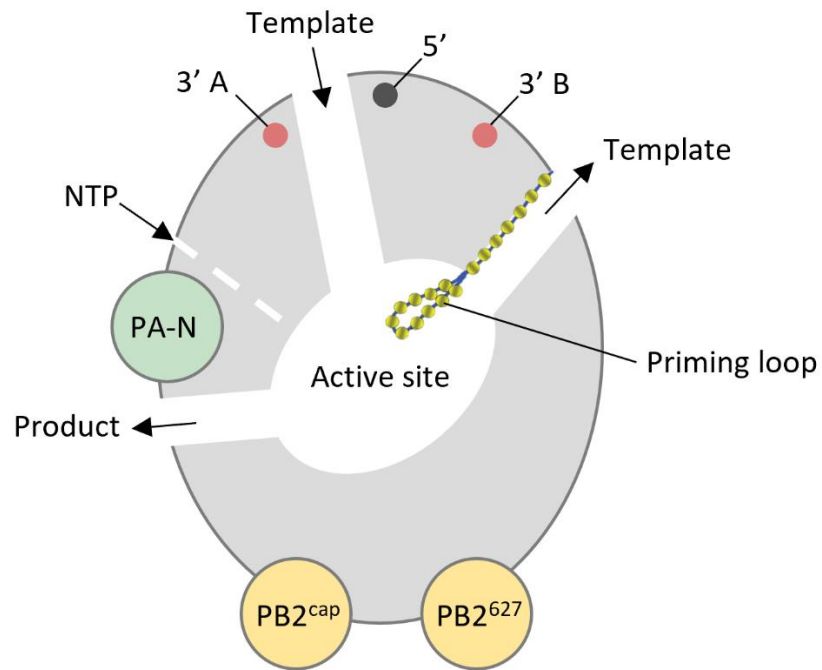


Figure 1.7: A schematic model of the IAV RdRP.

The connection of the catalytic core (active site) to the solvent via template and NTP entry channels, and template and product exit channels are shown. Priming loop as well as the 3' and 5' end binding sites are indicated.

1.5.4 Conformational rearrangement of the viral polymerase

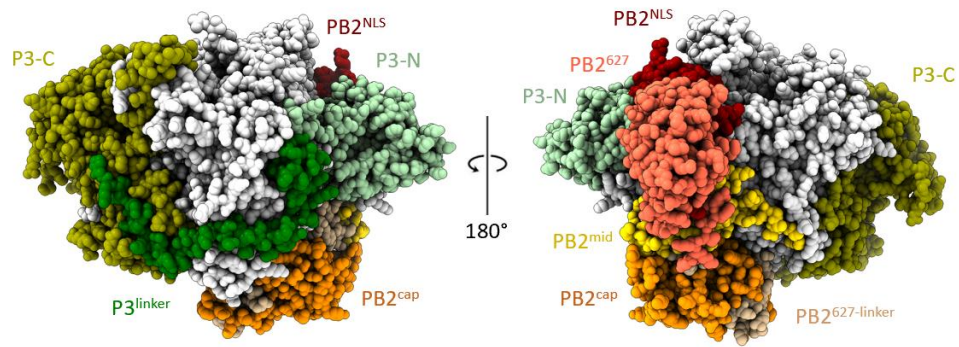
The PA-N and PB2-C domains are linked to the central RdRP catalytic core via flexible linkers and can adopt different positions relative to the RdRP catalytic core. Hence, several radically different conformations of the RdRP have been observed. In the structure of the RdRP apo form, the PB2^{cap} domain packs against the PB1^{palm} subdomain, whereas the PB2⁶²⁷ domain is located near the nascent RNA exit channel and the PA-N domain (Hengrung *et al.*, 2015; Thierry *et al.*, 2016) (**Figure 1.8A**). Moreover, the priming loop in the RdRP catalytic site is disordered (Hengrung *et al.*, 2015). This RdRP conformation is unable to bind to capped RNA due to the insertion of the mid-link domain into the cap-binding pocket of the PB2^{cap} domain, and is incompatible with ‘cap-snatching’ as the PA-N and PB2^{cap} domain are positioned away from each other (te Velthuis and Fodor, 2016; te Velthuis *et al.*, 2021).

In contrast, during different stages of transcription, the mid-link domain repositions the PB2^{cap} domain and the PB2⁶²⁷ domain opposite to the PA-N and next to the interface of the PB1^{palm} and PB1^{thumb} subdomains, respectively (Kouba *et al.*, 2019; Reich *et al.*, 2014; Wandzik *et al.*, 2020) (**Figure 1.8B–C**). Unlike in the RdRP apo form, the priming loop is ordered in the vRNA template-bound ‘pre-initiation’ and ‘post-cap-snatching’ structures captured during transcription (Fan *et al.*, 2019; Reich *et al.*, 2014). However, while the cap-binding pocket is free to bind to capped RNA and faces the PA-N in the ‘pre-initiation’ structure (**Figure 1.8B**), it rather faces the product exit channel in the ‘post-cap-snatching’ structure which also reveals a rotation of the PB2^{cap} domain (**Figure 1.8C**). The rearrangement of the flexible domains in the ‘pre-initiation’ structure is consistent with a RdRP movement that facilitates cap-snatching (**transcriptase**). By contrast the

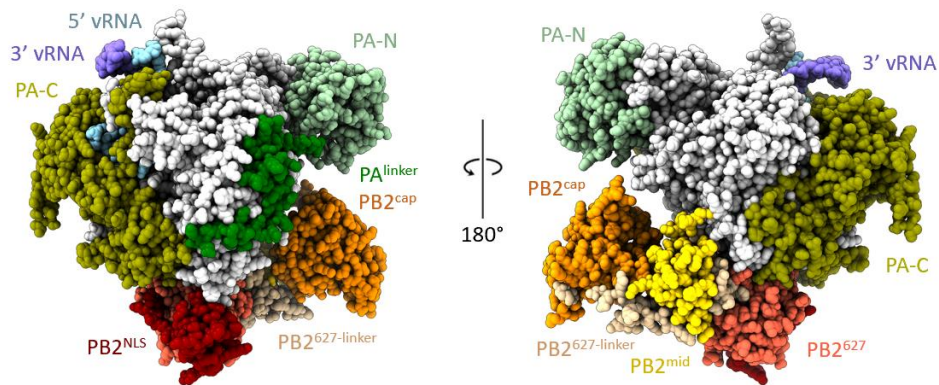
rearrangement of the flexible domains in the ‘post-cap-snatching’ structures suggests how the RdRP facilitates the insertion of the cleaved capped RNA primer into the RdRP catalytic site through the product exit channel (Reich *et al.*, 2014). In addition to these two structures, further conformational rearrangements were captured during transcription, particularly during transition from pre-initiation and initiation to elongation and then to termination (Kouba *et al.*, 2019; Wandzik *et al.*, 2020).

Recently, an entirely different RdRP conformation has been observed in a dimer of the RdRP bound by the host ANP32A protein (Carrique *et al.*, 2020). In this asymmetric dimer, one of the RdRPs is bound to the vRNA and acts as a replicating RdRP (**replicase**) while the other one acts as an encapsidating RdRP (**encapsidase**) that is involved in the assembly of the nascent RNA into an RNP. The arrangement of the PA-N and PB2^{cap} domains of the replicase is like that observed in the RdRP apo form, and the priming loop is also disordered. However, the arrangement of flexible domains of the encapsidase is unique compared not only to the RdRP apo form but also to the ‘pre-initiation’ and ‘post-cap-snatching’ structures of the RdRP. Specifically, the PB2^{cap} and mid-link domains pack against the PB1^{palm} and PB1^{thumb} subdomains and are separated from the PB2⁶²⁷ and PB2^{NLS} domains, which mostly associate with the PA-C domain (Carrique *et al.*, 2020) (**Figure 1.8D**).

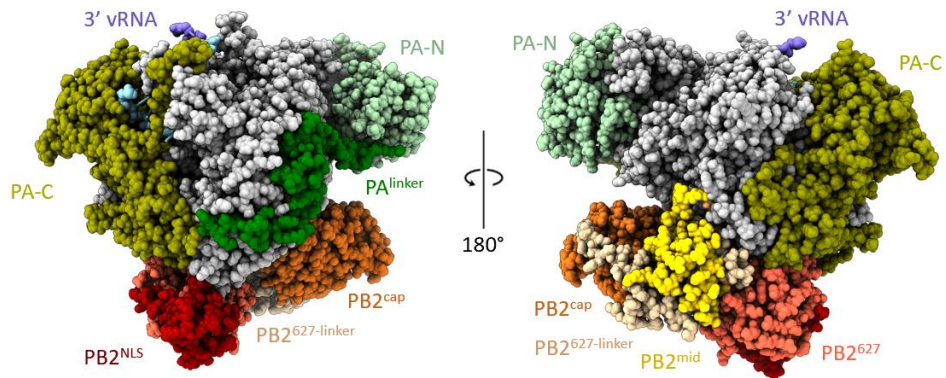
A ICV polymerase – apo



B IAV polymerase – pre-initiation



C IAV polymerase – post-cap-snatching



D ICV polymerase – encapsidating

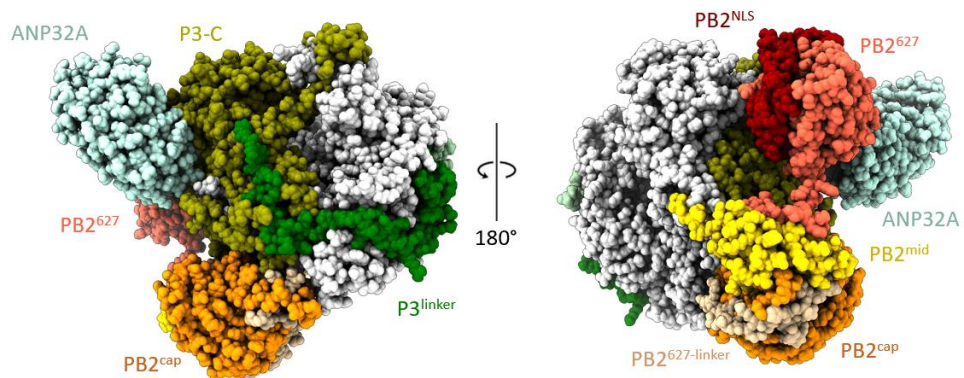


Figure 1.8: Structures of influenza virus RNA polymerase in different conformations.

Surface representations of the structures of the **(A)** apo form of the influenza C virus (ICV) RdRP (PDB: 5D9A), **(B)** pre-initiation conformation of the influenza A virus (IAV) RdRP (PDB: 4WSB), **(C)** post-cap-snatching conformation of the IAV RdRP (PDB: 6RR7), and **(D)** encapsidating ICV RdRP bound to ANP32A (PDB: 6XZR). Domains of the PA subunit and the PB2-C flexible peripheral domains are coloured.

1.6 Cellular Rab GTPases

Ras-related in brain (Rab) small G proteins constitute the largest family of the Ras related small GTPases and are involved in the regulation of intracellular vesicular trafficking with specialised function of the different Rab proteins in the secretory and endocytic pathways (Hutagalung and Novick, 2011; Zerial and McBride, 2001). Rab GTPases tether vesicles to the correct target organelle by interacting with actin filaments and microtubules, and by facilitating SNARE complex formation (Stenmark, 2009). Rab GTPases cycle between a biologically inactive GDP-bound cytosolic Rab and an active GTP-bound Rab, which is found inserted into membranes (Hutagalung and Novick, 2011; Stenmark, 2009). By exchanging the nucleotide while bound in the membrane the GTPase becomes activated for signalling roles and can interact with effector proteins that specifically facilitate traffic in its respective pathway. Activation and deactivation of Rabs depend on the opposite activities of GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). While GAP accelerates hydrolysis of GTP, GEF exchanges GDP for GTP, thereby activating Rabs (Stenmark, 2009).

Rabs generally possess a GTPase fold, composed of a six-stranded β -sheet flanked by five α -helices, common to all members of the Ras superfamily (Hutagalung and Novick, 2011). Additionally, all Rab proteins have a hypervariable C-terminal region of variable lengths which is anchored to the membrane (Li *et al.*, 2014). Because of the overall structural conservation, the differences between the inactive (**Figure 1.9A**) and active states (**Figure 1.9B**) are determined by defined switch 1 and switch 2 regions which interact with the γ -phosphate of GTP (Hutagalung and Novick, 2011). In an inactive GDP-bound conformation,

the switch regions tend to be disordered and undergo major changes to adopt a structurally well-ordered state upon binding GTP (Lee *et al.*, 2009). Furthermore, the Mg^{2+} cofactor, which is needed for high affinity nucleotide binding, is absent in an inactive Rab conformation (**Figure 1.9C**). This Mg^{2+} stabilises the interaction between GTP nucleotide and Rab in an active conformation, thereby facilitating its hydrolysis (Lee *et al.*, 2009). The active form is further stabilised by hydrogen bond interactions with the γ -phosphate of GTP mediated by serine residues in the phosphate-binding loop (P-loop) and switch 1 region as well as an extensive hydrophobic interface between the switch 1 and switch 2 regions (**Figure 1.9D**) (Dumas *et al.*, 1999). The residues involved in binding to guanine nucleotides are broadly conserved in GTPases and have been used in mutagenesis studies to generate Rab variants with altered nucleotide-binding and GTP hydrolytic properties (Lee *et al.*, 2009). The two most widely used mutations involve asparagine substitution of the P-loop serine/threonine (SN/TN mutant) and leucine substitution of the switch 2 glutamine (QL mutant). The SN/TN substitution disrupts the Mg^{2+} binding site and shifts the equilibrium toward the GDP-bound Rab (dominant negative mutant) (Farnsworth and Feig, 1991). In contrast, the QL substitution disrupts the catalytic site of Rab for the intrinsic GTP hydrolytic reaction and shifts the equilibrium toward the GTP-bound Rab (constitutively active mutant) (Uhlig *et al.*, 2006).

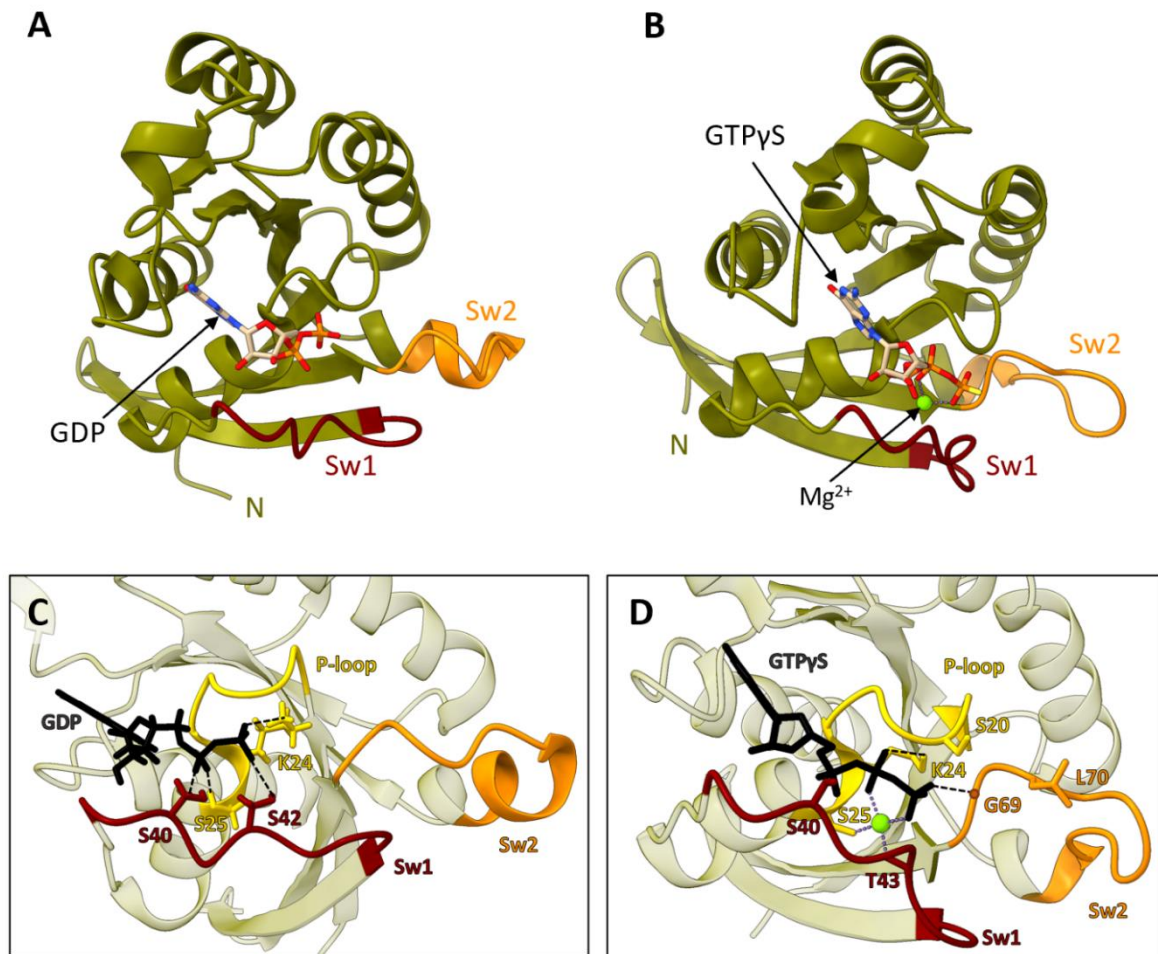


Figure 1.9: Structures of GDP- and GTP γ S-bound Rab11a.

A ribbon representation of the **(A)** wild type Rab11a bound to GDP (PDB: 1OIV) and **(B)** constitutively active Rab11a mutant (Q70L) bound to GTP γ S (a non-hydrolysable G-protein-activating analogue of GTP) (PDB: 1OIW). N-terminus (N), P-loop, and switch 1 and switch 2 regions are indicated (Sw1 and Sw2, respectively). GDP and GTP γ S are represented as stick models, and the conserved Mg²⁺ ion is drawn as a green sphere. **(C)** A close-up view of the GDP binding site showing interactions of Sw1 and P-loop with GDP in the absence of Mg²⁺ ion. The deficiency of stabilizing interactions resulting from the absence of the Mg²⁺ ion is restored by hydrogen bonds of Sw1 with the α -phosphate and the ribose of GDP, and of serine 25 (S25) from the P-loop with α -phosphate of GDP. Hydrogen bonds are in dotted lines. PDB: 1OIV. **(D)** A close-up view of the GTP γ S binding site showing the absence of interactions of the external oxygen of the γ -phosphate of GTP with either S20 from the P-loop or S42 from Sw1. Threonine 43 (T43) from Sw1 binds Mg²⁺ ion and the γ -phosphate

of GTP. Sw2 region diverges most from the GDP-bound Rab11a. Hydrogen bonds are in dotted lines. PDB: 1OIW.

1.6.1 The small GTPase Rab11a

Rab11 is the master regulator of the ERC where it mediates slow endocytic recycling from the *trans*-Golgi network to the cell surface via recycling endosomes (REs) (Amorim, 2019; Maxfield and McGraw, 2004). The mammalian genome encodes three Rab11 proteins: Rab11a, Rab11b, and Rab11c (better known as Rab25), which share high sequence identity (mouse proteins, Rab11a and Rab11b, 91% identity; Rab11a and Rab25, 62% identity; Rab11b and Rab25, 61% identity) (Welz *et al.*, 2014). While Rab11a and Rab11b are ubiquitously expressed in mammals (Goldenring *et al.*, 1996; Lai *et al.*, 1994), the expression of Rab25 is restricted to epithelial cells in lungs, kidney, and gastrointestinal tract (Goldenring *et al.*, 1993). Interestingly, even though Rab11a and Rab11b are both dependent on the microtubule cytoskeleton, it has been shown that, unlike Rab11a, the majority of Rab11b does not colocalise with transferrin receptor, suggesting its localisation in distinct vesicles compared to Rab11a (Lapierre *et al.*, 2003).

In healthy conditions, GTP-bound Rab11 recruits a series of molecular motor proteins using molecular adaptors called Rab11 family interacting proteins (Rab11-FIPs) (Horgan and McCaffrey, 2009). The formation of Rab11 complexes with distinct motor proteins enables bidirectional transport of Rab11 vesicles along microtubule tracks and actin-filament-based cytoskeletal structures (Schuh, 2011; Welz *et al.*, 2014). In mammalian cells, Rab11 interacts with molecular motor protein myosin Vb through its effector Rab11-FIP2, which acts as an adapter protein for transport along actin microfilaments (Hales *et al.*, 2002). Similarly, Rab11 interacts with microtubule tracks using different adapter proteins. Microtubules are generally organised with their minus ends at the MTOC, such as centrosome, whereas their plus ends are directed into the cytoplasm, towards the cell

periphery (Hutagalung and Novick, 2011). Rab11 has been shown to regulate trafficking in both directions by interacting with either kinesin (plus-end directed motors) or dynein (minus-end directed motors) through its effectors Rab11-FIP5 (Schonteich *et al.*, 2008) and Rab11-FIP3 (Horgan *et al.*, 2010), respectively. Rab11 containing vesicles tether to the plasma membrane using SEC15 or Munc13-4 while the fusion step at the plasma membrane involves SNARE proteins (Hutagalung and Novick, 2011; Stenmark, 2009; Zerial and McBride, 2001).

Recent evidence suggests that Rab11a is used by many viruses for their intracellular transport of viral components to their sites of egress (Bhagwat *et al.*, 2020; Chambers and Takimoto, 2010; Genoyer *et al.*, 2020; Hsiao *et al.*, 2015; Mannová and Forstová, 2003; Nanbo and Ohba, 2018; Stone *et al.*, 2016). For instance, Rab11a-containing REs (Rab11a-REs) have been shown to transport the accessory protein Vpu of human immunodeficiency virus 1 (HIV-1) from the cytoplasm towards the assembly site on the plasma membrane (Varthakavi *et al.*, 2006). Similarly, Rab11a-REs have been found to associate with viral ribonucleoprotein (vRNP) complexes of Sendai virus and human parainfluenza virus type I to promote their trafficking toward the site of egress on the plasma membrane (Genoyer *et al.*, 2020; Stone *et al.*, 2016). Moreover, it has been recently shown that Rab11a-REs have an important role in trafficking of the major matrix protein of Ebola virus, VP40, to the plasma membrane (Nanbo and Ohba, 2018). While it has been indeed demonstrated that this leads to the formation and subsequential budding of Ebola virus-like particles, it remained unclear whether the Rab11a-mediated recycling pathway is directly responsible for trafficking of the VP40 to the plasma membrane (Nanbo and Ohba, 2018). One of the first published reports on the involvement of Rab11a in IAV infection indicated its role in

the budding of the IAV virions from the plasma membrane (Bruce *et al.*, 2010). The evidence for this came from small interfering RNA (siRNA) depletion of Rab11a or disruption of its expression by overexpressing either dominant negative or constitutively active Rab11a proteins, both of which had a negative impact on viral production (Bruce *et al.*, 2010; Eisfeld *et al.*, 2011). Thereafter, several studies have provided insights into involvement of Rab11a in IAV genome trafficking and assembly and proposed that these processes are intimately related.

1.6.2 Rab11a and influenza A virus genome transport

During IAV infection, Rab11a delivers vRNPs to the cell membrane where the assembly of progeny virions takes place. Microscopy-based approaches using live cell imaging systems of cells infected with fluorescently tagged IAV, overexpressing GFP-NP, split-GFP or injected with antibodies targeting vRNPs showed that vRNPs colocalise in a Rab11a-dependent manner in discrete puncta, originating from REs, which enlarge as infection progresses (Amorim *et al.*, 2011; Avilov *et al.*, 2012a; Chou *et al.*, 2013; Eisfeld *et al.*, 2011; Han *et al.*, 2021; Lakdawala *et al.*, 2014; Momose *et al.*, 2011). Furthermore, in the absence of Rab11a, vRNPs show aberrant kinetics (Amorim *et al.*, 2011; Avilov *et al.*, 2012a; Avilov *et al.*, 2012b; Eisfeld *et al.*, 2011; Momose *et al.*, 2011; Naito *et al.*, 2007). These data led to the suggestion that vRNPs use the microtubule-based cytoskeleton in synchrony with Rab11a-REs to facilitate their fast movement to the plasma membrane. Importantly, a large body of evidence shows that the composition of Rab11a-vesicles of infected cells is different to that in uninfected cells, with many vesicles devoid of transferrin receptor (de

Castro Martin *et al.*, 2017) and enriched in cholesterol (Kawaguchi *et al.*, 2015). Together these results indicate that IAV infection impairs ERC vesicular movement and function, and that Rab11a works in a distinct manner. In corroboration, data show a modest reduction in viral titres upon depolymerisation of microtubules (Amorim *et al.*, 2011; Momose *et al.*, 2011), and a decrease in Rab11-FIPs in infected cells (Bhagwat *et al.*, 2020; Vale-Costa and Amorim, 2016), suggesting that association between Rab11a-REs and microtubules is compromised upon infection. Taken together, these studies suggest that, upon nuclear export, newly synthesised vRNPs initially accumulate around the MTOC where they interact with GTP-bound Rab11a before being distributed onto the microtubule network throughout the cytoplasm via Rab11-REs destined for the cell periphery. A schematic presentation of this model is shown in **Figure 1.10 (1)**.

The emergence of electron microscopy, however, brought the model of vRNP trafficking along microtubules via Rab11-REs into question. Ultrastructural examination of infected cells by electron microscopy revealed that clustered vesicles, positive for vRNPs and Rab11a, have discrete locations and are comprised of single and double membranes (Vale-Costa *et al.*, 2016). This unique rearrangement of membranes was observed independently by another research group who referred to these viral inclusions as irregular coated vesicles (ICVs) (de Castro Martin *et al.*, 2017). Subsequent live-cell imaging of infected cells showed that ICVs assemble in the vicinity of the endoplasmic reticulum exit sites (ERES), suggesting that their formation is spatially regulated (Alenquer *et al.*, 2019). In addition to an altered vesicular morphology, electron microscopy also revealed that IAV infection subverts the ER during vRNP transport from the nuclear periphery to the plasma membrane (Alenquer *et al.*, 2019). Moreover, Alenquer *et al.* showed that the movement

of IAV inclusions matches that of ER and that nocodazole treatment does not hinder viral inclusion formation but rather severely impairs fusion and fission events (Alenquer *et al.*, 2019). Collectively, this led to an alternative vRNP trafficking model in which once progeny vRNPs reach the cytosol they first bind to the remodelled ER from where Rab11a-coated ICVs loaded with vRNPs are subsequently released and directed to the plasma membrane via the extensions of the remodelled ER. A schematic presentation of this model is shown in **Figure 1.10 (2)**.

That being said, Bhagwat *et al.* have recently shown that while neither microtubule- nor actin network-depolymerizing treatment has an impact on vRNP movement, the colocalization of Rab11a and vRNPs changes drastically depending on the type of the depolymerising drug (Bhagwat *et al.*, 2020). Namely, in the absence of microtubules the colocalization of vRNPs and Rab11a-REs decreases. In contrast, in the absence of actin filaments vRNP strongly associates with Rab11a-REs which predominantly transport via the microtubule network. These data are in agreement with an earlier study showing that aberrant motion of vRNPs is observed only when cells are treated with both microtubule- and actin network-depolymerizing drug (Avilov *et al.*, 2012a). These data suggest that the intracellular transport of IAV vRNPs requires a membrane scaffold either in the form of Rab11a-REs or the ER and imply that membrane promiscuity may be advantageous for IAV genome assembly.

1.6.3 Rab11a and influenza A virus genome assembly

It has been demonstrated that vRNPs form a supramolecular genomic complex with a 7+1 arrangement inside virions (Dadonaite *et al.*, 2019; Fournier *et al.*, 2012; Noda *et al.*, 2006; Noda and Kawaoka, 2012). However, the cellular locations and the mechanisms whereby the influenza genome complex is formed remain less understood. Currently accepted models link genome transport to genome assembly (Amorim, 2019; Dou *et al.*, 2018; Eisfeld *et al.*, 2015; Gerber *et al.*, 2014; Hutchinson and Fodor, 2013; Lakdawala *et al.*, 2016; Pohl *et al.*, 2016). The dispersed (vesicular collision) model implies that cytosolic Rab11a-vesicles carrying the vRNPs collide transiently, allowing the establishment of RNA-RNA interactions. Several microscopy-based approaches support this model with evidence of the formation of viral inclusions that expand as IAV infection progresses and can accommodate a large number of vRNP segments (Amorim *et al.*, 2011; Avilov *et al.*, 2012a; Chou *et al.*, 2013; Lakdawala *et al.*, 2014). Although this model is discrepant with the lack of Rab11a at the plasma membrane (Eisfeld *et al.*, 2011) and in virions (Hutchinson *et al.*, 2014), the viral inclusions observed by fluorescent microscopy match the clustered vesicles identified by electron microscopy (Vale-Costa *et al.*, 2016), thereby being the basis supporting the dispersed vesicular collision model.

However, recent observations of the involvement of the ER in vRNP transport and a large body of accumulating evidence on infection-specific alterations to the Rab11a cycle shed light on alternative mechanisms (Alenquer *et al.*, 2019; de Castro Martin *et al.*, 2017; Kawaguchi *et al.*, 2015; Vale-Costa *et al.*, 2016). The concentration of viral material in ICVs may serve to compartmentalize different vRNP segments, separating them from the rest of the cytosol, thereby creating designated sites for genome assembly. This led a second

model for IAV genome assembly, called the compartmentalized model. In this model, the assembly of eight vRNP segments occurs near the ERES where the progeny vRNPs initially concentrate before being loaded onto Rab11a-coated ICVs for posterior delivery to the plasma membrane. In further support of this model are viral inclusions observed by electron microscopy which were shown to have liquid properties (Alenquer *et al.*, 2019), thereby being able to exchange viral material dynamically. Alternatively, it is also possible that Rab11a vesicles in ICVs collide to establish vRNP intersegment interactions (Amorim, 2019).

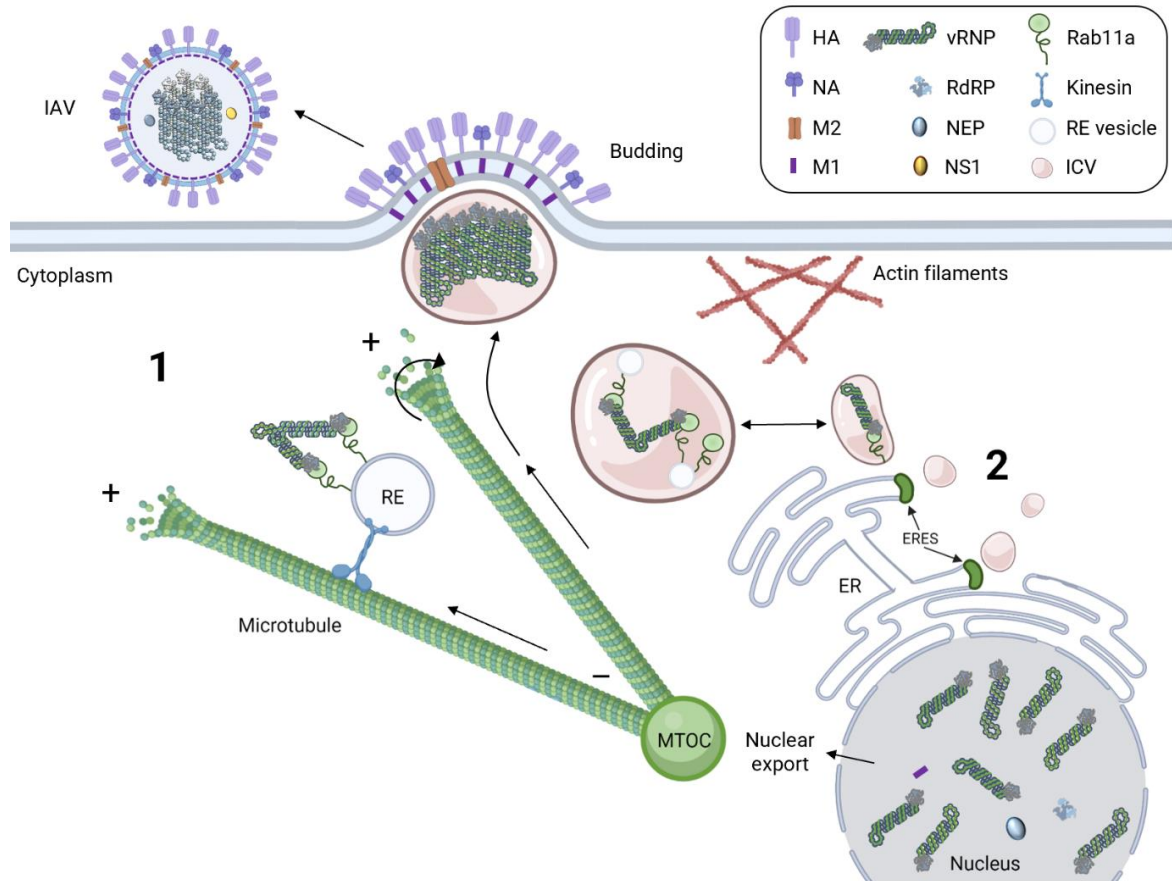


Figure 1.10: Influenza A virus genome transport and assembly.

After viral genome replication and vRNP assembly in the nucleus, vRNP complexes must be exported from the nucleus and transported to the cell membrane for packaging into progeny virions. Proteins including M1 are translated in the cytosol and bind the transmembrane proteins at the cell surface. Transmembrane proteins are translated in the rough ER and reach the lipid membrane using the classic secretory pathway. vRNPs are found inside virions as a supramolecular genomic complex displaying a 7 + 1 arrangement. Current models suggest that the genomic complex is formed before reaching the cell surface by the establishment of RNA-RNA interactions promoted as **(1)** Rab11a-containing REs transporting vRNPs collide throughout the cytosol (dispersed vesicular collision model) or **(2)** in designated locations (compartmentalized model). Parts of the figure were made using BioRender.

1.7 Aims of this study

I aimed to contribute to our understanding of the mechanism of the interaction between the IAV RdRP and the small GTPase Rab11a. My aims were:

- 1) Determine whether the viral RdRP is a mediator of the interaction between IAV vRNP and Rab11a.
- 2) Investigate how IAV infection alters the Rab11a cycle and interactome.
- 3) Investigate whether Rab11a has a role in determining the host specificity of influenza viruses.
- 4) Identify the exact binding site of the IAV RdRP on Rab11a.
- 5) Establish a workflow to study the interaction between IAV vRNPs and Rab11a in the context of live infected cells using a novel correlative imaging scheme for biological samples at cryogenic temperatures.

2 Materials and methods

2.1 Materials and reagents

2.1.1 DNA oligonucleotides

All DNA oligonucleotides were purchased from Sigma-Aldrich® desalted and lyophilised at a scale of 0.025 µmole. All DNA oligonucleotides were dissolved to 100 mM in nuclease-free water (Invitrogen™) and used as PCR primers.

Table 2.1: DNA oligonucleotides.

<i>Oligonucleotide</i>	<i>Sequence</i>
Rab11a_pGEX_F	5'-GGGGCCCCCTGGGATCCCCGGAATTCATGGGCACTCGTGACGAC-3'
Rab11a_pGEX_R	5'-GTCAGTCACGATGCGGCCGCTCGAGTTAGATGTTCTGGCAGCACTG-3'
Rab11a_Q70L_F	5'-GCACGGTAACGCTCAAGACCAGCGGTGTCC-3'
Rab11a_Q70L_R	5'-GGACACCGCTGGTCTTGAGCGTTACCGTGC-3'
Rab11_1-173_R	5'-GTCAGTCACGATGCGGCCGCTCGAGTTAGTAGATCTCGGTCAGGATGG-3'
Rab11_I44A_G4 5A_V46A_F	5'-TGGAATCCAAGTCCACCGCCGCTCTCGAGTTCGCTACCCGTTC-3'
Rab11_I44A_G4 5A_V46A_R	5'-GAACGGGTAGCGAACTCGAGAGCGGCGGTGGACTTGGATTCCA-3'
mRuby_Rab11_ F	5'-GGCACCGGTGGCACCAGGGACG-3'
mRuby_Rab11_ R	5'-GTCCTCGAGtcattaGATGTTCTGG-3'
NT60_PB2-C_F	5'-GGGGCCCCCTGGGATCCCCGGAATTCGGCGAAGTGCCTAACGACGAC-3'
NT60_PB2-C_R	5'-GTCACGATGCGGCCGCTCGAGCTTCATGACCAGGACCACGTC-3'

Fj_PB2-C_F	5'-GGGGCCCCTGGGATCCCCGGAATTCGGCGAAGTGC GTAACGACGAC-3'
Fj_PB2-C_R	5'-GTCACGATGCGGCCGCTCGAGCTTCATGACCAGGACCACGTC-3'
PB2-C_E627K_F	5'-GCTGCTCCCCCTAAGCAGTCCAGGATG-3'
PB2-C_E627K_R	5'-CATCCTGGACTGCTTAGGGGGAGCAGC-3'
PB2-C_K627E_F	5'-GCTGCTCCCCCGAGCAGTCCAGGATG-3'
PB2-C_K627E_R	5'-CATCCTGGACTGCTCGGGGGGAGCAGC-3'
NT60_627-NLS_F	5'-GGGGCCCCTGGGATCCCCGGAATTCGAGATCAACGGTCCCGAGTCC-3'
Viet_PB2-C_A274T_F	5'-CGTGCAACCGTTAGCACCGATCCGCTGGCAAGC-3'
Viet_PB2-C_A274T_R	5'-GCTTGCCAGCGGATCGGTGCTAACGGTTGCACG-3'
Viet_PB2-C_K526R_F	5'-GCGAAACCCAGGGCACCGAACGCCTGACCATTACCTATAGCA-3'
Viet_PB2-C_K526R_R	5'-TGCTATAGGTAATGGTCAGGCGTTCGGTGCCCTGGGTTTCGC-3'
Viet_PB2-C_I553V_F	5'-CTGGTTAATACCTATCAGTGGGTCAATTCGCAATTGGGAAACCGTG-3'
Viet_PB2-C_I553V_R	5'-CACGGTTTCCCAATTGCGAATGACCCACTGATAGGTATTAACCAG-3'
Viet_PB2-C_V584I_F	5'-CCGTTCCAGAGCCTGATCCCGAAAGCAGCACGT-3'
Viet_PB2-C_V584I_R	5'-CGTGCTGCTTTCGGGATCAGGCTCTGGAAC-3'
Viet_PB2-C_L607V_F	5'-TGTTTCAGCAGATGCGTGATGTTGTGGGCACCTTTG-3'
Viet_PB2-C_L607V_R	5'-CAAAGGTGCCCAACAACATCACGCATCTGCTGAAACA-3'
Viet_PB2-C_A622T_F	5'-GATTATCAAACCTGCTGCCGTTTACCGCAGCACCGCCTAAACAG-3'

Viet_PB2- C_A622T_R	5'-AGGCGGTGCTGCGGTAAACGGCAGCAGTTTGATAATC-3'
Viet_PB2- C_K627E_F	5'-GCAGCAGCACCGCCTGAGCAGAGCCGTATGCA-3'
Viet_PB2- C_K627E_R	5'-ACTGCATACGGCTCTGCTCAGGCGGTGCTGCTGC-3'

2.1.2 RNA oligonucleotides

All RNA oligonucleotides were designed by Dr Narin Hengrung and Dr Jeremy Keown, and purchased from Dharmacon Inc. All RNA oligonucleotides were dissolved to 4 mM in nuclease-free water (Invitrogen™) and used to bind to purified influenza virus RNA-dependent RNA polymerases.

Table 2.2: RNA oligonucleotides.

5' IAV vRNA	5'-AGUAGAAACAAGGCC-3'
3' IAV vRNA	5'-GGCCUGCUUUUGCU-3'
5' IAV cRNA	5'-pAGCAAAAGCAGGCC-3'
3' IAV cRNA	5'-GGCCUUGUUUCUACU-3'
5' IBV vRNA	5'-pAGUAGUAACAAGAGG-3'
3' IBV vRNA	5'-CCUCUGCUUCUGCU-3'
5' ICV vRNA	5'-AGCAGUAGCAAGGGG-3'
3' ICV vRNA	5'-CCCCUGCUUCUGCU-3'

2.1.3 Synthetic genes

All synthetic genes were purchased from GeneArt (ThermoFisher Scientific).

Table 2.3: Synthetic genes.

<i>Gene</i>	<i>Notes</i>
Rab11a	Residues 1-216 from Rab11a (UniProtKB: P62491-1). Gene optimised for <i>Spodoptera frugiperda</i> expression.
pET151/D-TOPO Viet PB2-C	Residues 247-736 from the influenza A/Vietnam/1203/2004 (H5N1) PB2 subunit (Genbank: AY818126.1) cloned in pET151/D-TOPO bacterial vector. Gene optimised for <i>Escherichia coli</i> expression.

2.1.4 Plasmids

All pHW2000 plasmids for generation of recombinant IAV were received from Prof. Ervin Fodor (Hoffmann *et al.*, 2000). pEGFP-C2 Rab11a plasmid was received from Dr Maria Amorim (unpublished). pHR-SFFV, pMD2.G, and psPAX2 lentiviral plasmids were received from Dr Benjamin Bishop (Elegheert *et al.*, 2018).

Table 2.4: Plasmids.

<i>Plasmid</i>	<i>Notes</i>
pHW2000 PB1 WSN	Mammalian expression of influenza A/WSN/1933 (H1N1) PB1 protein (GenBank: LC333183) and PB1 vRNA.
pHW2000 PB2 WSN	Mammalian expression of influenza A/WSN/1933 (H1N1) PB2 protein (GenBank: J02179) and PB2 vRNA.

pHW2000 PA WSN	Mammalian expression of influenza A/WSN/1933 (H1N1) PA protein (GenBank: LC333184) and PA vRNA.
pHW2000 NP WSN	Mammalian expression of influenza A/WSN/1933 (H1N1) NP protein (GenBank: LC333186) and NP vRNA.
pHW2000 HA WSN	Mammalian expression of influenza A/WSN/1933 (H1N1) HA protein (GenBank: J02176) and HA vRNA.
pHW2000 NA WSN	Mammalian expression of influenza A/WSN/1933 (H1N1) NA protein (GenBank: J02177) and NA vRNA.
pHW2000 M WSN	Mammalian expression of influenza A/WSN/1933 (H1N1) M protein (GenBank: MF039638) and M vRNA.
pHW2000 NS WSN	Mammalian expression of influenza A/WSN/1933 (H1N1) NS protein (GenBank: LC333189) and NS vRNA.
pHW2000 PA(XbaI)-GFP WSN	Mammalian expression of influenza A/WSN/1933 (H1N1) PA protein fused to the GFP tag at its C-terminus and PA-GFP vRNA. Described in Lakdawala <i>et al.</i> (2014).
pEGFP-C2 Rab11CA	Mammalian expression of constitutively active (CA) mutant of Rab11a fused to the EGFP tag at its N-terminus.
pGEX-6P-1 Rab11a/ pGEX-6P-1 Rab11CA/ pGEX-6P-1 Rab11_I44A_G45A_V46A	Bacterial vector for expression of N-terminally GST-tagged Rab11a with a human rhinovirus (HRV) 3C protease cleavage site. Rab11a gene was amplified by PCR from a synthetic Rab11a gene using primers Rab11a_pGEX_F and Rab11a_pGEX_R. The PCR product and pGEX-6P-1 backbone were digested with EcoRI and XhoI, followed by ligation with In-Fusion HD Cloning Kit. PCR site-directed mutagenesis was performed to generate the constitutively active (CA) Rab11a using primers Rab11a_Q70L_F and Rab11a_Q70L_R to introduce a single amino acid substitution at position 70. Further PCR site-directed mutagenesis was performed to generate Rab11CA with mutations at positions 44, 45, and

	46 in switch I region using Rab11_I44A_G45A_V46A_F and Rab11_I44A_G45A_V46A_R primers.
pGEX-6P-1 Rab11_1-173	Bacterial vector for expression of N-terminally GST-tagged Rab11a (residues 1-173 only) with a HRV 3C protease cleavage site. Rab11a gene was amplified by PCR from a synthetic Rab11a gene using primers Rab11a_pGEX_F and Rab11a_1-173_R. The PCR product and pGEX-6P-1 backbone were digested with EcoRI and XhoI, followed by ligation with In-Fusion HD Cloning Kit.
pHR-SFFV mRuby-Rab11CA	Lentiviral transfer plasmid (Addgene plasmid number: 113900) containing the minimally necessary <i>cis</i> -acting HIV components: the 5' and 3' long terminal repeats, the polypurine tract, the Rev response element, and the psi packaging signal. The SFFV promoter is cloned between Sall and XbaI restriction sites. mRuby gene is cloned between EcoRI and AgeI restriction sites. Rab11CA gene was amplified by PCR from pEGFP-C2 Rab11CA plasmid using primers mRuby_Rab11_F and mRuby_Rab11_R. The PCR product was purified using the PCR purification kit by Zymo Research. Purified PCR product and pHR-SFFV mRuby backbone were digested with AgeI and XhoI, followed by ligation with In-Fusion HD Cloning Kit.
pMD2.G	Lentiviral envelope plasmid (Addgene plasmid number: 12259) containing the vesicular stomatitis virus G envelope protein (VSV-G).
psPAX2	Lentiviral second-generation packaging plasmid (Addgene plasmid number: 12260) containing the minimally necessary HIV genes required for virus production: <i>gag</i> , <i>pol</i> , <i>rev</i> , and <i>tat</i> .

pGEX-6P-1 NT60 PB2-C/ pGEX-6P-1 Fj PB2-C/ pGEX-6P-1 NT60 PB2-C K627E/ pGEX-6P-1 Fj PB2-C E627K	Bacterial vector for the expression of N-terminally GST-tagged PB2-C (residues 247-736 from the PB2 subunit) with a HRV 3C protease cleavage site. PB2-C gene was amplified by PCR from either pFL PB2-pA plasmid expressing the influenza A/Northern Territories/60/1968 (H3N2) or influenza A/duck/Fujian/01/2002 (H5N1) PB2 using NT60_PB2-C_F and NT60_PB2-C_R, and Fj_PB2-C_F and Fj_PB2-C_R primers, respectively. The PCR product and pGEX-6P-1 backbone were digested with EcoRI and XhoI, followed by ligation with In-Fusion HD Cloning Kit. Site-directed mutagenesis was performed to generate NT60 PB2-C K627E and Fj PB2-C E627K using primers PB2-C_E627K_F and PB2-C_E627K_R, and PB2-C_K627E_F and PB2-C_K627E_R, respectively, to introduce a single amino acid substitution at position 627.
pGEX-6P-1 NT60 627-NLS	Bacterial vector for expression of N-terminally GST-tagged 627-NLS (residues 538-736 from the PB2 subunit) with a HRV 3C protease cleavage site. 627-NLS gene was amplified by PCR from pFL PB2-pA plasmid expressing the influenza A/Northern Territories/60/1968 (H3N2) PB2 using NT60_PB2-C_F and NT60_627-NLS_R primers. The PCR product and pGEX-6P-1 backbone were digested with EcoRI and XhoI, followed by ligation with In-Fusion HD Cloning Kit.
pET151/D-TOPO Viet PB2-C	Bacterial vector for expression of N-terminally 6xHis and V5 tagged PB2-C (residues 247-736) from the influenza A/Vietnam/1203/2004 (H5N1) PB2 subunit (Genbank: AY818126.1). Site-directed mutagenesis was performed to generate different mutants containing either a single, double, or triple amino acid substitution using Viet_PB2-C_* primers.

2.2 Cells

All tissue culture media were purchased from Sigma-Aldrich®, unless otherwise specified. Human Embryonic Kidney 293T (HEK 293T) cells, Madin-Darby Canine Kidney (MDCK) cells, adenocarcinomic human alveolar basal epithelial (A549) cells, human bone osteosarcoma epithelial (U2OS), and *Spodoptera frugiperda* 9 (Sf9) insect cells were sourced from the Cell Bank of the Sir William Dunn School of Pathology, University of Oxford.

2.2.1 Cell culture

HEK 293T, MDCK, A549, and U2OS cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10%(v/v) foetal calf serum (FCS), unless otherwise specified. Cells were kept at 37°C, 5% CO₂. Cells were passaged by 1:10 dilution thrice per week from a confluent density of approximately $1 \cdot 10^7$ cells ml⁻¹. Cells were washed using Dulbecco's Phosphate-Buffered Saline (DPBS) and trypsinised with TrypLE™ Express Enzyme (Gibco) for 1–3 min.

Sf9 insect cells were grown in Sf-900 II serum free medium (Gibco) supplemented with 1X PenStrep (Gibco). Cell density was maintained between 0.5 and 2 million cells ml⁻¹. Cells were kept at 27°C, 110 rpm shaking incubator.

2.2.2 Generation of a stable cell line expressing mRuby-Rab11CA

Lentiviral transduction system (Elegheert *et al.*, 2018) was used to generate a stable U2OS cell line expressing mRuby-Rab11CA. The experiment was performed at the Division of

Structural Biology, University of Oxford, at Biosafety Level 2 under the supervision of Dr Benjamin Bishop who provided all reagents and HEK 293T Lenti-X cells.

HEK 293T Lenti-X cells were transfected with pHR-SFFV mRuby-Rab11CA, pMD2.G, and psPAX2 (**Table 2.4**) using polyethylenimine (PEI) in DMEM according to the manufacturers' instructions. Briefly, 11 µg of each plasmid and 82.5 µl of PEI were mixed with 750 µl of serum-free DMEM. The DNA-PEI transfection mix was incubated at RT for 10 min and used to transfect approximately $1 \cdot 10^7$ HEK 293T Lenti-X cells. HEK 293T Lenti-X cells were topped up to 15 ml with DMEM media supplemented with 2%(v/v) FCS and kept at 37°C, 5% CO₂ for 3 days. After 3 days, lentiviral particles were collected and filtered using a 0.45 µm syringe filter. Filtered solution containing viral particles was mixed with 15 ml DMEM media supplemented with 2%(v/v) FCS to which 30 µl of hexadimethrine bromide was added to increase the efficiency of transduction. U2OS cells were then transduced with 15 ml of lentivirus-containing medium and incubated at 37°C, 5% CO₂ for 3 days. After 3 days, old DMEM media supplemented with 2%(v/v) FCS was aspirated, cells were detached using trypsin (Gibco® TrypLE™) and resuspended in fresh DMEM media supplemented with 10%(v/v) FCS. Polyclonal mRuby-Rab11CA U2OS cells were expanded for single cell fluorescence activated cell sorting (FACS) to isolate a monoclonal cell line.

A single cell FACS was performed by Dr Robert Hedley at The Flow Cytometry Facility (University of Oxford).

Cell suspension containing approximately $1 \cdot 10^7$ mRuby-Rab11CA U2OS cells ml⁻¹ was prepared in FACS buffer (PBS + 2%(v/v) FCS). mRuby-expressing cells were FACS sorted on the BD FACSAria™ III cuvette cell sorter (Beckinson Dickinson) using 561 nm laser into 96-

well cell culture-treated, flat-bottom microplates (Corning®) containing conditioned medium [40%(v/v) fresh DMEM, 40%(v/v) filtered DMEM collected from the log phase of mRuby-Rab11CA U2OS polyclonal cell line, 20%(v/v) FCS]. 4',6-diamidino-2-phenylindole (DAPI) was used to determine DNA content. Wild type U2OS cells were used as a negative control. Data was analysed in BD FlowJo software version 10.5.3 (Beckinson Dickinson).

Single clones of mRuby-Rab11CA U2OS cell line were expanded, and 10 clones were further analysed by FACS as described above to determine their homogeneity. Clones were grown in DMEM supplemented with 10%(v/v) FCS and aliquots were stored at -80°C.

2.3 Influenza virus

2.3.1 Influenza virus reverse genetics

Table 2.5: Influenza viruses.

<i>Virus</i>	<i>Notes</i>
WSN	Influenza A/WSN/1933 (H1N1) wild-type virus.
WSN PA-GFP	Influenza A/WSN/1933 (H1N1) recombinant virus with GFP tag fused at the C-terminus of the viral PA polymerase subunit.

The 8-plasmid system (Hoffmann *et al.*, 2000) was used to generate either a wild type (WSN) or a recombinant fluorescently-tagged (WSN PA-GFP) IAV. For the WSN virus, pHW2000 PB1 WSN, pHW2000 PB2 WSN, pHW2000 PA WSN, pHW2000 NP WSN, pHW2000 HA WSN, pHW2000 NA WSN, pHW2000 M WSN, and pHW2000 NS WSN were

used. For the WSN PA-GFP virus pHW2000 PB1 WSN, pHW2000 PB2 WSN, pHW2000 PA(XbaI)-GFP WSN, pHW2000 NP WSN, pHW2000 HA WSN, pHW2000 NA WSN, pHW2000 M WSN, and pHW2000 NS WSN were used. HEK 293T cells were transfected with 1.0 µg of each plasmid using Lipofectamine® 2000 Transfection Reagent (ThermoFisher Scientific), according to the manufacturer's instructions, and plated in 6 cm dishes. For a negative control sample, the plasmid encoding PA was omitted. Cells were kept at 37°C, 5% CO₂ for 6 h before replacing DMEM media supplemented with 10%(v/v) FCS with fresh DMEM media supplemented with 0.5%(v/v) FCS. Cells were then kept at 37°C, 5% CO₂ for another 48 hours. After 48 h, the supernatants from HEK 293T cells were collected and cell debris was removed by centrifugation at 2000 rpm (Hettich ROTINA 420, rotor 5624). Virus stock was stored at -80°C until further use. Viral titres were measured using plaque assay as described in section 2.3.2.

2.3.2 Plaque assay

MDCK cells were seeded in 6-well plates and grown to ~80% confluency. Serial 10-fold dilutions of generated virus were made in DMEM supplemented with 0.5%(v/v) FCS and 200 µl of each dilution was applied to the cells for 1 h at RT. After the incubation, virus solution was aspirated, and 2 ml of agarose overlay [2%(w/v) agarose in DPBS mixed with an equal volume of DMEM supplemented with 0.5%(v/v) FCS] was applied on the cells. Plates were incubated for 15 min at RT to allow the agarose overlay to set. Cells were then incubated at 37°C, 5% CO₂ for 72 h before being stained with Coomassie Brilliant Blue stain (0.1% Coomassie R250(w/v), 10%(v/v) acetic acid, 40%(v/v) methanol).

2.4 Confocal microscopy

2.4.1 Sample preparation

The expression of PA-GFP gene was analysed by confocal microscopy optical imaging. MDCK, U2OS, and U2OS mRuby-Rab11CA cells were grown on a circular glass coverslip (12Cir-1.5, ThermoFisher Scientific) to ~80% confluency in 24-well cell culture plates (Greiner Bio-One) before being infected with 100 µl of virus. MDCK cells were infected with WSN PA-GFP virus at an MOI of 0.1 or no virus (mock infected), U2OS cells with either WSN or WSN PA-GFP virus at an MOI of 5 or no virus (mock infected), and U2OS mRuby-Rab11CA cells with WSN PA-GFP virus at an MOI of 5 or no virus (mock infected). Cells were kept in the 37°C, 5% CO₂ incubator for the indicated times before being fixed with pre-chilled 4%(v/v) paraformaldehyde in DPBS for 15 min at RT. After fixation, wells were washed 3 times with DPBS prior to permeabilising the cells in 0.5%(v/v) Triton X-100 in DPBS for 15 min at RT. Cells were then blocked for 1 h at RT using DPBS blocking buffer [1%(m/v) BSA, 0.2%(m/v) gelatine, 0.01%(v/v) Tween-20] for 1 h at RT. Immunofluorescent staining was performed, where indicated, using anti-IAV NP primary antibody (1:250 diluted in DPBS blocking buffer) (Varečková *et al.*, 1995) for 1 h at RT followed by a Cy3 conjugated goat anti-rabbit secondary antibody (Sigma, 1:1000 diluted in DPBS blocking buffer) for 1 h at RT. Glass coverslips were mounted onto Mowiol (Calbiochem) containing DAPI dye (1:1000) and stored at 4°C until further use.

2.4.2 Imaging

Samples were visualised on an Olympus FluoView Spectral FV-1000 Laser Scanning Microscope (Olympus) using either a 60X or 100X, 1.40 oil objective. Samples were excited

with a 488 nm argon laser (GFP), a 559 nm solid state laser (Cy3 and mRuby), and a 405 nm solid state laser (DAPI). All images were acquired on a FLUOVIEW Software (Olympus) and processed using ImageJ-win32 (Schneider *et al.*, 2012).

2.5 Molecular cloning

2.5.1 PCR and site-directed mutagenesis

All PCRs were ran in ProFlex PCR System Applied Biosystems thermal cycler (ThermoFisher Scientific).

PCR gene amplifications were performed using Phusion® High-Fidelity DNA Polymerase (New England Biolabs). 50 µl reactions were set up using 10 ng plasmid DNA, 2.5 µl of each 10 µM forward and 10 µM reverse primer, 1 µl of 10 mM dNTPs, 1.5 µl of 100%(v/v) DMSO, and 10 µl of 5X Phusion HF Reaction Buffer (New England Biolabs). Thermocycling conditions: 1) 98°C for 30 seconds, 2) 98°C for 10 seconds, 3) 60-68°C for 25 seconds, 4) 72°C for 40 seconds, 5) cycle to step 2 for 30 times, 6) 72°C for 5 minutes, 7) 4°C infinite hold.

PCR site-directed mutagenesis was performed using *PfuTurbo* DNA Polymerase (Agilent). 50 µl reactions were set up using 100 ng plasmid DNA, 1 µl of each 10 µM forward and 10 µM reverse primer, 1 µl of 10 mM dNTPs, 1.5 µl of 100%(v/v) DMSO, and 5 µl of 10X Pfu Reaction Buffer (Agilent). Thermocycling conditions: 1) 95°C for 3 minutes, 2) 95°C for 30 seconds, 3) 63-68°C for 30 seconds, 4) 68°C for 14 minutes, 5) cycle to step 2 for 18 times, 6) 68°C for 5 minutes, 7) 4°C infinite hold. PCR products were digested with DpnI restriction

enzyme for 1 hour at 37°C to eliminate template DNA before their transformation into *E. coli* DH5α cells. Amino acid substitutions were confirmed by sequencing.

2.5.2 Bacterial culture

Chemically competent *Escherichia coli* cells were sourced from The Division of Structural Biology, University of Oxford. *E. coli* bacterial cultures were grown in either lysogeny broth (LB) or 2x YT media at 37°C, 180 rpm (Mixer HC, STARLAB). LB media was supplemented with 100 µg ml⁻¹ ampicillin, 2x YT media was supplemented with and 100 µg ml⁻¹ ampicillin and 50 µg ml⁻¹ chloramphenicol.

2.5.3 Transformations

Chemically competent *E. coli* DH5α and BL21(DE3) cells were generated by and received from Dr Haitian Fan. Chemically competent *E. coli* Rosetta(DE3) cells were generated by and received from Alaa Baazaoui.

Chemically competent *E. coli* DH5α cells were used for transformation of fused pFL PB2-pA and pUCDM PB1 PA vectors, pGEX-6P-1 Rab11a constructs, pGEX-6P-1 PB2-C constructs, pGEX-6P-1 627-NLS, and pET151/D-TOPO Viet PB2-C constructs with ampicillin selection. Chemically competent *E. coli* BL21(DE3) cells were used for transformation of pGEX-6P-1 Rab11a constructs with ampicillin selection. Chemically competent *E. coli* Rosetta(DE3) cells were used for transformation of pGEX-PB-C constructs, pGEX-6P-1 627-NLS, and pET151/D-TOPO Viet PB2-C constructs with ampicillin and chloramphenicol selection.

Generally, an aliquot of 100 μ l competent cells was thawed on ice for 20 min before being transformed with 1 μ g of plasmid. The mixture was incubated on ice for 15 min followed by a heat shock at 42°C for 40 seconds. After the heat shock, cells were chilled on ice and recovered in 500 μ l LB broth at 37°C, 1000 rpm (Mixer HC, STARLAB) for 2 h. 50 μ l of *E. coli* DH5 α and BL21(DE3) cells were then plated on a pre-warmed LB agar plate supplemented with 100 μ g ml⁻¹ ampicillin and kept at 37°C overnight. *E. coli* Rosetta(DE3) cells were supplemented with 100 μ g ml⁻¹ ampicillin and 50 μ g ml⁻¹ chloramphenicol.

E. coli BL21(DE3) and *E. coli* Rosetta(DE3) bacterial 50%(v/v) glycerol stocks were produced and stored at -80°C.

2.5.4 Isolation of plasmid DNA

Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (Qiagen) using manufacturer's instructions.

2.6 Protein expression and purification

The tobacco etch virus (TEV) and human rhinovirus (HRV) 3C proteases were expressed and purified by either Dr Haitian Fan or the author of this thesis. Glutathione S-transferase (GST) protein was expressed and purified by Dr Haitian Fan.

2.6.1 Purification buffers

Table 2.6: Protein purification buffers.

Buffer A	50 mM HEPES-NaOH pH 7.5, 500 mM NaCl, 10%(v/v) glycerol, 0.05% OTG(w/v), 1 mM DTT
Buffer B	50 mM HEPES-NaOH pH 7.5, 500 mM NaCl, 10%(v/v) glycerol, 0.05%(w/v) OTG
Buffer C	25 mM HEPES-NaOH pH 7.5, 500 mM NaCl, 5%(v/v) glycerol
Buffer D	50 mM HEPES-NaOH pH 7.5, 500 mM NaCl, 10%(v/v)) glycerol, 1 mM DTT, 25 mM glutathione
Buffer E	50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5%(v/v) glycerol, 5 mM β -mercaptoethanol
Buffer F	50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5%(v/v) glycerol, 1 mM β -mercaptoethanol

2.6.2 Expression and purification of the influenza virus RdRP

Table 2.7: Influenza virus polymerases investigated.

Virus	Polymerase details	Polymerase abbreviation	Baculovirus made by	Protein purified by
A/Northern Territory/60/1968 (H3N2)	Wild type	NT60 RdRP	Dr Narin Hengrung	Hana Veler
A/Northern Territory/60/1968 (H3N2)	PB2 Δ 535-667	NT60 PB2 Δ 538-679 RdRP	Dr Haitian Fan	Hana Veler
A/Brevig Mission/1/1918 (H1N1)	Wild type	1918 RdRP	Dr Jeremy Keown	Hana Veler

A/duck/Fujian/01/2002 (H5N1)	Wild type	Fj RdRP	Dr Haitian Fan	Dr Haitian Fan
B/Panama/45/1990	Wild type	IBV RdRP	Dr Narin Hengrung	Dr Jeremy Keown
C/Johannesburg/1/1966	Wild type	ICV RdRP	Dr Narin Hengrung	Hana Veler

Sf9 cell suspension cultures at $2 \cdot 10^6$ cells ml^{-1} were infected with recombinant baculoviruses expressing influenza virus polymerases (**Table 2.7**) at an MOI of 0.5-1.0 and incubated for 72 h at 27°C, 110 rpm shaking incubator. Cells were harvested by centrifugation at 800g for 20 min at 4°C. Cell pellets were re-suspended in buffer A supplemented with 100 $\mu\text{g ml}^{-1}$ mg ribonuclease A (RNaseA) and 1X cOmplete™ EDTA-free protease inhibitor (Roche), and lysed by sonication (3x 30 seconds on/30 seconds off). The lysate was clarified by centrifugation at 35,000g for 45 min at 4°C, and the supernatant was incubated with pre-equilibrated IgG Sepharose beads (GE Healthcare) (1 ml bead slurry per 1 l of culture) at 4°C for 3 h. After binding, the beads were washed with buffer B. The RdRP was released using TEV protease (0.5 mg) at 4°C overnight in buffer A and cleared from the beads by centrifugation at 1,000g for 5 min at 4°C. Released RdRP was concentrated to 500 μl using an Amicon Ultra centrifugal filter unit (Merck Millipore, NMWL of 100 kDa) and further purified by a size exclusion chromatography (SEC) through a Superdex® 200 increase 10/300 column (GE Healthcare) at 4°C equilibrated with a buffer C. Purified RdRP proteins were concentrated as above, and protein concentration was determined by measuring the ratio of absorbance at 260 nm and 280 nm ($A_{260/280}$) using NanoDrop™

2000/2000c Spectrophotometer (ThermoFisher Scientific). Concentrated proteins were aliquoted, flash-frozen using liquid nitrogen, and stored at -80°C.

To generate either vRNA- or cRNA-bound RdRP, 0.04 mg of purified RdRP was incubated on ice with a 1.2-fold molar excess of 5' and 3' vRNA and cRNA promoters (**Table 2.2**) for 5 min. Protein was further purified by SEC and the presence of RNA promoters was confirmed by measuring $A_{260/280}$.

2.6.3 Bacterial expression and purification of Rab11a

Table 2.8: Rab11a proteins investigated.

Rab11a protein	Protein details	Protein abbreviation
N-terminal GST-tagged full length Rab11a	Wild type	GST-Rab11a
N-terminal GST-tagged full length Rab11a	Constitutively active (CA) Rab11a mutant	GST-Rab11CA
N-terminal GST-tagged full length Rab11a	CA Rab11a mutant with additional mutations at positions 44-46 in switch I region	GST-Rab11CA Ala44-46
N-terminal GST-tagged truncated Rab11a	CA Rab11a mutant lacking the C-terminal 43 hypervariable residues	GST-Rab11CA 1-173
Full length Rab11a	Wild type	Rab11a
Full length Rab11a	Constitutively active (CA) Rab11a mutant	Rab11CA

Truncated Rab11a	CA Rab11a mutant lacking the C-terminal 43 hypervariable residues	Rab11CA 1-173
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GST-tagged Rab11a proteins (**Table 2.8**) were expressed in *E. coli* BL21(DE3) cells. A 10 ml overnight LB pre-culture supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin was inoculated with a single colony. This pre-culture was then used to inoculate a 1 l LB culture at a ratio of 1:100 supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin. The culture was grown at 37°C, 180 rpm shaking incubator until OD₆₀₀ reached ~0.6. At that point, expression was induced with 1 mM IPTG and cells were incubated at 37°C, 180 rpm shaking incubator for ~6 h.

Cells were harvested by centrifugation at 4,000*g* for 20 min at 4°C. The pellet was re-suspended in buffer A supplemented with 1 mg ml⁻¹ lysozyme (Sigma-Aldrich®) and lysed by sonication (3x 30 seconds on/30 seconds off). The lysate was clarified by centrifugation at 35,000*g* for 45 min at 4°C. The supernatant was incubated with pre-equilibrated Glutathione Sepharose 4B beads (GE Healthcare) (2 ml slurry per 1 l culture) on a rotor at 4°C for 4 h. After binding, the glutathione sepharose beads were washed three times with buffer B. Rab11a, Rab11CA, and Rab11CA 1-173 were eluted in buffer A by overnight cleavage of the GST tag at 4°C using 0.5 mg HRV 3C protease. GST-Rab11a, GST-Rab11CA, GST-Rab11CA Ala44-46, and GST-Rab11CA 1-173 were eluted in 10 ml buffer D overnight at 4°C and cleared from the beads by centrifugation at 1,000*g* for 5 min at 4°C. Where a further elution was required, beads were resuspended in 10 ml buffer D and remaining GST-bound proteins were eluted for additional 2 hours at 4°C.

Cleaved and eluted proteins were concentrated to 500 μ l using an Amicon Ultra centrifugal filter unit (Merck Millipore, NMWL of 3 kDa) and further purified by a SEC through a Superdex® 200 increase 10/300 column (GE Healthcare) connected to an Äkta chromatography system (GE Healthcare) at 4°C equilibrated with buffer C. Purified proteins were concentrated as above, and protein concentration was determined by measuring $A_{260/280}$. Concentrated protein was aliquoted, flash-frozen using liquid nitrogen, and stored at -80°C.

2.6.4 Bacterial expression and purification of PB2-C and 627-NLS domain

Table 2.9: Influenza A virus PB2 proteins investigated.

Virus	Residues in the PB2 subunit	PB2 details	Protein abbreviation
A/Northern Territory/60/1968 (H3N2)	246-736	Wild type	NT60 PB2-C
A/Northern Territory/60/1968 (H3N2)	246-736	K627E	NT60 PB2-C K627E
A/Northern Territory/60/1968 (H3N2)	537-736	Wild type	NT60 627-NLS
A/duck/Fujian/01/2002(H5N1)	246-736	Wild type	Fj PB2-C
A/duck/Fujian/01/2002(H5N1)	246-736	E627K	Fj PB2-C E627K

A/Viet Nam/1203/2004 (H5N1)	246-736	Wild type	Viet PB2-C
A/Viet Nam/1203/2004 (H5N1)	246-736	Single amino acid substitutions: A274T, K526R, I553V, V584I, L607V, A622T, and K627E. Double amino acid substitutions: A274T + K526R and A274T + L607V. Triple amino acid substitutions: A274T + K526R + L607V.	Viet PB2-C mutants

All PB2 proteins shown in **Table 2.9** were expressed in *E. coli* Rosetta(DE3) cells. A 10 ml overnight 2x YT pre-culture supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin and 50 $\mu\text{g ml}^{-1}$ chloramphenicol was inoculated with a single colony. This pre-culture was then used to inoculate a 1 l 2x YT culture at a ratio of 1:100 supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin and 50 $\mu\text{g ml}^{-1}$ chloramphenicol. The culture was grown at 37°C, 180 rpm shaking incubator until OD₆₀₀ reached ~0.6. At that point, expression was induced with 0.5 mM IPTG and the temperature was reduced to 18°C. After 16 h, the bacteria were pelleted by centrifugation at 4000g for 20 min at 4°C.

PB2-C NT60, PB2-C NT60 K627E, PB2-C Fj, PB2-C Fj E627K, and NT60 627-NLS were purified as Rab11a described in section 2.6.3. Cells expressing Viet PB2-C proteins were harvested by centrifugation at 4,000g for 20 min at 4°C. The pellet was re-suspended in buffer E supplemented with 100 $\mu\text{g ml}^{-1}$ RNase A, protease inhibitors (Roche, complete mini, EDTA-

free) and 1 mg ml⁻¹ lysozyme (Sigma-Aldrich®), and lysed by sonication (3x 30 seconds on/30 seconds off). The lysate was clarified by centrifugation at 35,000g for 45 min at 4°C. The supernatant was incubated with pre-equilibrated Ni-NTA Agarose matrix (Qiagen) (1 ml slurry per 1 l culture) on a rotor at 4°C for 1 h. After binding, the Ni-NTA Agarose matrix was washed three times with buffer E. Viet PB2-C proteins were eluted in 10 ml buffer E supplemented with 500 mM imidazole by rotating at 4°C for 30 min and cleared from the beads by centrifugation at 1,000g for 5 min at 4°C. Viet PB2-C proteins were then concentrated to 500 µl using an Amicon Ultra centrifugal filter unit (Merck Millipore, NMWL of 30 kDa) and further purified by a SEC through a Superdex® 200 increase 10/300 column (GE Healthcare) connected to an Äkta chromatography system (GE Healthcare) at 4°C equilibrated with buffer F. Viet PB2-C proteins were concentrated as above, and protein concentration was determined by measuring A_{260/280}. Concentrated protein was aliquoted, flash-frozen using liquid nitrogen, and stored at -80°C.

2.7 Protein analysis

2.7.1 SDS polyacrylamide gel electrophoresis

Protein samples were mixed with a 4X SDS loading dye [500 mM Tris-HCl pH 6.8, 4%(w/v) SDS, 40%(w/v) glycerol, 2 mM DTT, bromophenol blue] and heated for 5 min at 95°C prior to being loaded onto a 12% SDS-PAGE gel. Stacking gel solution contained 125 mM Tris-HCl pH 6.8, 0.1%(w/v) SDS, 0.1%(w/v) APS, 0.01%(v/v) TEMED and 4%(v/v) acrylamide/bis-acrylamide (29:1). Resolving gel solution contained 375 mM Tris-HCl pH 8.8, 0.1%(w/v) SDS, 0.1%(w/v) APS, 0.01%(v/v) and 12%(v/v) acrylamide/bis-acrylamide (29:1). Gels were run

in a mini-protean gel apparatus (Bio-Rad) at 150–220 V for ~1 h. A Precision plus protein all blue standards' (Bio-Rad) pre-stained protein marker was used as a molecular weight marker. Gels were stained with PageBlue Protein Staining Solution (ThermoFisher Scientific).

2.7.2 Western blots

The expression levels of proteins were detected by Western blot. Protein samples were separated by 8–12% SDS-PAGE before being transferred onto a nitrocellulose membrane (Amersham Biosciences) using a Mini Trans-Blot cell (BioRad) at 100 V for 75 min at 4°C. Generally, the membrane was blocked in a Phosphate buffered saline (PBS) blocking buffer [2.5%(w/v) powdered milk dissolved in PBS] for 1 h at RT followed by immunoblotting with primary antibody (diluted in blocking buffer) and a horseradish peroxide conjugated secondary antibody (diluted in blocking buffer), both incubated with a membrane for 1 h at RT. The membrane was washed 3 times after each antibody incubation with PBS. Proteins were visualised by chemiluminescence using Amersham ECL western blotting detection reagent (GE Healthcare).

2.8 Glutathione-S-transferase (GST) pull-down assay

0.2 mg of purified GST-tagged Rab11a proteins (**Table 2.8**) or GST tag were incubated with pre-equilibrated Glutathione Sepharose 4B beads (GE Healthcare) (100 μ l slurry per 1 ml sample volume) at 4°C for 3 h in binding buffer [50 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 10%(v/v) glycerol, 8 mM $MgCl_2$, 10 μ M GTP- γ -S (Abcam)]. After binding, the glutathione sepharose beads were washed three times in 1 ml of binding buffer. After the final wash, beads were resuspended in 1 ml binding buffer to which either 0.06 mg of purified heterotrimeric RdRP or 0.08 mg of purified PB2 protein was added and incubated at 4°C for 1 h. After binding, the glutathione sepharose beads were washed five times in 1 ml of the above binding buffer. After the final wash, beads were resuspended in 500 μ l binding buffer supplemented with 1 mM DTT and protein complexes were released by cleavage of the GST tag at 4°C for 1 h using HRV 3C protease (0.1 mg). Targeted protein complexes were cleared from the beads by centrifugation at 1,000g for 5 min at 4°C and remaining beads were washed three times in 1 ml binding buffer. GST pull-down protein fractions were mixed with a 4X SDS loading buffer [500 mM Tris-HCl pH 6.8, 4%(w/v) SDS, 40%(w/v) glycol, 2 mM DTT, bromophenol blue] and heated for 5 min at 95°C prior to being loaded onto a 12% SDS-PAGE gel for analysis. Proteins were visualised using PageBlue Protein Staining Solution (ThermoFisher Scientific).

2.9 Sample preparation for viral RNA and protein analysis

2.9.1 WSN infection of HEK 293T cells and cell lysis

About $4 \cdot 10^6$ HEK 293T cells in DMEM supplemented with 10%(v/v) FCS were seeded on 10 cm tissue-culture treated culture dishes (Corning®) and incubated in a 37°C, 5% CO_2

incubator for 24 h. After the incubation, DMEM medium was aspirated, and cells were washed with DPBS. Cells were then infected with WSN virus (**Table 2.5**) at an MOI of 5 or mock infected in DMEM supplemented with 0.5%(v/v) FCS and incubated in a 37°C, 5% CO₂ incubator for 12 h. After the incubation, cells were released by pipetting and transferred into 15 ml conical-bottom centrifuge Falcon tubes. Culture dishes were washed with 5 ml cold DPBS to remove remaining cells which were then transferred into Falcon tubes. Cells were pelleted by centrifugation at 1,000 rpm (Hettich ROTINA 420, rotor 5624) for 5 min at 4°C. The pellets were washed with 5 ml cold DPBS and spun at 1,000 rpm (Hettich ROTINA 420, rotor 5624) for 5 min at 4°C. Washed pellets were resuspended in 1 ml cold DPBS and cells were transferred into microcentrifuge tubes. Cells were harvested by centrifugation at 1,000 rpm (Thermo Scientific™ Fresco™ 21 Microcentrifuge, rotor 75003424) for 5 min at 4°C and pellets were lysed on a rotor at 4°C for 1 h in 550 µl lysis buffer [50 mM HEPES-NaOH pH 7.5, 200 mM NaCl, 0.5%(v/v) IGEPAL® CA-630 (Sigma-Aldrich®), 1 mM β-mercaptoethanol, 1x PMSF, 1x protease inhibitor (Roche, complete mini, EDTA-free)]. Whole cell lysates (WCL) were collected by centrifugation at 13,000 rpm (Thermo Scientific™ Fresco™ 21 Microcentrifuge, rotor 75003424) for 5 min at 4°C. 50 µl of infected or mock infected WCL was kept while the rest were used in a GST pull-down assay (see next section).

2.9.2 GST pull-down assay of cell lysates

0.2 mg of purified GST-Rab11CA or GST tag (expressed and purified by Haitian Fan) were incubated with pre-equilibrated Glutathione Sepharose 4B beads (GE Healthcare) (100 µl slurry per 1 ml sample volume) at 4°C for 3 h in binding buffer [50 mM HEPES-NaOH pH

7.5, 300 mM NaCl, 10%(v/v) glycerol, 8 mM MgCl₂, 10 µM GTP-gamma-S (Abcam)]. After binding, the glutathione sepharose beads were washed three times and resuspended in 750 µl binding buffer to which 500 µl of infected or mock infected WCL was added and incubated at 4°C for 1 h (WSN infected WCL + GST-Rab11CA, WSN infected WCL + GST tag, mock infected WCL + GST-Rab11CA). After binding, the glutathione sepharose beads were washed five times in 1 ml of the above binding buffer. Beads were resuspended in 130 µl binding buffer supplemented with 1 mM DTT and protein complexes were released by cleavage of the GST tag at 4°C for 1 h using HRV 3C protease (0.1 mg). Targeted protein complexes were cleared from the beads by centrifugation at 1,000g for 5 min at 4°C and beads were washed three times in 1 ml binding buffer. Protein was spun at 13,000g for 5 min at 4°C to remove any remaining beads. 30 µl of cleaved sample along with other GST pull-down protein fractions was mixed with a 4X SDS loading dye [500 mM Tris-HCl pH 6.8, 4%(w/v) SDS, 40%(w/v) glycerol, 2 mM DTT, bromophenol blue] and heated for 5 minutes at 95°C prior to being loaded onto a 12% SDS-PAGE gel for analysis. The remaining 100 µl of cleaved sample was used for either RNA extraction (section 2.10.1) or mass spectrometry (section 2.11.1) to analyse viral RNA and protein, respectively.

2.10 Viral RNA analysis

2.10.1 RNA extraction

Total RNA was extracted from cells using TRI Reagent (Sigma-Aldrich®) according to manufacturer's instructions. Approximately 6·10⁶ cells were lysed in 1 ml of TRI Reagent and incubated for 5 min at RT. 200 µl of chloroform was added and samples were vigorously agitated, followed by centrifugation at 17,000g for 15 min at 4°C. The clear

upper aqueous phase was transferred to fresh Eppendorf tubes and supplemented with 1 μl of Glycogen, RNA grade (20 mg ml^{-1}) (ThermoFisher Scientific) and 500 μl of 100%(v/v) isopropanol. RNA was precipitated by centrifugation at $17,000g$ for 15 min at 4°C . RNA pellets were washed in 70%(v/v) ethanol and dissolved in 50 μl of nuclease-free water (Invitrogen™). Samples were stored at -20°C .

2.10.2 Primer extension analysis

Primer extension assay was done by Jane Sharps.

For oligonucleotide radiolabelling, 1 μl of oligonucleotide at $10\text{ }\mu\text{M}$ was mixed with 1 μl [γ - ^{32}P]ATP ($\sim 6000\text{ Ci/mmol}$, 10 mCi/ml , Perkin Elmer), 1 μl PNK enzyme (Fermentas) and 1 μl PNK buffer A in a total reaction volume of 10 μl . This was incubated at 37°C for 1 h. The oligonucleotides were isolated using a QiaQuick Nucleotide Removal Kit (Qiagen) following manufacturer's instructions, eluting in 30-50 μl nuclease-free water (Ambion).

The RNA content of samples was analysed by primer extension. 1-4 μl isolated RNA were mixed with the appropriate radiolabelled primers; usually 0.25 μl vRNA binding primer and 0.25 μl m/cRNA binding primer in a total reaction volume of 5 μl . Where total cellular RNA was analysed, a 5S rRNA binding primer was also included: 0.1 μl radiolabelled 5S rRNA primer and 0.9 μl non-radiolabelled 5S rRNA primer. This was incubated at 95°C for 4 min before placed on ice. The samples were then heated to 50°C and 5 μl of superscript containing master mix [20 mM DTT, 1x First Strand buffer (Invitrogen), 10 U/ μl SuperScript III (Invitrogen), 1 mM dNTPs] were added per reaction. The reaction was incubated at 50°C

for 1 h, followed by a 10 min incubation at 70°C. The samples were then analysed by RNA polyacrylamide gel electrophoresis.

Samples were heated to 95°C for 3 min, then mixed with 10 µl of formamide loading dye (9 ml formamide, 200 µl 0.5 M EDTA pH 8, bromophenol blue, xylene blue) and heated again to 95°C for 3 min. Samples were then run on 6% acrylamide/bis-acrylamide (19:1) gels, containing 7 M urea and 1X TBE buffer (90 mM Tris base, 90 mM boric acid, 2.5 mM EDTA) polymerised with 0.1%(w/v) ammonium persulphate and 0.01%(v/v) TEMED. The gels were run at 1300 V for 1 h 10 min in 1x TBE. Bands were detected on phosphor screen imaging plates (Fujifilm), which were scanned on a Fuji FLA-5000 scanner for quantitation.

2.11 Viral protein analysis

2.11.1 Sample preparation for proteomics analysis

All chemicals used for preparation of liquid chromatography and tandem mass spectrometry (LC-MS/MS) samples were of mas spec grade and purchased from Sigma-Aldrich®. Protein samples (section 2.9.2) were first denatured at RT by shaking at 800 rpm (Mixer HC, STARLAB) for 10 min in 4 M urea [8M stock prepared in 50 mM TEAB pH 8.5]. Cysteines were reduced in 10 mM TCEP pH 7.0 at RT for 30 min and alkylated with 50 mM 2-Chloroacetamide (0.5 M stock made in 8 M stock urea) at RT in the dark for 30 min. Samples were then pre-digested for 2 h in a 37°C shaking heat block using Lys-C at a ratio of 1 µg of Lys-C per 100 µg of protein sample. After incubation, urea was diluted to 2 M in 50 mM TEAB, and CaCl₂ at a final concentration of 2 mM was added to the samples. Finally, samples were trypsin digested overnight in a 37°C shaking heat block using 1 µg of trypsin

for 40 µg of protein sample. Trypsin reaction was quenched with 5%(v/v) formic acid and supernatants were collected by centrifugation at 14,000 rpm (Thermo Scientific™ Fresco™ 21 Microcentrifuge, rotor 75003424) for 30 min at 4°C. Samples were stored at -20°C.

2.11.2 Mass spectrometry

LC-MS/MS and data analyses were performed by Dr Marjorie Fournier at The Advanced Proteomics Facility (University of Oxford).

Peptides were separated by nano liquid chromatography (Thermo Scientific Ultimate RSLC 3000) coupled in line a Q Exactive mass spectrometer equipped with an Easy-Spray source (Thermo Fischer Scientific). Peptides were trapped onto a C18 PepMac100 precolumn (300 µm i.d.x5mm, 100 Å, ThermoFischer Scientific) using Solvent A (0.1%(v/v) formic acid, HPLC grade water). The peptides were further separated onto an Easy-Spray RSLC C18 column (75 µm i.d., 50 cm length, Thermo Fischer Scientific) using a 60 min linear gradient [15%-35% solvent B [0.1%(v/v) formic acid in acetonitrile]] at a flow rate 200 nl min⁻¹. The raw data were acquired on the mass spectrometer in a data-dependent acquisition mode (DDA). Full-scan MS spectra were acquired in the Orbitrap (Scan range 350-1500 m/z, resolution 70,000; AGC target, 3e⁶, maximum injection time, 50 ms). The 10 most intense peaks were selected for higher-energy collision dissociation (HCD) fragmentation at 30% of normalised collision energy. HCD spectra were acquired in the Orbitrap at resolution 17,500, automatic gain control (AGC) target 5e⁴, maximum injection time 60 ms with fixed mass at 180 m/z. Charge exclusion was selected for unassigned and 1⁺ ions. The dynamic exclusion was set to 40 s.

2.11.3 Database searching

Tandem mass spectra were searched using SEQUEST HT within Proteome discoverer PD1.4 (Thermo Fischer Scientific, version 1.4.0.288) against a database containing 20418 protein entries combining protein sequences from *Homo sapiens* in which the endogenous Rab11a protein sequence has been replaced by GST-tagged Rab11a, IAV A/WSN/1933 (H1N1), and common contaminants. During database searches, cysteines (C) were considered to be fully carbamidomethylated (+57,0215, statically added), methionine (M) to be fully oxidised (+15,9949, dynamically added), and all N-terminal residues and lysine (K) to be acetylated (+42,0106, dynamically added). Two missed cleavages were permitted. Peptide mass tolerance was set at 50 ppm and 0.02 Da on the precursor and fragment ions, respectively. Protein identification was filtered at false discovery rate (FDR) below 1%.

2.11.4 Quantitative proteomics and statistical analyses

GST-Rab11a interacting protein partners were identified by quantitative proteomic and statistical analyses, after sorting out proteins significantly enriched in GST-Rab11a protein dataset versus GST (negative control) in control and infected conditions. The quantitative analysis was based on a label-free quantitation method using normalized spectral abundance factor (NSAF), as a measure of relative protein abundance within the protein mixture. Spectral abundance factor (SAF) and NSAF values were calculated as previously described (Florens *et al.*, 2006), as the number of spectral counts (PSM) that identify a protein, divided by the protein length (L), the PSM/L value represents the SAF, which is then divided by the sum of PSM/L for all proteins in the experiment. NSAF values were

calculated after contaminants proteins being removed such as keratins, endoproteinase Lys-C, trypsin and immunoglobulins. For better visualization of the data, NSAF values were multiplied by 100 (NSAF·100). The statistical analysis was performed on NSAF values from three biological replicate experiments, using a power law global error model (PLGEM) (Pavelka *et al.*, 2008). The signal-to-noise (STN) ratio calculated from PLGEM analysis indicates the level of enrichment for each protein identified in GST-Rab11a versus GST pull down. The p-value indicates the significance of the enrichment. Before log transformation of the data, zero values were replaced by a minimum value in the dataset.

The mass spectrometry proteomics data is currently being deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol *et al.*, 2019).

2.12 Electron microscopy (EM)

2.12.1 Cryo-EM sample preparation

vRNA-bound NT60 RdRP was purified as described in section 2.6.2. 50 ml of *E. coli* BL21(DE3) cells expressing GST-Rab11a were harvested by centrifugation at 4,000g for 10 min at 4°C. The pellet was re-suspended in buffer A (**Table 2.6**) and lysed by sonication (3x 30 seconds on/30 seconds off). The lysate was clarified by centrifugation at 4,000g for 30 min at 4°C. The supernatant was incubated in buffer A with pre-equilibrated Glutathione Sepharose 4B beads (GE Healthcare) (100 µl slurry) on a rotor at 4°C for 4 h. After binding, the glutathione sepharose beads were washed three times with buffer B (**Table 2.6**). After the final wash, beads were resuspended in 1 ml buffer B to which 0.06 mg of purified vRNA-bound NT60 RdRP protein was added and incubated at 4°C for 2 h. After binding, the

glutathione sepharose beads were washed five times in 1 ml of buffer B. After the final wash, beads were resuspended in 1 ml buffer A and protein complex was released by cleavage of the GST tag at 4°C overnight using 3C protease (0.1 mg). Targeted protein complex was cleared from the beads by centrifugation at 1,000g for 5 min at 4°C. Protein was then spun at 13,000g for 5 min at 4°C to remove any remaining beads. Protein surface charges were neutralised as described elsewhere (Fan *et al.*, 2019) prior to re-injection on a Superdex™ 200 Increase 10/300 GL column at 4°C equilibrated with a buffer consisting of 25 mM HEPES-NaOH pH 7.5, 150 mM NaCl. The fractions of interest were pooled together and concentrated to 0.24 mg ml⁻¹ using an Amicon Ultra centrifugal filter unit (Merck Millipore, NMWL of 100 kDa). 3.5 µl of complex was applied on glow discharged gold Quantifoil R2/1 grids (2 µm holes, 1.0 µm spacing, 200 mesh) (Electron Microscopy Sciences) before plunging into liquid nitrogen-cooled liquid ethane using Vitrobot Mark IV (FEI) (ThermoFisher Scientific). The Vitrobot was set to plunge at 20°C, 100% humidity, -25 blot-force, 3 s blotting time. Samples were stored at liquid nitrogen temperatures until further use.

2.12.2 Cryo-EM data collection and processing

All data were collected and processed by Dr Loïc Carrique at The Division of Structural Biology (University of Oxford).

Data were collected on a 300 keV Titan Krios G3i microscope (ThermoFisher Scientific) using K2 summit direct electron detector (Gatan) fitted with a GIF Quantum energy filter. Automated data collection was performed using EPU2.1 software (ThermoFisher Scientific)

at a normal magnification of 105k, given a calibrated pixel size of 1.34 Å, over a defocus range between -2 to -3.5 µm. Movies were collected in counting mode with total dose of around 30 e Å⁻² spread over 32 frames. The 2,059 movies were aligned using MotionCor2-1.1.0 with a 5 by 5 patch-based alignment, keeping all the frames and dose weighting up to the total exposure. The contrast transfer function (CTF) of full dose non-weighted micrographs was estimated using Gctf-v1.06 (Zhang, 2016). Non-weighted micrographs were imported in cryoSPARC(v2.9) (Punjani *et al.*, 2017) where particles were automatically picked using the blob picker and 2D classified. Selected classes were used as a template for automatic picking with the template picker and 2D classified. 3 ab-initio models were generated using the selected 2D classes (335,747 particles) and refined using heterogenous refinement. Each class was refined independently using non-uniform refinement. Particles from the best class were imported into RELION(3.0.4) (Scheres, 2012), re-extracted on dose weighted micrographs and refined to 3.54 Å. Bayesian polishing, CTF refinement/particle and beam-tilt correction were performed improving map resolution to 3.38 Å. 3D classification without alignment across 10 classes was performed and the selected class showing extra density for the PB2 C-terminal domain was refined to 4.19 Å. The structure was modelled by rigid body fitting individual domains of the model into the locally filtered map using UCSF Chimera (Pettersen *et al.*, 2004) and PHENIX rigid body refinement (Adams *et al.*, 2010).

2.13 Size exclusion chromatography coupled with a small-angle X-ray scattering (SEC-SAXS)

2.13.1 SEC-SAXS sample preparation

Rab11CA 1-173 was purified as described in section 2.6.3, buffer exchanged, and concentrated to 6 mg ml⁻¹. Viet PB2-C was purified as described in section 2.6.4, buffer exchanged, and concentrated to 10 mg ml⁻¹. Both proteins were buffer exchanged to SEC-SAXS running buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1%(v/v) glycerol, 1 mM β -mercaptoethanol, 8 mM MgCl₂, 10 μ M GTP γ S). 50-70 μ l of each sample was prepared: Viet PB2-C, Rab11CA 1-173, Viet PB2-C:Rab11CA 1-173 = 1:0.5, Viet PB2-C:Rab11CA 1-173 = 1:1, Viet PB2-C:Rab11CA 1-173 = 1:1.5, Viet PB2-C:Rab11CA 1-173 = 1:2. All samples were kept at 4°C overnight before being transferred to Diamond Light Source.

2.13.2 SEC-SAXS experiments and data analyses

The SEC-SAXS experiments were performed at beamline B21, Diamond Light Source (Didcot, UK) by Dr Katsuaki Inoue (Cowieson *et al.*, 2020). Samples were loaded onto a Shodex KW403-4F column (4.6 mm ID x 300 mm long) which was connected into Agilent 1200 HPLC system (Waters) at a flow rate of 0.16 ml min⁻¹. The SEC separated sample was exposed to X-rays in a 1.6 mm diameter, 10 μ m thick quartz capillary flow cell, followed by data collection every 3 seconds with EIGER 4M (Dectris) detector. The X-ray beam size was 1 mm (horizontal) x 0.5 mm (vertical) at the sample position and 0.08 mm (horizontal) x 0.07 mm (vertical) at the focal point (the detector surface). The wavelength of X-ray was 0.95 Å and the sample-detector distance was 3.7 m. The measurement temperature was 20°C. Raw SAXS 2D images were processed with DAWN (<https://dawnsci.org/>) processing

pipeline to produce normalised, integrated 1D un-subtracted SAXS curves. The background subtraction, averaging of the data, and all other subsequent analysis were performed using programs in the ASTAS 3.0.4 package (Petoukhov *et al.*, 2012).

2.14 Full field cryo-X-ray microscopy

2.14.1 Cryo-soft X-ray tomography (cryo-SXT) sample preparation

A549, U2OS, and U2OS cells stably expressing mRuby-Rab11CA were seeded onto 3.05 mm gold transmission electron microscopy (TEM) grids (Quantifoil AU G200F1 finder) and grown in DMEM supplemented with 10%(v/v) FCS. To encourage cell attachment, grids were soaked in 100%(v/v) FCS prior to cell culture and kept in the 37°C, 5% CO₂ incubator overnight. Prior to infection, conventional brightfield microscopy was used to check the confluency and distribution of individual cells on TEM grids. At ~60% confluency media was aspirated from wells and cells were washed with DPBS. Cells were then infected with either WSN or WSN PA-GFP virus at an MOI of 5, or mock infected. Plates were incubated for however hours needed at 37°C, 5% CO₂ before being frozen in liquid nitrogen-cooled liquid ethane using a Leica EM GP2 plunge freezer. Twenty minutes before plunging, MitoTracker™ Deep Red (ThermoFisher Scientific) was added in the virus solution and in the media (mock infected) at 25 nM final concentration. NucBlue™ Fixed Cell ReadyProbes™ Reagent (Thermo Fisher Scientific) was also added at the same time according to manufacturers' instructions. Plates were put back in the 37°C, 5% CO₂ incubator to allow cells to uptake the dyes. Prior to plunge freezing, 2 µl of 250 nm gold nanoparticle fiducial markers (BBi Solutions) were added for later use in data processing of

tilt series reconstruction and excess liquid was blotted away. Leica EM GP2 was set to plunge at -170°C, 70% humidity, 1-2 s blotting time. Samples were stored at liquid nitrogen temperatures until further use.

2.14.2 Cryo-structured illumination microscopy (cryo-SIM)

All data were collected at beamline B24, Diamond Light Source (Didcot, UK). Vitrified samples on TEM grids were transferred to the cryo-SIM where cells, objectives and microscope were kept at liquid nitrogen-cooled temperature. Mosaic images were generated using a brightfield imaging to evaluate the location (likely to allow data collection on both this instrument and the TXM) and overall state (no obvious grid surface or cell sample disruption) of the individual cells. Samples containing A549 and U2OS cells of interest were then imaged in green (488 nm Omicron Deepstar polarised illumination beam using 525 nm filter and 100 ms exposure), far red (647 nm Omicron Deepstar polarised illumination beam using 655 nm filter and 2-5 ms exposure), and blue (405 nm Omicron Deepstar polarised illumination beam using 452 nm filter and 60-100 ms exposure) fluorescence to identify individual cells within the population that were both infected (or mock infected) with fluorescent virus, and expressed fluorescently labelled nucleus, and mitochondria. Samples containing U2OS cells stably expressing mRuby-Rab11CA were also imaged in red (561 nm COBOLT Jive™ polarised illumination beam using 605 nm filter and 50-80 ms exposure) to identify individual cells within the population that were expressing fluorescent Rab11CA. 3D-SIM data was collected using opensource software packages Cockpit and python-microscope (Micron, Oxford University) on number of each representative cells.

2.14.3 Cryo-SXT data collection and reconstruction

Data were collected and processed at the Diamond Light Source beamline with an UltraXRM-S/L220c X-ray microscope (Carl Zeiss X-ray Microscopy, Inc.) operating at 500 eV. This instrument is fitted with a capillary condenser, a 40 nm zone plate objective (25 nm for the BSC-1 work) and a 1024B Pixis CCD camera (Princeton instruments). Samples were loaded into the microscope chamber in batches of four. To locate the areas of interest and to assess sample's structural integrity and alignment potential, the grids were first examined using an in-line visible light (20x objective). To provide an overview of cells, whole grid squares were examined using low-dose X-ray exposures to collect mosaic images. Tilt series were collected from -65° to $+70^{\circ}$ with $0.5^{\circ}/1^{\circ}$ step size. Exposure times were in the range of a few seconds per projection. Background flat field images were collected to correct for fixed pattern noise present in the beam. All data were aligned and reconstructed automatically to tomograms using the in-house pipeline which employs Batchruntomo (Mastronarde and Held, 2017), which is a part of the IMOD package (Kremer *et al.*, 1996), via several scripts to allow parallel and near real-time processing. Specifically, both alignment and reconstruction were done in IMOD(4.9.10) (Kremer *et al.*, 1996) using weighted back projection protocol (Gilbert, 1972).

3 The influenza virus polymerase interacts directly with Rab11a

3.1 Introduction

In the course of IAV infection, upon successful viral entry, the eight vRNPs are trafficked into the nucleus of the host cell, where the RdRP performs the distinct processes of transcription of viral mRNA and replication of vRNA in the context of vRNP (te Velthuis and Fodor, 2016). Newly synthesised vRNPs are then exported to the cytoplasm where they initially accumulate around the microtubule organising centre before being distributed throughout the cytoplasm, concentrating in discrete puncta, originating from recycling endosomes, which have been shown to enlarge as infection progresses (Alenquer *et al.*, 2019; Amorim *et al.*, 2011; Chou *et al.*, 2013; de Castro Martin *et al.*, 2017; Einfeld *et al.*, 2011; Lakdawala *et al.*, 2014; Vale-Costa *et al.*, 2016). Each punctum accommodates different vRNPs, forming so-called irregularly coated vesicles (ICVs), the formation of which has been shown to require an active, GTP-bound form of the cellular GTPase Rab11a (Amorim *et al.*, 2011; de Castro Martin *et al.*, 2017; Einfeld *et al.*, 2011; Lakdawala *et al.*, 2016; Momose *et al.*, 2011). In uninfected cells, Rab11a is the master regulator of the recycling endosome, delivering endocytosed proteins and lipids to the cell surface upon binding to its effectors, the Rab11-family-interacting-proteins (Rab11-FIPs) (de Castro Martin *et al.*, 2017; Vale-Costa *et al.*, 2016). Infected cells, however, show alterations in Rab11a distribution as well as in recycling pathway efficiency, which has been proposed to be related to the fact that vRNPs outcompete Rab11-FIPs and prevent them from binding to Rab11a (Bhagwat *et al.*, 2020; Vale-Costa *et al.*, 2016).

Previously published reports suggest that Rab11a (directly or indirectly) interacts with vRNPs in infected cells (Amorim *et al.*, 2011; Einfeld *et al.*, 2011; Momose *et al.*, 2011). In corroboration, different research groups have independently observed Rab11a-dependent ICVs, IAV-infection specific organelles, beneath the plasma membrane from which vRNPs, but not Rab11, reach the plasma membrane where virion assembly, budding and release occur (Alenquer *et al.*, 2019; de Castro Martin *et al.*, 2017; Vale-Costa *et al.*, 2016). A pull-down assay in cells expressing the individual vRNP components further revealed that only the RdRP PB2 subunit precipitated with GFP-Rab11a (Amorim *et al.*, 2011), suggesting the PB2 as the mediator of the interaction between vRNP and Rab11a. This result was later confirmed by Förster resonance energy transfer (FRET) microscopy (Avilov *et al.*, 2012a). Despite active research in this area, most of the studies focused on investigating the morphology and cellular spatial arrangements of Rab11a vesicles carrying vRNPs at an ultrastructural level (Alenquer *et al.*, 2019; Bhagwat *et al.*, 2020; Chou *et al.*, 2013; de Castro Martin *et al.*, 2017; Han *et al.*, 2021; Vale-Costa *et al.*, 2016) while the precise mechanism of the interaction between vRNPs and Rab11a at a structural level was never addressed.

The aim of this study was to further study how the viral RdRP is a mediator of the interaction between vRNP and Rab11a by using recombinantly expressed proteins in a glutathione S-transferase (GST) pull-down assay. Using heterotrimeric RdRP lacking the PB2⁶²⁷ domain, and individually expressed PB2-C protein, I also characterise a potential role of the PB2 subunit in this interaction. A more detailed understanding of the molecular basis of this interaction could lead to the development of a next-generation antiviral drug

compound that would disrupt the interaction of Rab11a with the IAV RdRP, thereby affecting the propagation of the IAV.

3.2 Results

3.2.1 IAV RdRP directly interacts with Rab11a

In order to determine, whether the viral RdRP mediates the interaction between Rab11a and vRNP, I used recombinantly expressed RdRP and Rab11a proteins in a GST pull-down assay. Firstly, I expressed and purified recombinant heterotrimeric RdRP from the influenza A/Northern Territory/60/1968 (H3N2) virus using baculovirus available in the lab containing polymerase genes cloned by Dr Narin Hengrung (**Figure 3.1A**). For simplicity, I will refer to the influenza A/Northern Territory/60/1968 (H3N2) virus RdRP as NT60 RdRP. SDS-PAGE analysis of purified NT60 RdRP from cell lysates using the protein-A tag on PB2 subunit separated by a tobacco etch virus (TEV) cleavage site confirmed the presence of proteins with molecular weights that correspond to RdRP subunits (83 kDa PA, 85 kDa PB2, 86 kDa PB1) (**Figure 3.1B**). NT60 RdRP was further purified by a size exclusion chromatography (SEC) as both a putative monomeric and putative dimeric complex, confirming previous observations that IAV RdRP can form dimers in solution (Fan *et al.*, 2019) (**Figure 3.1C**). To confirm the presence of NT60 RdRP in both elution peaks, I analysed SEC fractions 3-9 by SDS-PAGE (**Figure 3.1D**). NT60 RdRP was detected in fractions 4-6, which correspond to the putative RdRP dimer (peak 1), as well as in fractions 7-8, which correspond to the putative RdRP monomer (peak 2). Then, I recombinantly expressed Rab11a as a fusion protein with a GST affinity tag at its N-terminus separated by a HRV 3C

protease (3C) cleavage site (**Figure 3.2A**). Since the interaction between vRNPs and Rab11a requires an active, GTP-bound conformation of Rab11a (Amorim *et al.*, 2011; Eisfeld *et al.*, 2011; Momose *et al.*, 2011), I generated a constitutively active mutant of Rab11a (Rab11CA) by PCR site-directed mutagenesis, replacing glutamine (Q) at position 70 to leucine (L) to favour the GTP-bound form (Uhlig *et al.*, 2006). Both, wild type Rab11a and Rab11CA were then purified as fusion proteins with an N-terminal GST tag to enable their immobilisation on glutathione sepharose beads. The correct molecular weight of GST-Rab11a fusion protein (~50 kDa) was confirmed by SDS-PAGE (**Figure 3.2B**). Interestingly, both wild type and constitutively active GST-Rab11a were eluted in a main peak with a shoulder peak (**Figure 3.2C**), which could represent two different conformations of Rab11a, a compact and an extended conformation (Receveur-Brechot and Durand, 2012). To confirm the presence of Rab11a and Rab11CA in the main elution peak, I analysed both purified proteins by SDS-PAGE (**Figure 3.2D**). I then designed a GST pull-down assay where either GST-Rab11a or GST-Rab11CA was immobilised on Glutathione-Sepharose beads to test its interaction with heterotrimeric RdRP. Purified proteins were incubated in a buffer containing $MgCl_2$ and GTPgammaS (GTP γ S), a non-hydrolysable analogue of guanosine triphosphate (GTP), as both Mg^{2+} ion and GTP are essential for Rab11a activation (Pasqualato *et al.*, 2004). A GST tag protein was included as a negative control. SDS-PAGE analysis of pull-down fractions showed that NT60 RdRP binds to immobilised GST-Rab11a (**Figure 3.3A, bound**), and remains bound to Rab11a released from the beads using 3C protease (**Figure 3.3A, cleaved**). As a control, I incubated purified NT60 RdRP with immobilised GST tag which showed no NT60 RdRP binding (**Figure 3.3B, bound**). Similarly, SDS-PAGE analysis of pull-down fractions showed NT60 RdRP bound to immobilised GST-

Rab11CA (**Figure 3.3C, bound**) as well as to Rab11CA released from the beads (**Figure 3.3C, cleaved**). As Rab11CA appeared to consistently bind higher levels of RdRP, I used it in all subsequent pull-down assays in this study.

To further assess the association between RdRP and Rab11a, I purified recombinant heterotrimeric RdRP from a different IAV subtype, i.e., from the influenza A/Brevig Mission/1/1918 (H1N1) virus (hereafter referred to as 1918 RdRP), cloned and expressed by Dr Jeremy Keown. SDS-PAGE analysis of pull-down fractions showed that 1918 RdRP binds to immobilised GST-Rab11CA (**Figure 3.4A, bound**), and remains bound to Rab11CA after the cleavage (**Figure 3.4A, cleaved**). As expected, there was no non-specific binding of 1918 RdRP to the immobilised GST tag (**Figure 3.4B, bound**).

Finally, I tested the ability of the NT60 RdRP to form a complex with Rab11a when bound to either vRNA or cRNA promoter. Firstly, NT60 RdRP was bound to vRNA promoter, which comprised a 15-nt-long RNA corresponding to the 5' end of vRNA and a 14-nt-long RNA corresponding to the 3' end of vRNA. The presence of vRNA promoter was confirmed by measuring the ratio of absorbance at 260 nm and 280 nm ($A_{260/280}$). In contrast to RNA-free NT60 RdRP which $A_{260/280}$ was 0.67, the $A_{260/280}$ of 0.99 confirmed the presence of vRNA promoter. NT60 RdRP formed a complex with Rab11CA when bound to vRNA (**Figure 3.5A, cleaved**) while there was no non-specific binding of vRNA-bound NT60 RdRP to immobilised GST tag (**Figure 3.5B, bound**). Then, NT60 RdRP was bound to cRNA promoter, which comprised a 14-nt-long RNA corresponding to the 5' end of cRNA and a 15-nt-long RNA corresponding to the 3' end of cRNA. The $A_{260/280}$ of 0.97 was used to confirm the presence of cRNA promoter. NT60 RdRP also formed a complex with Rab11CA when bound

to cRNA promoter (**Figure 3.5C, cleaved**). As expected, there was no non-specific binding of cRNA-bound NT60 RdRP to immobilised GST tag (**Figure 3.5D, bound**).

Taken together, these results show that RdRP and Rab11a form a complex *in vitro* and establish that the RdRP-Rab11a interaction is direct. This is consistent with previous reports showing that vRNPs interact with Rab11a through the RdRP (Amorim *et al.*, 2011; Momose *et al.*, 2011), and suggestions that this interaction is direct (Avilov *et al.*, 2012a).

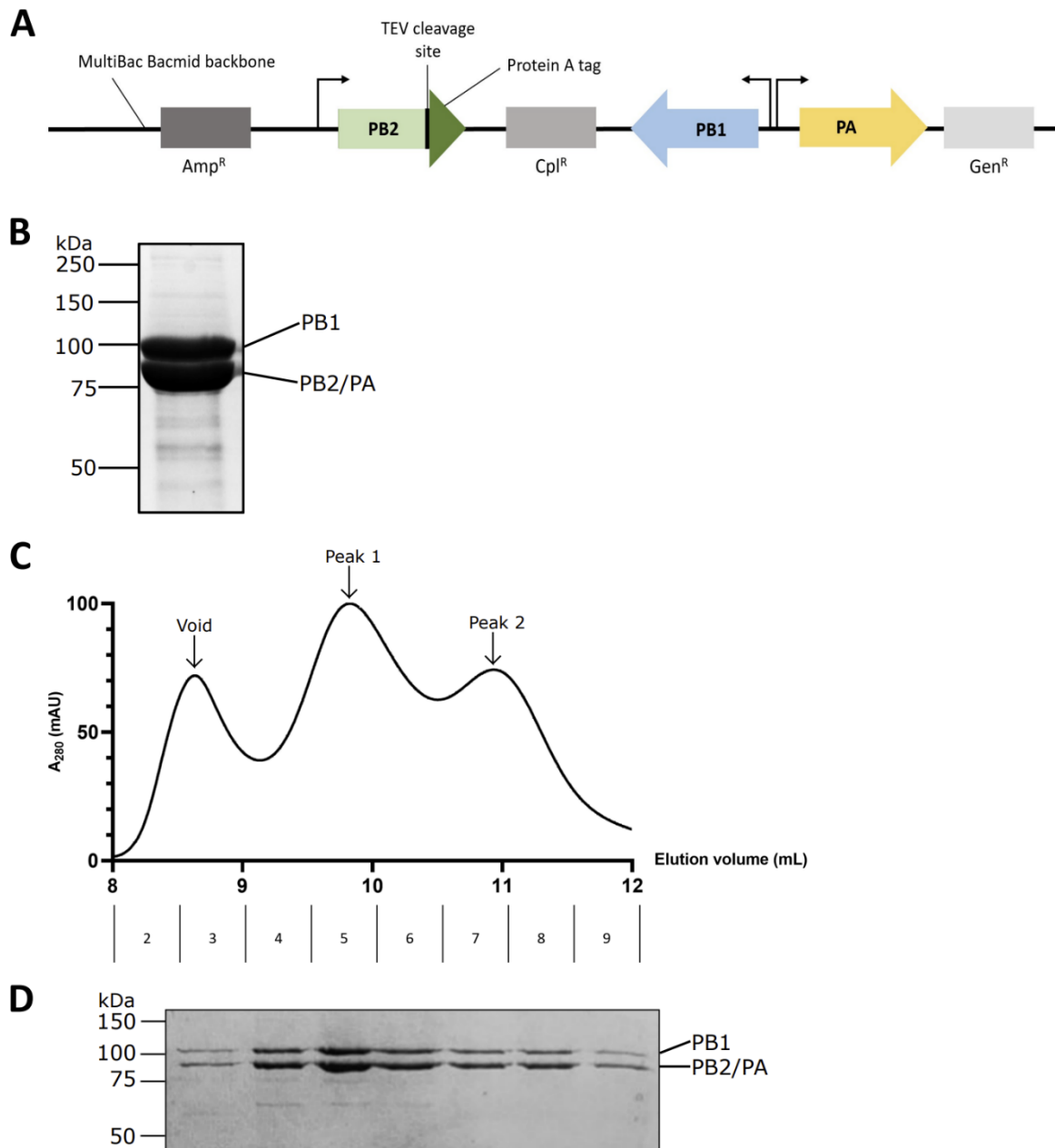


Figure 3.1: Design and purification of recombinant heterotrimeric influenza virus RdRP from influenza A/Northern Territory/60/1968 (H3N2) virus.

(A) A schematic of a recombinant baculovirus, generated by Dr Narin Hengrung, used for expression of the influenza virus RdRP genes in insect cells. In addition to polymerase genes, genes for ampicillin, chloramphenicol, and gentamicin resistance (Amp^R, Cpl^R, and Gen^R, respectively) are also annotated. Direction of the open reading frames are indicated with arrows. **(B)** Heterotrimeric IAV RdRP was expressed in insect cells and purified by

affinity chromatography using the protein-A tag on the PB2 subunit. Purified recombinant RdRP was analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. **(C)** Elution profile of size exclusion chromatography (SEC) on a Superdex® 200 increase 10/300 column of purified recombinant RdRP. Gel filtration chromatogram of RdRP shows the presence of a putative RdRP dimer (peak 1) and a putative monomer (peak 2) (Fan *et al.*, 2019). **(D)** SEC fractions 3-9 were collected and analysed by 12% SDS-PAGE reducing gel to confirm the presence of NT60 RdRP. Fractions 4-6 correspond to the putative dimer (peak 1) while fractions 7-9 correspond to the putative monomer (peak 2). Bands were visualised using Coomassie Brilliant Blue R-250 dye.

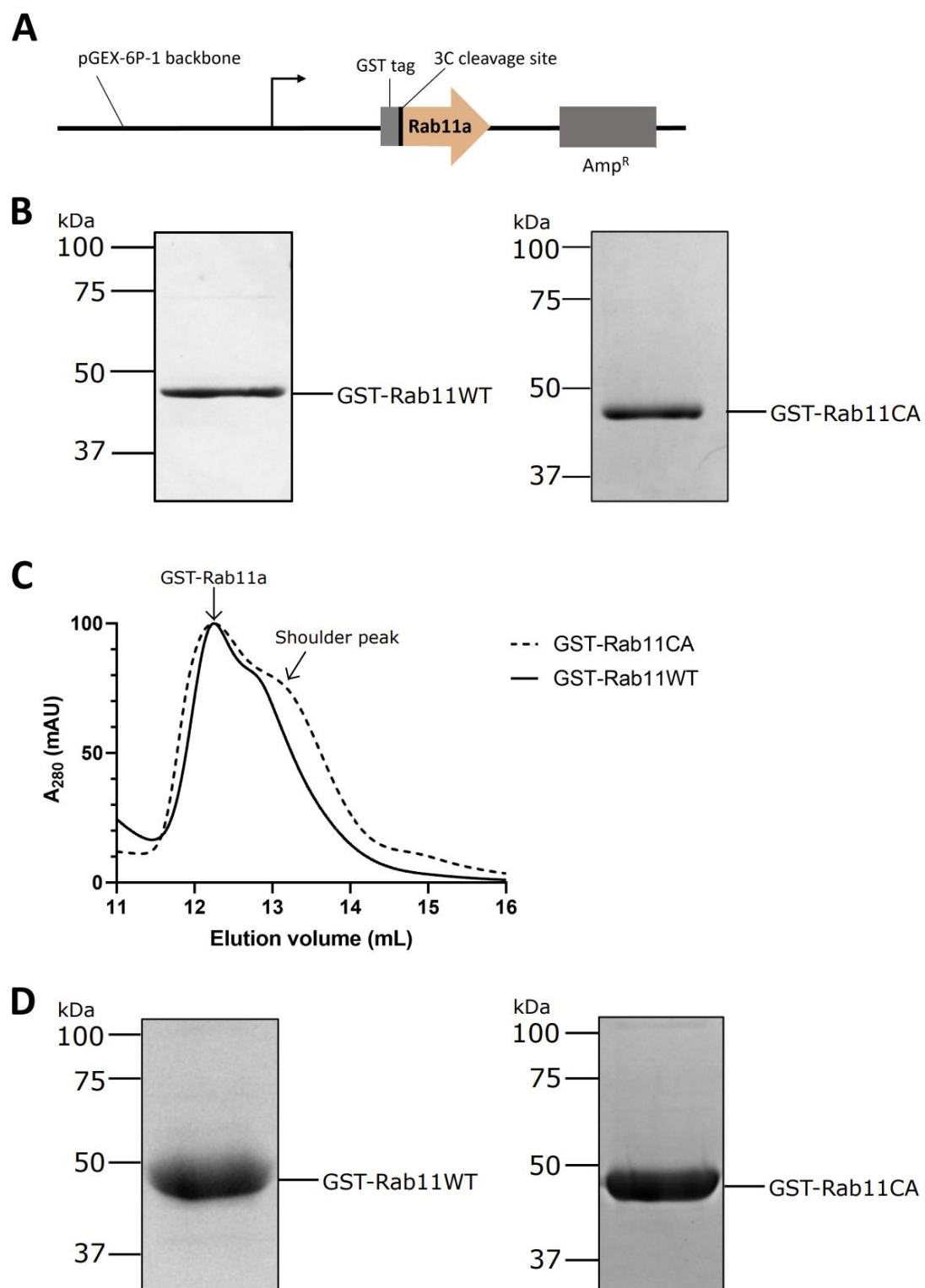


Figure 3.2: Design and purification of recombinant GST-tagged Rab11a.

(A) A schematic of Rab11a plasmid. Full length Rab11a was cloned into the vector pGEX-6P-1 with an N-terminal GST tag separated by a HRV 3C protease (3C) cleavage site.

Direction of the open reading frame is indicated with the arrow. **(B)** Recombinant Rab11a and Rab11CA were expressed in *E. coli* cells and purified by affinity chromatography as fusion proteins with a GST tag on the N-terminus. GST-Rab11a and GST-Rab11CA fusion proteins were then eluted under mild, non-denaturing conditions using reduced glutathione, and analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. **(C)** Elution profile of size exclusion chromatography (SEC) on a Superdex[®] 200 increase 10/300 column of eluted recombinant wild type GST-Rab11a (GST-Rab11WT) and GST-Rab11CA which both eluted in the main GST-Rab11a peak with a shoulder peak. **(D)** The main SEC peak of GST-Rab11WT was collected and analysed by 12% SDS-PAGE reducing gel (left) side by side with the collected main SEC peak of GST-Rab11CA (right). Bands were visualised using Coomassie Brilliant Blue R-250 dye.

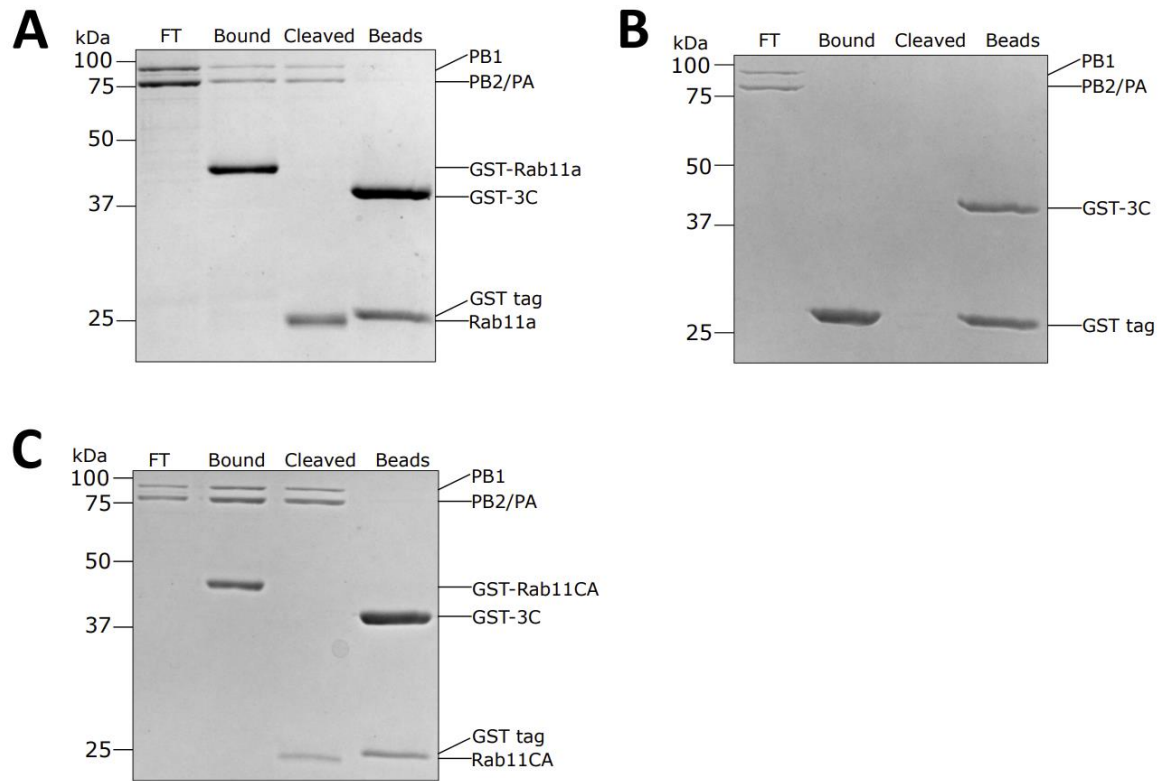


Figure 3.3: RdRP from influenza A/Northern Territory/60/1968 (H3N2) directly interacts with Rab11a.

Purified **(A)** GST-Rab11a, **(B)** GST tag, and **(C)** GST-Rab11CA were immobilised onto glutathione sepharose beads before being incubated with a purified NT60 RdRP. Complex formed was cleaved from the beads using 3C protease. Samples were analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after binding of the NT60 RdRP; *Lane Bound*, bound sample; *Lane Cleaved*, cleaved complex; *Lane Beads*, washed beads after cleavage.

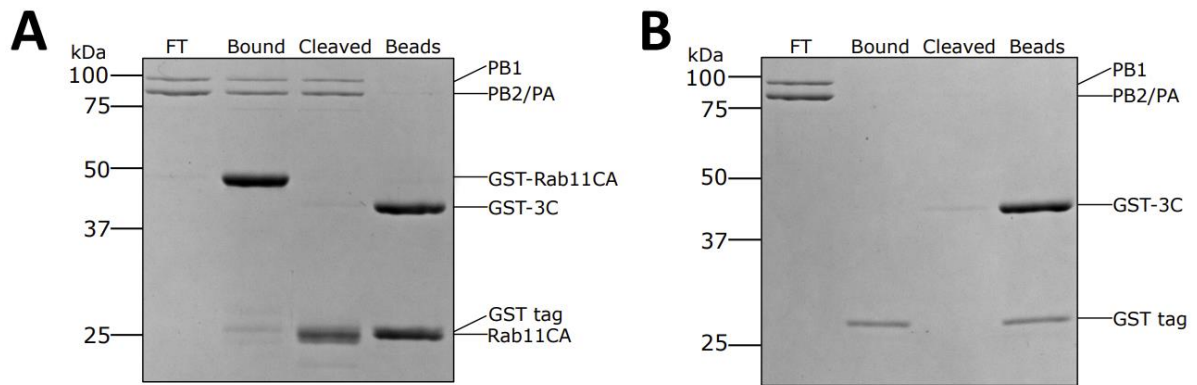


Figure 3.4: RdRP from influenza A/Brevig Mission/1/1918 (H1N1) directly interacts with Rab11a.

Purified **(A)** GST-Rab11CA and **(B)** GST tag were immobilised onto glutathione sepharose beads before being incubated with a purified 1918 RdRP. Complex formed was cleaved from the beads using 3C protease. Samples were analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after binding of the 1918 RdRP; *Lane Bound*, bound sample; *Lane Cleaved*, cleaved complex; *Lane Beads*, washed beads after cleavage.

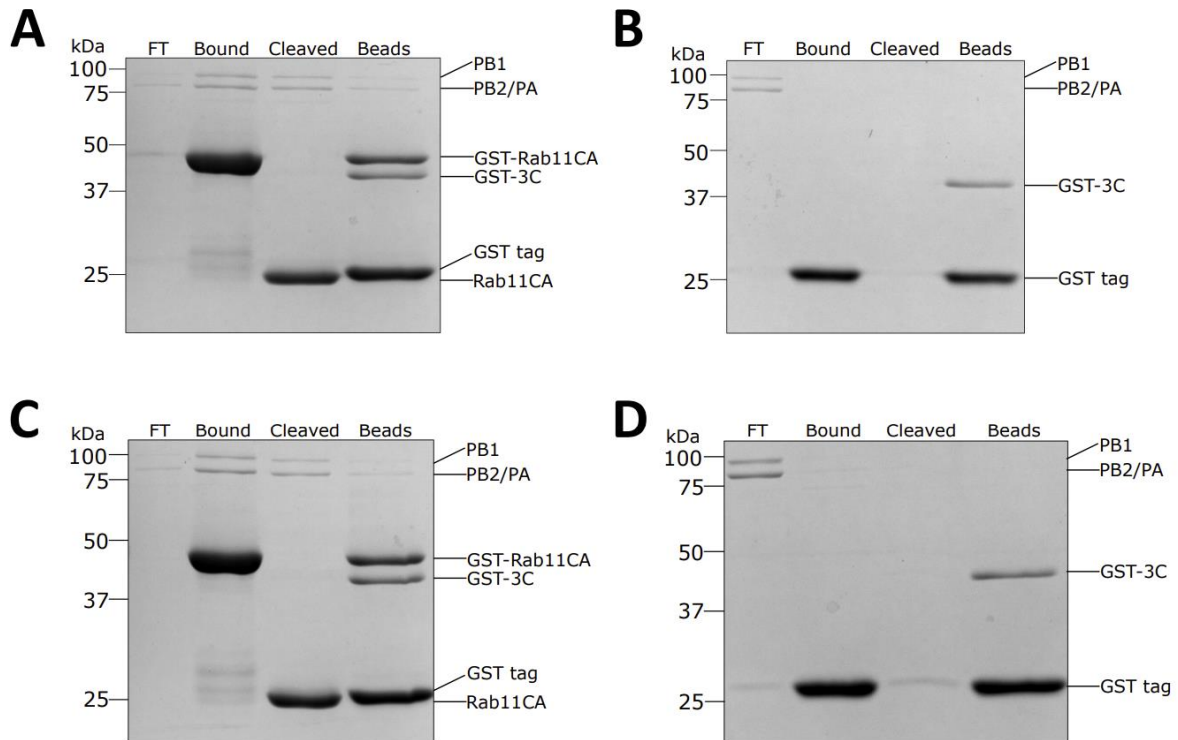


Figure 3.5: IAV RdRP bound to either vRNA or cRNA promoter binds to Rab11a.

NT60 RdRP was bound to a 15-nt-long RNA corresponding to the 5' end of vRNA and a 14-nt-long RNA corresponding to the 3' end of vRNA before being incubated with immobilised **(A)** GST-Rab11CA or **(B)** GST tag. NT60 RdRP was bound to a 14-nt-long RNA corresponding to the 5' end of cRNA and a 15-nt-long RNA corresponding to the 3' end of cRNA before being incubated with immobilised **(C)** GST-Rab11CA or **(D)** GST tag. In both cases, the complex formed was cleaved from the beads using 3C protease. Samples were analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after binding of the vRNA- or cRNA-bound NT60 RdRP; *Lane Bound*, bound sample; *Lane Cleaved*, cleaved complex; *Lane Beads*, washed beads after cleavage.

3.2.2 The 627-domain of the PB2 subunit is required for a direct interaction between IAV RdRP and Rab11a

Having shown that the RdRP directly interacts with Rab11a, I then wanted to address the question, whether this interaction occurs through the PB2 subunit of the RdRP, as it is currently proposed by some research groups (Amorim *et al.*, 2011; Avilov *et al.*, 2012a). I used a design of a RdRP that lacks the PB2⁶²⁷ domain (RdRP PB2_{Δ535–667}), which has been established by Dr Benjamin Nilsson-Payant (**Figure 3.6A**). The 627 domain of PB2 has been implicated to be involved in interactions with host factors by numerous studies (Ando *et al.*, 2016; Baker *et al.*, 2018; Naito *et al.*, 2007; Tarendeau *et al.*, 2007). RdRP that lacks the PB2⁶²⁷ domain was shown to purify as a heterotrimeric complex which is active *in vitro* (Nilsson *et al.*, 2017). A baculovirus containing polymerase genes of the NT60 RdRP PB2_{Δ535–667} was generated by Dr Haitian Fan and was already available in the laboratory. I expressed and purified this protein using the same affinity purification as for the wild-type NT60 RdRP. NT60 RdRP PB2_{Δ535–667} was then further purified by a SEC which showed both putative monomeric and dimeric peak (**Figure 3.6B**). This is reminiscent of NT60 RdRP and is in agreement with a distinct SEC profile of the IAV RdRP. The dimeric peak of NT60 RdRP PB2_{Δ535–667} was collected and analysed by SDS-PAGE side by side with the dimeric peak of wild type NT60 RdRP (**Figure 3.6C**). To confirm the lack of the PB2⁶²⁷ domain, I used an antibody raised against the N-terminal 180 amino acids of PB2 to immunoblot for the PB2 subunit (Carr *et al.*, 2006). Western blot confirmed the presence of a shorter PB2, consistent with the lack of the PB2⁶²⁷ domain in NT60 RdRP PB2_{Δ535–667} (**Figure 3.6D**). I then incubated purified NT60 RdRP PB2_{Δ535–667} with immobilised GST-Rab11CA and analysed the GST pull-down samples by SDS-PAGE. The RdRP lacking the PB2⁶²⁷ domain failed to form a

complex with Rab11CA (**Figure 3.7A, bound**). No NT60 RdRP PB2_{Δ535–667} bound to immobilised GST tag (**Figure 3.7B, bound**). These results demonstrate that the PB2⁶²⁷ domain is required for a direct interaction between RdRP and Rab11a.

To further analyse the importance of the PB2⁶²⁷ domain in the Rab11-RdRP binding, I generated a PB2 construct from the influenza NT60 RdRP including residues 537-736 which correspond to the PB2⁶²⁷ and PB2^{NLS} domains (hereafter referred to as the NT60 627-NLS) but lacking the flexible NLS peptide (residues 737-760) (**Figure 3.8A**). I cloned NT60 627-NLS as a fusion protein with an N-terminal GST tag separated by a 3C cleavage site (**Figure 3.8B**). Recombinant NT60 627-NLS protein was purified by affinity chromatography as a fusion protein and its tag was released using 3C protease. The correct molecular weight of NT60 627-NLS (~25 kDa) was confirmed by SDS-PAGE (**Figure 3.8C**) and purified the protein further by SEC which showed a single elution peak (**Figure 3.8D**). SEC peak was collected and analysed by SDS-PAGE to confirm the presence of NT60 627-NLS (**Figure 3.8E**). SDS-PAGE analysis of GST pull-down fractions showed that the NT60 627-NLS domain does not form a complex with Rab11CA (**Figure 3.9A, bound**). There was also no non-specific binding between NT60 627-NLS and immobilised GST tag (**Figure 3.9B, bound**). These results show that while the PB2⁶²⁷ domain is required for a direct interaction between RdRP and Rab11a, individually expressed 627-NLS domain does not bind to Rab11a. Based on these finding, I hypothesised that other PB2 domains are required to form a stable complex with Rab11a.

To test this hypothesis, I generated a PB2 construct from the influenza NT60 RdRP including residues 246-736, which correspond to the PB2-C but lacking a flexible NLS peptide (residues 737-760) (**Figure 3.10A**). As previously, I cloned NT60 PB2-C as a fusion protein

with an N-terminal GST tag separated by a 3C cleavage site (**Figure 3.10B**). The correct molecular weight of NT60 PB2-C protein (~55 kDa) was confirmed by SDS-PAGE (**Figure 3.10C**) and purified the protein further by SEC which showed a distinct elution peak (**Figure 3.10C**). SEC peak was collected and analysed by SDS-PAGE to confirm the presence of NT60 PB2-C (**Figure 3.10E**). SDS-PAGE analysis of GST pull-down fractions showed that NT60 PB2-C binds to immobilised GST-Rab11CA (**Figure 3.11A, bound**), and remains bound to Rab11CA released from the beads (**Figure 3.11A, cleaved**). There was no non-specific binding between NT60 PB2-C and immobilised GST tag (**Figure 3.11B, bound**).

Overall, these results show that the PB2⁶²⁷ domain is required for a direct interaction between IAV RdRP and Rab11a. Moreover, the results demonstrate that the PB2-C of the IAV RdRP directly interacts with Rab11a.

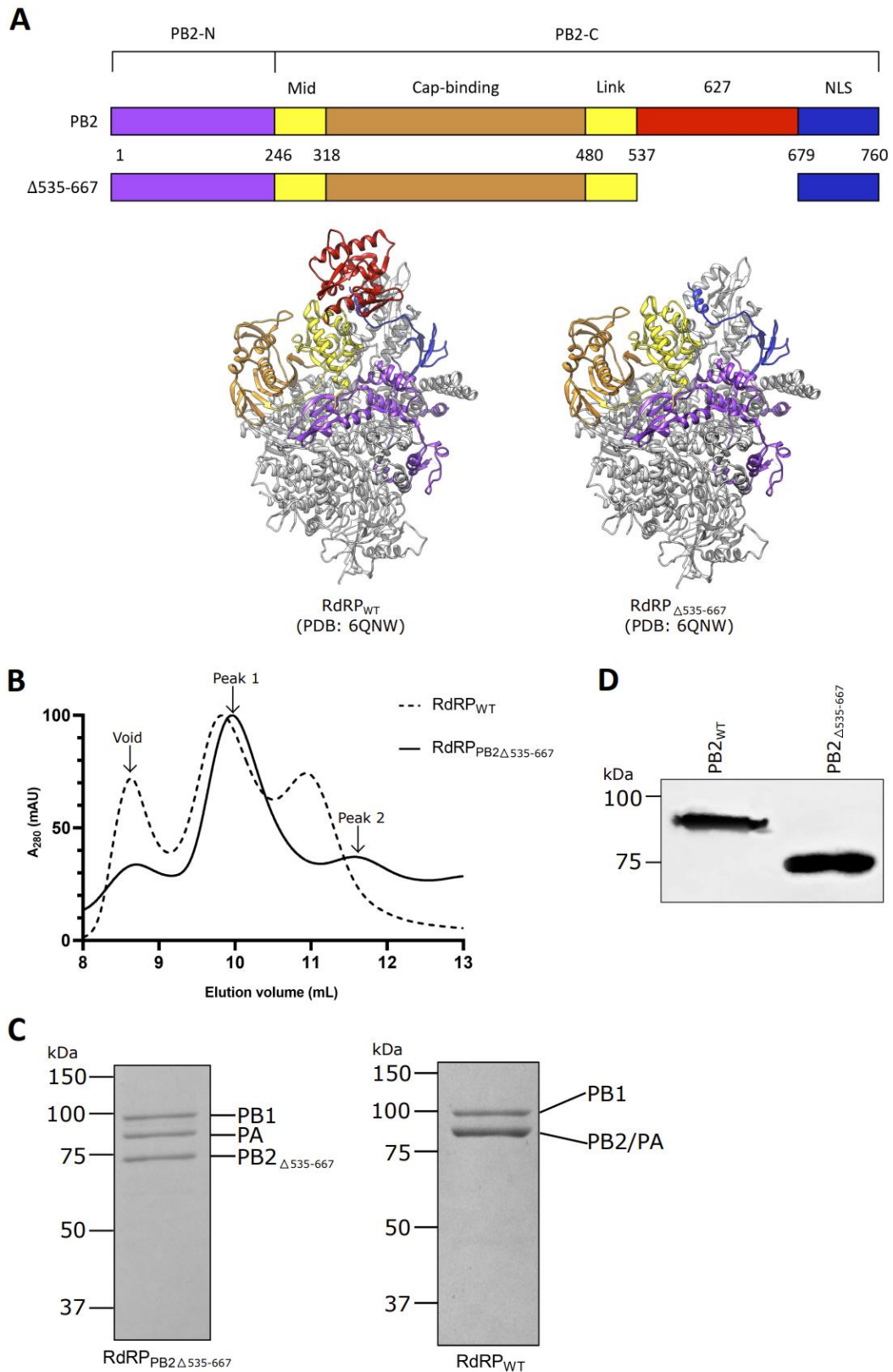


Figure 3.6: Design and purification of recombinant NT60 RdRP with the PB2⁶²⁷ domain deletion.

(A) A schematic of PB2 and truncated PB2 mutant with the PB2⁶²⁷ domain deletion showing PB2 domains with corresponding residue numbers in the NT60 RdRP, and a crystal structure of the NT60 RdRP (PDB: 6QNW). The colour of PB2-C domains in the structure corresponds to their colour in a schematic of PB2 while the rest of the polymerase is coloured in grey. **(B)** Elution profile of size exclusion chromatography (SEC) on a Superdex® 200 increase 10/300 column of purified recombinant NT60 RdRP PB2_{Δ535–667} compared to the wild-type NT60 RdRP. Gel filtration chromatogram of NT60 RdRP PB2_{Δ535–667} shows the presence of NT60 RdRP PB2_{Δ535–667} dimer (peak 1) and monomer (peak 2), a distinct characteristic of the IAV RdRP. **(C)** SEC peak 1 of NT60 RdRP PB2_{Δ535–667} (the dimer) was collected and analysed by 12% SDS-PAGE reducing gel (left) side by side with the collected SEC peak 1 (the dimer) of wild type NT60 RdRP (right). Bands were visualised using Coomassie Brilliant Blue R-250 dye. **(D)** PB2⁶²⁷ deletion in a purified NT60 RdRP PB2_{Δ535–667} was confirmed by Western blotting using a primary antibody targeting the N-terminal region (1-180) of PB2 (Carr *et al.*, 2006).

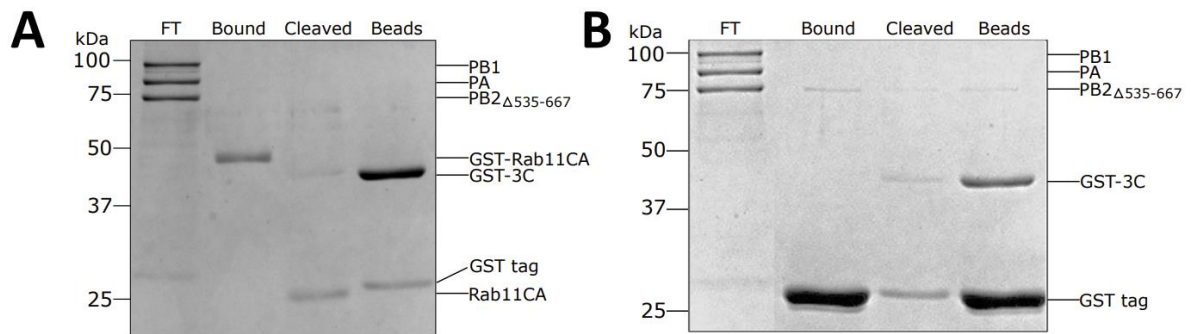


Figure 3.7: The 627-domain of the PB2 subunit is required for a direct interaction between IAV RdRP and Rab11a.

Purified **(A)** GST-Rab11CA and **(B)** GST tag were immobilised onto glutathione sepharose beads before being incubated with a purified NT60 RdRP PB2 Δ 535-667. Complex formed was cleaved from the beads using 3C protease. Samples were analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after binding of the NT60 RdRP PB2 Δ 535-667; *Lane Bound*, bound sample; *Lane Cleaved*, cleaved complex; *Lane Beads*, washed beads after cleavage.

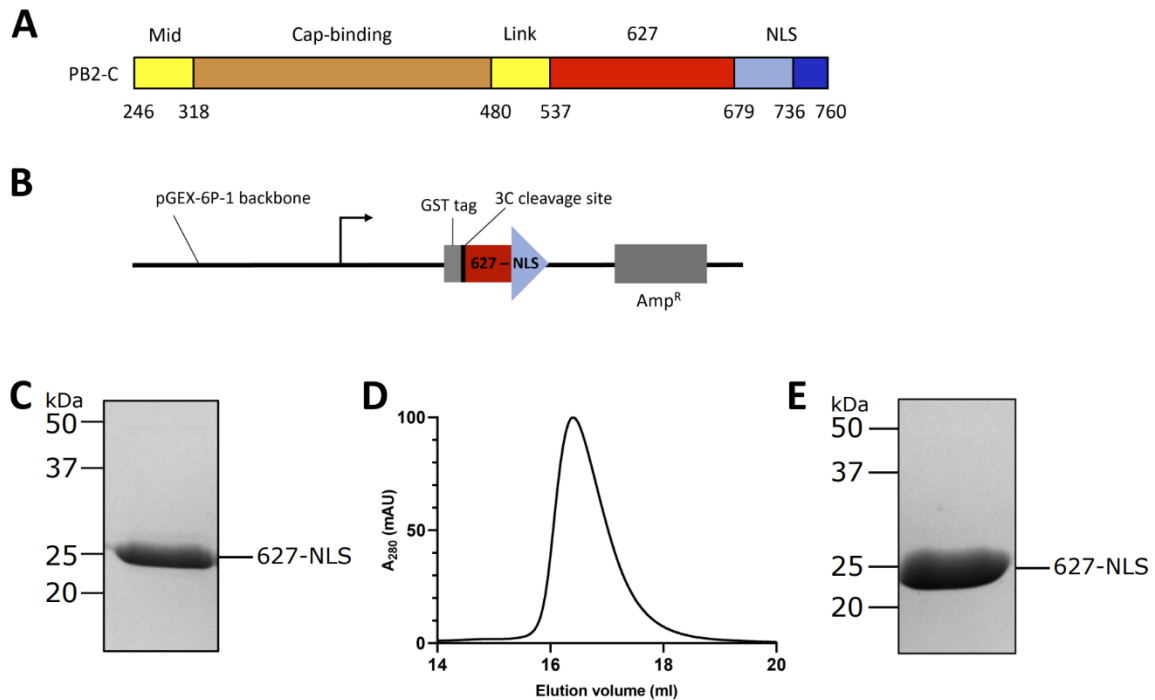


Figure 3.8: Design and purification of recombinant NT60 627-NLS domain.

(A) A schematic of PB2-C domains with corresponding residue numbers in the NT60 RdRP. **(B)** A schematic of NT60 627-NLS vector. Residues 537-736 (lacking the flexible NLS peptide, residues 737-760) of NT60 PB2-C were cloned into the vector pGEX-6P-1 with an N-terminal GST tag separated by a HRV 3C protease (3C) cleavage site. Direction of the open reading frame is indicated with the arrow. **(C)** Recombinant NT60 627-NLS was expressed in *E. coli* cells and purified by affinity chromatography as a fusion protein with a GST tag on the N-terminus. NT60 627-NLS protein was released using 3C protease and analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. **(D)** Elution profile of size exclusion chromatography (SEC) on a Superdex® 200 increase 10/300 column of purified NT60 627-NLS. Gel filtration chromatogram shows a distinct peak of purified NT60 627-NLS protein. **(E)** SEC peak of NT60 627-NLS was collected and analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye.

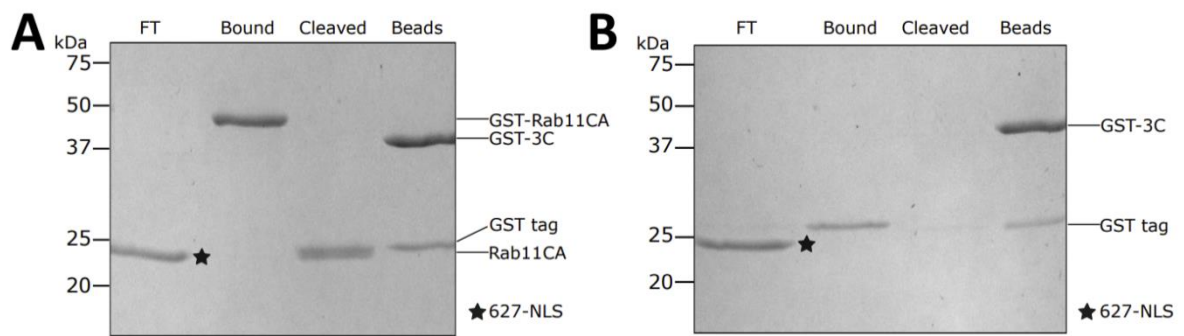


Figure 3.9: Individually expressed NT60 627-NLS domain does not bind to Rab11a.

Purified **(A)** GST-Rab11CA and **(B)** GST tag were immobilised onto glutathione sepharose beads before being incubated with a purified NT60 627-NLS. GST-Rab11CA was cleaved from the beads using 3C protease. Samples were analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after binding of the NT60 627-NLS; *Lane Bound*, bound sample; *Lane Cleaved*, cleaved complex; *Lane Beads*, washed beads after cleavage.

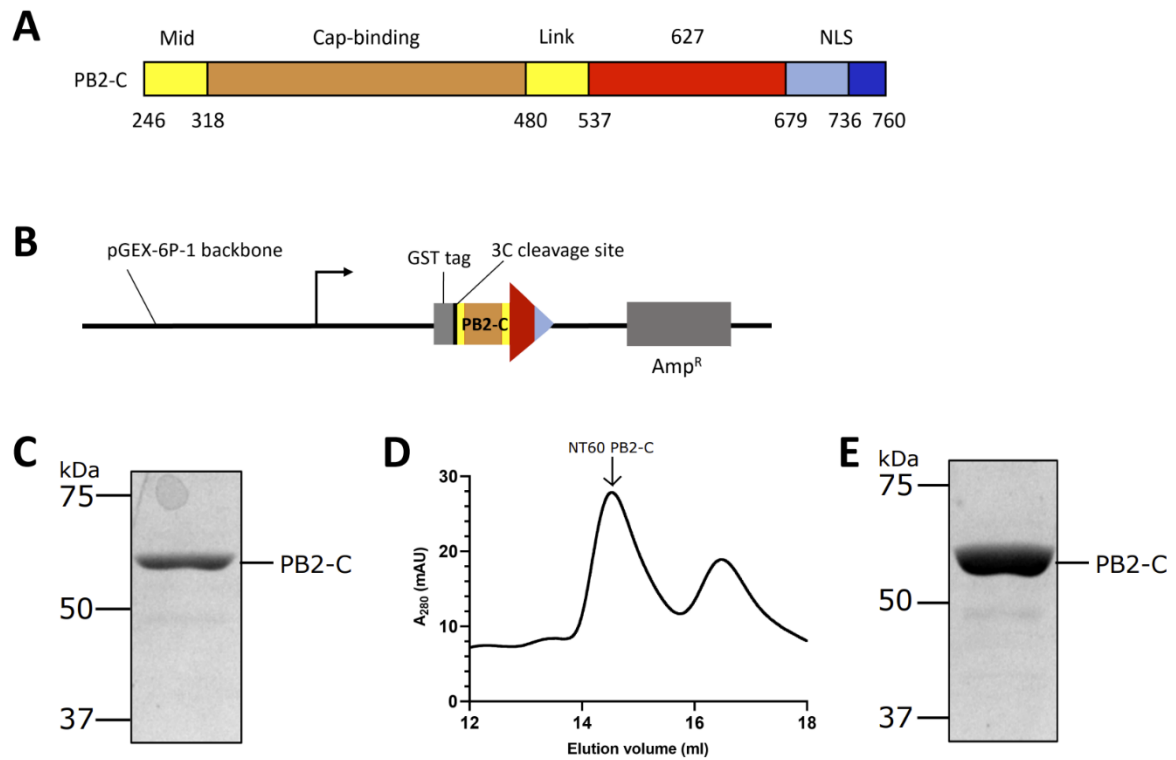


Figure 3.10: Design and purification of recombinant NT60 PB2-C.

(A) A schematic of PB2-C domains with corresponding residue numbers in the NT60 RdRP. **(B)** A schematic of NT60 PB2-C plasmid. Residues 246-736 (lacking the flexible NLS peptide, residues 737-760) of NT60 PB2-C were cloned into the vector pGEX-6P-1 with an N-terminal GST tag separated by a HRV 3C protease (3C) cleavage site. Direction of the open reading frame is indicated with the arrow. **(C)** Recombinant NT60 PB2-C was expressed in *E. coli* cells and purified by affinity chromatography as a fusion protein with a GST tag on the N-terminus. NT60 PB2-C protein was released using 3C protease and analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. **(D)** Elution profile of size exclusion chromatography (SEC) on a Superdex® 200 increase 10/300 column of purified NT60 PB2-C showing NT60 PB2-C purification peak. **(E)** SEC peak of NT60 PB2-C was collected and analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye.

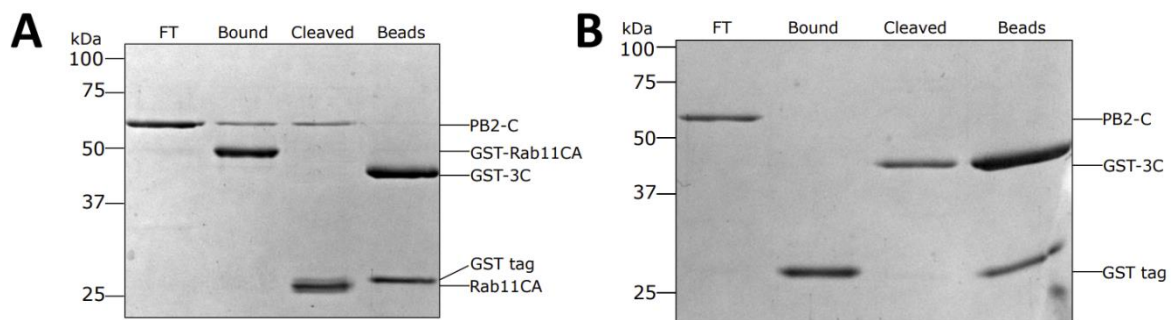


Figure 3.11: PB2 subunit of IAV RdRP mediates the interaction between RdRP and Rab11a.

Purified **(A)** GST-Rab11CA and **(B)** GST tag were immobilised onto glutathione sepharose beads before being incubated with a purified NT60 PB2-C. Complex formed was cleaved from the beads using 3C protease. Samples were analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after binding of the NT60 PB2-C; *Lane Bound*, bound sample; *Lane Cleaved*, cleaved complex; *Lane Beads*, washed beads after cleavage.

3.2.3 RdRPs from influenza B and C viruses do not bind to Rab11a *in vitro*

Influenza B virus (IBV) has been recently found to use the Rab11a-dependent pathway for its genome transport (Bhagwat *et al.*, 2020); however, there is currently no data on Rab11a involvement in the transport of the influenza C virus (ICV) genome. To address the question, whether RdRPs from IBV and ICV also bind to Rab11a, I expressed and purified RdRPs from the influenza B/Panama/45/1990 (hereafter referred to as IBV RdRP) and influenza C/Johannesburg/1/1966 (hereafter referred to as ICV RdRP) viruses using previously constructed baculoviruses in our laboratory.

SDS-PAGE analysis of GST pull-down fractions showed that IBV RdRP did not bind to immobilised GST-Rab11CA (**Figure 3.12A, bound**) as was also the case for ICV RdRP (**Figure 3.12B, bound**). Given that both RdRPs are structurally different from the IAV RdRP (Fan *et al.*, 2019; Hengrung *et al.*, 2015; Reich *et al.*, 2014; Thierry *et al.*, 2016), their RNA-free conformation might not be compatible to form a stable complex with Rab11a. Therefore, I tested the ability of IBV RdRP and ICV RdRP to form a complex with Rab11a when bound to vRNA promoter. Firstly, IBV RdRP was bound to vRNA promoter, which comprised a 15-nt-long RNA corresponding to the 5' end of vRNA and a 14-nt-long RNA corresponding to the 3' end of vRNA. Similarly, ICV RdRP was bound to vRNA promoter, which comprised a 15-nt-long RNA corresponding to the 5' end of vRNA and a 14-nt-long RNA corresponding to the 3' end of vRNA. The $A_{260/280}$ of 0.96 and 0.94 was used to confirm the presence of IBV and ICV vRNA promoter, respectively. Intriguingly, SDS-PAGE analysis of GST pull-down fractions showed that neither vRNA-bound IBV RdRP (**Figure 3.12C, bound**) nor vRNA-bound ICV RdRP (**Figure 3.12D, bound**) forms a complex with immobilised GST-Rab11CA.

These results demonstrate that regardless of the presence of the vRNA promoter RdRP from either influenza B or C virus does not bind to Rab11a *in vitro*. These results suggest that there might be other cellular factors involved in the trafficking of the IBV and ICV genome, if they indeed utilise Rab11a-dependent transport.

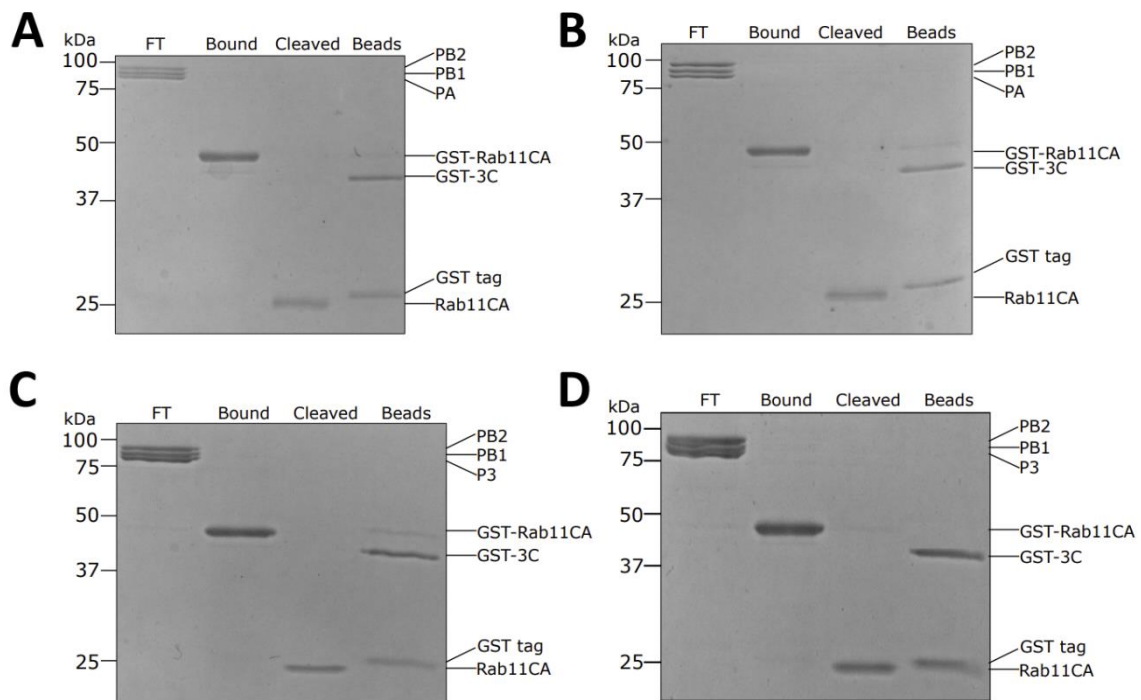


Figure 3.12: RdRP of IBV and ICV does not directly interact with Rab11a.

SDS-PAGE reducing gel analysis of a GST pull-down assay to investigate binding of an **(A)** apo IBV RdRP, **(B)** apo ICV RdRP, **(C)** vRNA-bound IBV RdRP, and **(D)** vRNA-bound ICV RdRP to Rab11a. Purified RdRPs were incubated with an immobilised GST-Rab11CA before 3C protease was added to release Rab11a. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after binding of the RdRP; *Lane Bound*, bound sample; *Lane Cleaved*, cleaved complex; *Lane Beads*, washed beads after cleavage.

3.3 Discussion

In this study, the interaction between the influenza virus RdRP and cellular GTPase Rab11a was investigated in a GST pull-down assay. The RdRP was previously proposed to mediate the interaction between the IAV vRNP and Rab11a (Amorim *et al.*, 2011; Avilov *et al.*, 2012a; Momose *et al.*, 2011). Two independent research groups have shown that the interaction between RdRP and Rab11a likely occurs via the PB2 subunit of the RdRP (Amorim *et al.*, 2011; Avilov *et al.*, 2012a); however, it remained unclear whether this interaction is direct. The results presented in this chapter prove that the IAV RdRP directly interacts with Rab11a. This is demonstrated by releasing immobilised Rab11a from the Glutathione-Sepharose beads in a complex with recombinant RdRP. This was not the case in the absence of Rab11a where immobilised GST tag was used as a negative control. Both wild type and a constitutively active mutant of Rab11a (Rab11CA) formed a complex with purified RdRP in this pull-down assay. Intriguingly, Rab11CA appeared to consistently bind higher levels of RdRP and was therefore chosen for all subsequent pull-down assays in this study. The Q70L substitution disrupts the catalytic site of Rab11a for the intrinsic GTP hydrolytic reaction and shifts the equilibrium toward the GTP-bound Rab11a (Uhlig *et al.*, 2006). Given that a non-hydrolysable GTPγS was used in the assay, this mutation should, therefore, not make a difference. However, it is important to note that endogenous GTP could have been incorporated during expression and purification of either Rab11a or Rab11CA in which case the Q70L substitution would favour the GTP form (Jagoe *et al.*, 2006). Therefore, it would be informative to repeat this assay without the presence of a non-hydrolysable GTPγS to determine whether Rab11CA indeed behaves differently than wild type Rab11a. Additionally, it would be also informative to include a dominant-negative

mutant of Rab11a, which exists preferentially in the GDP-bound state and has been shown to fail to associate with IAV vRNPs (Amorim *et al.*, 2011; Eisfeld *et al.*, 2011; Farnsworth and Feig, 1991; Momose *et al.*, 2011).

Furthermore, it was also shown here that RdRP-Rab11a interaction is direct across different IAV subtypes, as evident from a pull-down assay using RdRP from two different influenza A subtypes, i.e., influenza A/Northern Territory/60/1968 (H3N2) virus and influenza A/Brevig Mission/1/1918 (H1N1) virus. This is consistent with observations by other research groups who used different IAV subtypes to study interactions with Rab11a by either visualising infected cells (Amorim *et al.*, 2011; Ganti *et al.*, 2021) or measuring viral titres from infected cells using plaque assay (Han *et al.*, 2021). Considering that the influenza virus RdRP adopts strikingly different conformations, depending on the nature of the viral RNA template (Fan *et al.*, 2019; Hengrung *et al.*, 2015; Pflug *et al.*, 2014; Thierry *et al.*, 2016), I sought to find out whether purified IAV RdRP in the presence of vRNA and cRNA promoter also binds to Rab11a. My data show that RdRP forms a complex with Rab11a not only when it is bound to the vRNA promoter but also in the presence of the cRNA promoter. This is a surprising result as Rab11a is thought to be involved in the vesicular trafficking of vRNPs only and in a subsequent assembly of the viral genome, as described in recent manuscripts (Amorim, 2019; Dou *et al.*, 2018; Staller and Barclay, 2020). Although there is no unequivocal evidence showing that Rab11a differentially binds to vRNPs and cRNPs, it is commonly accepted in the literature that cRNPs are not exported from the nucleus, and thus never encounter Rab11a. While the study presented in this chapter failed to differentiate between vRNA- and cRNA-bound RdRP, it is important to highlight that our GST pull-down assay has limitations. Namely, the RdRP used in our assay

was bound to short RNAs in the absence of NP, which is also part of vRNP. Given that NP stabilises the vRNP (Coloma *et al.*, 2020) and that short RNAs might have different effects on the RdRP conformation in the presence and absence of NP, RdRP could have remained in a conformation which is compatible to form a stable complex with Rab11a regardless of which short RNA promoter is present. Therefore, it would be highly informative to repeat the GST pull-down experiment using either isolated vRNPs and cRNPs or whole cell lysates of an IAV infected cells. This idea will be explored further in Chapter 4.

In this chapter it was also demonstrated that the PB2 subunit of the IAV RdRP mediates the interaction between RdRP and Rab11a, which has been suggested previously in a GFP pull-down assay (Amorim *et al.*, 2011) as well as by FRET microscopy (Avilov *et al.*, 2012a). This is demonstrated in our study by a GST pull-down assay using purified heterotrimeric IAV RdRP that lacks the PB2⁶²⁷ domain which fails to bind to Rab11a. Moreover, these data further show that the PB2⁶²⁷ domain is required for a direct RdRP-Rab11a interaction. This is the first time that a particular domain of the RdRP has been found to be important for the interaction with Rab11a. While PB2⁶²⁷ domain has been recently reported to be involved in a direct interaction with a host protein ANP32A in the cell nucleus (Carrique *et al.*, 2020), the data presented in this study now shed light on its involvement in the vRNP cytoplasmic transport for the first time. To support these data, I generated and purified the NT60 627-NLS domain, and analysed its binding to Rab11a in a GST pull-down assay. Surprisingly, the isolated NT60 627-NLS domain failed to bind to Rab11a. I hypothesized that while the 627-NLS domain may be indeed important for the interaction with Rab11a, the isolated NT60 627-NLS domain is insufficient to bind to Rab11a on its own. To test this hypothesis, I generated and purified NT60 PB2-C protein and showed that it binds to

Rab11a. In light of all these findings, we propose here that the PB2⁶²⁷ domain is important for a direct interaction between IAV RdRP and Rab11a while other domains of the PB2 subunit are needed to stabilise the RdRP–Rab11a complex.

A recent study has shown that IBV also uses Rab11a-dependent pathway for its genome transport as suggested by imaging of A549 GFP-Rab11a cells infected with the IBV (Bhagwat *et al.*, 2020). Based on this finding, it is tempting to speculate that RdRP of the IBV also interacts directly with Rab11a. Thus, I sought to address the question, whether RdRP from either IBV or ICV binds directly to Rab11a. Interestingly though, neither of them formed a complex with Rab11a, either in the RNA-free apo or in a vRNA-bound conformation. These results suggest that if influenza B and C viruses indeed utilise a Rab11a cycle for their vRNP transport, the interaction between their RdRP and Rab11a is not direct. An alternative explanation could be that while the interaction between their RdRPs and Rab11a is nevertheless direct, the RdRP conformation in our assay is incompatible to form a stable complex with Rab11a *in vitro*. Sequence alignments of NT60 RdRP and 1918 RdRP show that 1918 RdRP has 96.18% identity (similarity) for PB2. In contrast, PB2 of IBV RdRP and ICV RdRP shows 38.22% and 23.21% identity (similarity) to PB2 of the NT60 RdRP, respectively, suggesting that the chemistry of the interaction surface is different. Perhaps, the addition of short vRNA promoters did not have the effect on their RdRP conformation, thereby preventing them to form a stable complex with Rab11a. The appropriate approach to address these two possibilities would be to repeat the GST pull-down assay using whole cell lysates from infected cells and analyse the co-precipitants by mass spectrometry.

In summary, I have established a GST pull-down assay to study the interaction between influenza virus polymerase and cellular GTPase Rab11a. Using this assay, my work provides the first evidence that the IAV RdRP interacts directly with Rab11a and that the PB2⁶²⁷ domain of the PB2 subunit is involved in this interaction. These findings further expand our understanding of the cytoplasmic transport of the influenza virus vRNPs.

4 Influenza virus infection alters Rab11a interactome

4.1 Introduction

A role of Rab11a in the influenza A virus (IAV) life cycle was initially identified in the budding and release in IAV virions (Bruce *et al.*, 2010). Further studies have identified a role for Rab11a in the migration of the IAV genome from the nucleus to the plasma membrane. The utilisation of Rab11a by the influenza virus has been shown to alter its distribution (Amorim *et al.*, 2011; Avilov *et al.*, 2012b; Einfeld *et al.*, 2011; Momose *et al.*, 2011), ultrastructural environment (Alenquer *et al.*, 2019; Amorim *et al.*, 2011; de Castro Martin *et al.*, 2017; Kawaguchi *et al.*, 2015; Vale-Costa *et al.*, 2016), function (Kawaguchi *et al.*, 2015; Vale-Costa *et al.*, 2016), and binding partners (Bhagwat *et al.*, 2020; Vale-Costa *et al.*, 2016). Together, a large body of evidence shows that influenza virus infection impairs the function of Rab11a by influencing its binding partners leading to its redistribution within the cytoplasm.

In steady state conditions without influenza virus infection Rab11a has a critical role in regulating the endocytic recycling compartment (ERC), one of the systems the cell uses for delivering endocytosed material from the trans-Golgi network (TGN) to the cell surface. Rab11a cycles between an inactive GDP-bound form and an active GTP-bound form. Upon activation, Rab11a undergoes conformational changes enabling interaction with Rab11 family interacting proteins (Rab11-FIPs). In turn, these binding partners bridge Rab11a interaction with cytoplasmic motor proteins to promote transport along microfilaments and microtubules (Vale-Costa and Amorim, 2017). In mammalian cells, Rab11a indirectly interacts with myosin Vb through Rab11-FIP2, which acts as an adapter protein for

transport along actin microfilaments (Hales *et al.*, 2002). Similarly, Rab11a moves along microtubule tracks either via kinesin (plus-end directed motors) or dynein (minus-end directed motors) motor proteins. Intriguingly, influenza virus proteins may influence the association of Rab11a with cytoskeletal motor proteins by competing with Rab11-FIPs. One study found that IAV vRNP segments potentially outcompete Rab11-FIPs for binding to Rab11a (Vale-Costa *et al.*, 2016), which may in turn reduce the association of Rab11a with specific motor proteins. Supporting this hypothesis, another study quantified Rab11a-interacting proteins using immunoprecipitation and mass spectrometry analyses, and identified a reduction in dynein motor protein upon influenza virus infection (Bhagwat *et al.*, 2020). However, it remained ambiguous whether the observed reduction in dynein was a result of an underlying reduction of Rab11a association with the corresponding dynein adaptor protein Rab11-FIP3 as the latter was not detected.

The aim of this study was to analyse, whether vRNPs interact with Rab11a *in vitro*. By using co-immunoprecipitation and mass spectrometry, this study also sought to better understand how IAV infection alters the Rab11a cycle and its interactome. Using purified Rab11a with mutations in the region which binds to Rab11-FIPs, I also show that IAV modulates host recycling by outcompeting Rab11-FIPs for interaction with Rab11a. A better understanding of the molecular mechanisms underlying the transport of IAV vRNP segments inside the host cell might provide the knowledge required to explain the assembly of segmented viral genomes.

4.2 Results

4.2.1 Rab11a has a selective specificity for vRNP but not cRNP

The findings described in Chapter 3 show that the RdRP interacts directly with Rab11a *in vitro*. In a cellular context, however, this interaction is thought to occur in the context of vRNP. To analyse, whether vRNPs interact with Rab11a *in vitro*, I infected human embryonic kidney 293T (HEK 293T) cells with influenza A/WSN/1933 (H1N1) virus (hereafter referred to as WSN), harvested whole cell lysates at 12 hours post infection (hpi) and incubated the cell lysates with GST-Rab11CA.

SDS-PAGE analysis of GST pull-down fractions showed the presence of a 56 kDa protein corresponding to the size of influenza nucleoprotein (NP) bound to the immobilised GST-Rab11CA (**Figure 4.1A, bound**), as well as bound to Rab11CA released from the beads (**Figure 4.1A, cleaved**). As a control, I incubated WSN-infected whole cell lysate with immobilised GST tag. The 56 kDa protein appeared in the bound fraction (**Figure 4.1B, bound**), indicating non-specific binding to the beads. There was no 56 kDa protein bound to immobilised GST-Rab11CA incubated with mock infected whole cell lysate (**Figure 4.1C, bound**). I confirmed that the 56 kDa protein is indeed NP by western blot (**Figure 4.1D**). To assess whether NP co-precipitated with Rab11CA on its own or in the context of a vRNP, I also blotted for the PB1 protein, a constituent of RdRP. Western blot confirmed the presence of PB1 bound to immobilised GST-Rab11CA (**Figure 4.1D, bound**), and, to a lower extent, bound to Rab11CA released from the beads (**Figure 4.1D, cleaved**). In contrast to NP, PB1 did not bind the immobilised GST tag (**Figure 4.1E, bound**).

To support our hypothesis that vRNP co-precipitated with Rab11CA, I addressed whether viral RNAs co-precipitated. I extracted total RNA from bound fractions of WSN infected and mock infected whole cell lysates incubated with GST-Rab11CA. The accumulation of positive- and negative-sense viral RNA was then analysed by primer extension assay by Jane Sharps using NA segment-specific primers (**Figure 4.1F**). As controls, we included total RNA extracted from WSN infected and mock infected whole cell lysates. The 5S rRNA was monitored as a control. mRNA, cRNA and vRNA were detected in the WSN infected lysate and were not detected in the mock infected lysate (**Figure 4.1F, total RNA**). We detected vRNA in the GST-Rab11CA bound fraction of WSN infected lysates; however, critically we did not detect cRNA or mRNA (**Figure 4.1F, RNA bound to GST-Rab11CA**). On average, 14% of total vRNA from cell lysates was recovered in the GST pull-down assay using Rab11CA while cRNA, mRNA, and 5S rRNA were around background levels (**Figure 4.1G**). These data demonstrate that more vRNA is recovered than the other RNAs, suggesting a specificity of interaction of Rab11a with vRNPs.

Overall, these data demonstrate that vRNPs co-precipitate with GST-Rab11CA. Moreover, they show that Rab11a differentially binds to vRNP rather than cRNP.

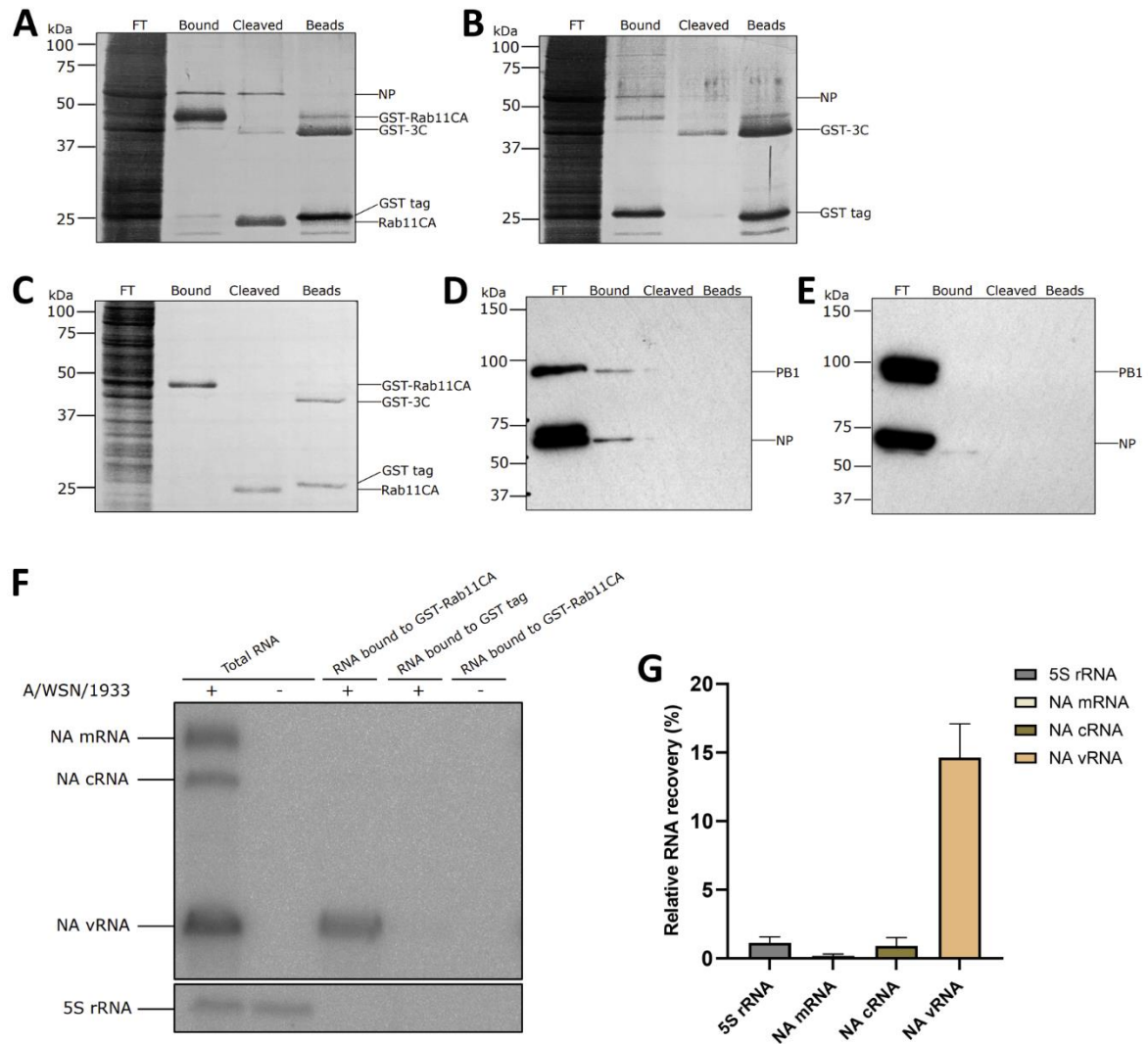


Figure 4.1: Rab11a selectively binds vRNPs over cRNPs.

Human embryonic kidney 293T (HEK 293T) cells were infected with influenza A/WSN/1933 (H1N1) virus at an MOI of 5 and whole cell lysates were isolated at 12 hours post infection. Lysates were incubated with immobilised **(A)** GST-Rab11CA or **(B)** GST tag. The complex formed was cleaved from the beads using 3C protease. Samples were analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after binding of the whole cell lysate; *Lane Bound*, bound sample; *Lane Cleaved*, cleaved complex; *Lane Beads*, washed beads after cleavage. **(C)** Same as in **A** but using mock infected human HEK 293T cells. **(D)** Pull-down samples from panel **A** were analysed by 8% SDS-PAGE reducing gel. The presence of the NP protein and the PB1 subunit of the IAV RdRP was confirmed by Western blotting. **(E)** Pull-down samples from panel **B**

were analysed by 8% SDS-PAGE reducing gel. The presence of the NP protein and the PB1 subunit of the IAV RdRP was confirmed by Western blotting using rabbit influenza A virus NP protein antibody (GeneTex, GTX125989) and rabbit influenza A virus PB1 protein antibody (GeneTex, GTX125923), respectively. **(F)** Accumulation of mRNA, cRNA, and vRNA was accessed by primer extension of NA RNAs. Primer extension assay was performed by Jane Sharps. **(G)** Quantitative analyses of average RNA recovery bound to the GST-Rab11CA. Data are shown as mean $\pm$ SD (n = 3 independent experiments/group). Quantitative analyses were performed by Prof Ervin Fodor.

4.2.2 vRNPs can be pulled down from infected cell lysates with GST-Rab11a

I further analysed co-precipitants of GST-Rab11CA in infected and mock infected cells by mass spectrometry in order to confirm the presence of all vRNP proteins (PA, PB1, PB2, and NP) and to determine whether infection with IAV alters the Rab11a interactome. To this end, proteins present in the cleaved fraction after the GST pull-down assay were digested with trypsin and analysed by label-free absolute quantification after liquid chromatography and tandem MS (LC-MS/MS) by the Advanced Proteomics Facility (University of Oxford). Sequest in Proteome Discoverer (version 1.4) was used to identify the proteins present from the detected peptides by Dr Marjorie Fournier. Quantitative proteomics analysis was performed based on normalised spectral counts (NSAF) (Zybailov *et al.*, 2006) and statistical analysis on NSAF values was performed using the power law global error model (PLGEM) (Pavelka *et al.*, 2004). Common contaminants were excluded from the analysis. Only host proteins that were detected in all three biological replicates were included in the analysis.

To determine whether vRNPs precipitated with GST-Rab11CA, NSAF values were used as a measure of relative viral protein abundance within the protein mixture (**Table 4.1**). Mass spectrometry analyses and protein identifications of proteins that co-precipitated with GST-Rab11a from infected lysates revealed the presence of NP. Furthermore, all three RdRP subunits (PB1, PB2, and PA) were detected in Rab11a interactome of infected cells. Interestingly, NS1, NEP, and M1 proteins also co-precipitated with GST-Rab11a. In addition to these viral proteins, HA, and NA proteins were also detected; however, their abundance was very low, suggesting their detection is coming from background signals. These data suggest that vRNPs co-precipitated with GST-Rab11CA.

Next, statistical analysis was performed on NSAF values from three biological replicate experiments, using a power law global error model (PLGEM). After PLGEM analysis, 52 proteins were found significantly abundant in at least one of the three pull-down samples at a p-value cut-off of 0.05 (**Table 4.2**). When comparing the enrichment of proteins between GST tag interactome and Rab11a interactome from mock infected lysates, 42 unique proteins were significantly enriched in Rab11a interactome at a p-value cut-off of 0.05 (**Table 4.2, compare Mock GST-Rab11CA vs. WSN GST tag**). Notably, Rab11b was the most abundant protein after Rab11a and showed strikingly equivalent high abundance to Rab11a. Another member of the mammalian Rab11 family, i.e., Rab25, was also highly abundant in the Rab11a interactome as well were other Rabs (Rab7b, Rab8a, Rab10, and Rab13) and Rab11-FIPs (Rab11-FIP1, Rab11-FIP2, Rab11-FIP4, and Rab11-FIP5). Furthermore, 38 unique proteins were significantly enriched in Rab11a interactome of infected lysates compared to GST tag interactome at a p-value cut-off of 0.05 (**Table 4.2, compare WSN GST-Rab11CA vs. WSN GST tag**). Both Rab11b and Rab25 were similarly abundant in Rab11a interactome of infected lysates as were in Rab11a interactome of mock infected lysates. Moreover, other Rabs (Rab7b, Rab8a, Rab10, and Rab13) and Rab11-FIPs (Rab11-FIP1, Rab11-FIP2, Rab11-FIP4, and Rab11-FIP5) were also significantly abundant. Intriguingly, NS1 and NEP were the only viral proteins which were significantly abundant in Rab11a interactome, NS1 being more abundant than NEP.

Comparison of the Rab11a interactomes of uninfected and infected cells revealed that viral infection induced changes in Rab11a interactome. Around 60% of significantly abundant proteins in Rab11a interactome were commonly found in both control and infected conditions. Additionally, 13 and 9 proteins were specifically identified before (**Table 4.3**)

and after infection (**Table 4.4**), respectively. Gene ontology (GO) analysis using the Manteia software (Tassy and Pourquié, 2014) showed that the interactome of Rab11a upon IAV infection is linked to various biological processes with those relating to the vRNP assembly having the highest GO enrichment score (**Figure 4.2**). Among others, protein targeting to endoplasmic reticulum (ER) and cellular membrane were also detected which is in agreement with the role of Rab11a in the cytoplasmic trafficking of the IAV vRNPs. Interestingly, while Rab11-FIP effector proteins are less abundant in the Rab11a interactome of infected cells compared to Rab11a interactome of uninfected ones, no notable changes were observed in the abundance of either cytoplasmic dynein 1 (DYNLT1) or kinesin (KIF1A) motor protein (**Table 4.2, compare Mock GST-Rab11CA vs. WSN GST-Rab11CA**). These data suggest that IAV infection potentially alters the microtubule based cytoskeletal motor stoichiometry of Rab11a by decreasing the association of Rab11-FIPs with motor proteins, which is in agreement with studies reporting that IAV competes with Rab11-FIP adaptor proteins for binding to Rab11a (Bhagwat *et al.*, 2020; Vale-Costa *et al.*, 2016).

Taken together, these findings demonstrate the IAV infection has a qualitative effect on the Rab11a interactome. Our mass spectrometry data confirms the interaction of vRNP with Rab11a and suggest a previously unreported interaction between Rab11a and viral NS1; whether this interaction is direct or indirect was not investigated. Moreover, the results of this study also suggest that influenza virus infection reduces the abundance of Rab11-FIP.

Table 4.1: List of viral proteins detected in a pull-down assay.

Average relative abundance of viral proteins detected in WSN infected GST tag pull-down sample, mock infected GST-Rab11CA pull-down sample, and in WSN infected GST-Rab11CA pull-down sample from three biological replicate experiments. The quantitative analysis was based on a label-free quantitation method using normalized spectral abundance factor (NSAF), as a measure of relative protein abundance within the protein mixture. For better visualization of the data, NSAF values were multiplied by 100 (NSAF·100). NSAF values are coloured coded; red and green colours represent the highest and the lowest abundance of a protein, respectively. Quantitative proteomics and statistical analyses were performed by Dr Marjorie Fournier.

		WSN + GST tag	Mock + GST- Rab11CA	WSN + GST- Rab11CA
Accession number	Annotation	NSAF·100	NSAF·100	NSAF·100
P15682	NP	2.301443	0.059144	3.236639
Q82506	NS1	0.703806	0	1.637752
P03430	PB1	0.271068	0.007137	0.309071
P03427	PB2	0.376865	0.047608	0.305128
P15659	PA	0.297601	0.005126	0.356318
P05777	M1	0.41756	0	0.215983
Q89733	NEP	0.090068	0	0.344974
P03470	NA	0.012116	0	0.009441
P03454	HA	0.028724	0	0.017308

Table 4.2: List of PLGEM proteins with different abundance.

The statistical analysis was performed on NSAF values from three biological replicate experiments, using a power law global error model (PLGEM) (Pavelka *et al.*, 2008). PLGEM proteins with their average relative abundance in WSN infected GST tag pull-down sample, mock infected GST-Rab11CA pull-down sample, and in WSN infected GST-Rab11CA pull-down sample with a p-value cut-off of 0.05 are gathered in the table. The quantitative analysis was based on a label-free quantitation method using normalized spectral abundance factor (NSAF), as a measure of relative protein abundance within the protein mixture. For better visualization of the data, NSAF values were multiplied by 100 (NSAF·100). NSAF values are coloured coded; red and green colours represent the highest and the lowest abundance of a protein, respectively. Quantitative proteomics and statistical analyses were performed by Dr Marjorie Fournier.

Accession number	Annotation	WSN + GST tag	Mock + GST-Rab11CA	WSN + GST-Rab11CA
		NSAF·100	NSAF·100	NSAF·100
P0000	Rab11a	0.40139135	32.76383835	31.5841978
Q82506	NS1	0.70380627	0	1.63775241
Q89733	NEP	0.090068329	0	0.34497421
Q15907	Rab11b	0.43269251	31.56807127	30.0993658
P57735	Rab25	0.151858461	4.150585376	4.00364213
Q96AH8	Rab7b	0	0.037234604	0.0590844
P61006	Rab8a	0.078401815	0.198020134	0.18411537
P61026	Rab10	0.081145879	0.204950839	0.19972978
P51153	Rab13	0.040167471	0.178898794	0.16648708
Q6WKZ4	Rab11-FIP1	0	0.13751477	0.10516634
Q7L804	Rab11-FIP2	0	0.108601783	0.07317388
Q86YS3	Rab11-FIP4	0	0.021688714	0.00677395
Q9BXF6	Rab11-FIP5	0	0.14918222	0.08843237
P63172	DYNLT1	0	0.024212155	0.02195513
Q12756	KIF1A	0	0.049036467	0.04372511
P56134	ATP5MF	0	0.049720347	0.0459043
P57678	GEMIN4	0.614834239	0.451264807	0.53132968
P57764	GSDMD	0.078170237	0.17650441	0.16558482

Q15370	ELOB	0	0.039607734	0
O75506	HSBP1	0	0.035999651	0
Q9P1F3	ABRACL	0	0.023922705	0.05327166
Q5XG85	LOC554249	0	0.029106101	0
Q9Y6A4	CFAP20	0	0.024216128	0
Q9C005	DPY30	0	0.019573123	0
Q53GQ0	HSD17B12	0	0.023748994	0.01970859
O95232	LUC7L3	0	0.021637558	0.00849108
Q96SW2	CRBN	0	0.020683253	0.00976245
Q96CN4	EVI5L	0	0.022110007	0.01942814
Q9NV79	PCMTD2	0	0.015534045	0.00508054
Q15121	PEA15	0	0.014905686	0
O15116	LSM1	0	0.014569467	0
Q8WWC4	MAIP1	0	0.016060868	0.00852553
Q96ER3	SAAL1	0	0.01394821	0.01160807
Q14011	CIRBP	0	0.011265925	0
P62873	GNB1	0	0.013746214	0
P11488	GNAT1	0	0.013353465	0
Q6P589	TNFAIP8L2	0	0.014869421	0
Q96BM9	ARL8A	0	0.010417952	0
Q5JSH3	WDR44	0	0.012135528	0.00472618
P62166	NCS1	0	0.009117431	0
O00399	DCTN6	0	0.010198627	0.00965302
P63173	RPL38	0	0	0.02620106
P42677	RPS27	0	0	0.02183422
Q9BQ48	MRPL34	0	0	0.0195209
O60925	PFDN1	0	0	0.0150334
Q9H7C9	AAMDC	0	0	0.0150334
P51668	UBE2D1	0	0	0.0124767
O95400	CD2BP2	0	0	0.01265397
O00519	FAAH	0	0.012797385	0.01173737
Q8IWF6	DENND6A	0	0.161460715	0.13377998
Q9ULA0	DNPEP	0	0.14721721	0.0607087
P14373	TRIM27	0	0.057103604	0.03364526
P35268	RPL22	0	0.051651967	0.06236838

Table 4.3: List of PLGEM proteins specifically identified before influenza infection.

As in Table 4.2 but showing PLGEM proteins with their average relative abundance in mock infected GST-Rab11CA pull-down sample with a p-value cut-off of 0.05 only. Proteins are listed in a descending order with the most abundant protein being first. Quantitative proteomics and statistical analyses were performed by Dr Marjorie Fournier.

Accession number	Annotation	Description	NSAF-100
O75506	ELOB	Elongin-B	0.039607734
Q5XG85	LOC554249	Putative UPF0633 protein LOC554249	0.029106101
Q9Y6A4	CFAP20	Cilia- and flagella-associated protein 20	0.024216128
O75506	HSBP1	Heat shock factor-binding protein 1	0.023922705
Q9C005	DPY30	Protein dpy-30 homolog	0.019573123
Q15121	PEA15	Astrocytic phosphoprotein PEA-15 protein	0.014905686
Q6P589	TNFAIP8L2	Tumour necrosis factor alpha-induced protein 8-like protein 2	0.014869421
O15116	LSM1	U6 snRNA-associated Sm-like protein LSm1	0.014569467
P62873	GNB1	Guanine nucleotide-binding protein subunit beta-1	0.013746214
P11488	GNAT1	Guanine nucleotide-binding protein subunit alpha-1	0.013353465
Q14011	CIRBP	Cold-inducible RNA-binding protein	0.011265925
Q96BM9	ARL8A	ADP-ribosylation factor-like protein 8A	0.010417952
P62166	NCS1	Neuronal calcium sensor 1 protein	0.009117431

Table 4.4: List of PLGEM proteins specifically identified after influenza infection.

As in Table 4.2 but showing PLGEM proteins with their average relative abundance in WSN infected GST-Rab11CA pull-down sample with a p-value cut-off of 0.05 only. Proteins are listed in a descending order with the most abundant protein being first. Quantitative proteomics and statistical analyses were performed by Dr Marjorie Fournier.

Accession number	Annotation	Description	NSAF·100
Q82506	NS1	Influenza virus non-structural protein 1	1.63775241
Q89733	NEP	Influenza virus nuclear export protein	0.34497421
P63173	RPL38	60S ribosomal protein L38	0.02620106
P42677	RPS27	40S ribosomal protein S27	0.02183422
Q9BQ48	MRPL34	39S ribosomal protein L34	0.0195209
O60925	PFDN1	Prefoldin subunit 1	0.0150334
Q9H7C9	AAMDC	Mth938 domain-containing protein	0.0150334
O95400	CD2BP2	CD2 antigen cytoplasmic tail-binding protein 2	0.01265397
P51668	UBE2D1	Ubiquitin-conjugating enzyme E2 D1	0.0124767

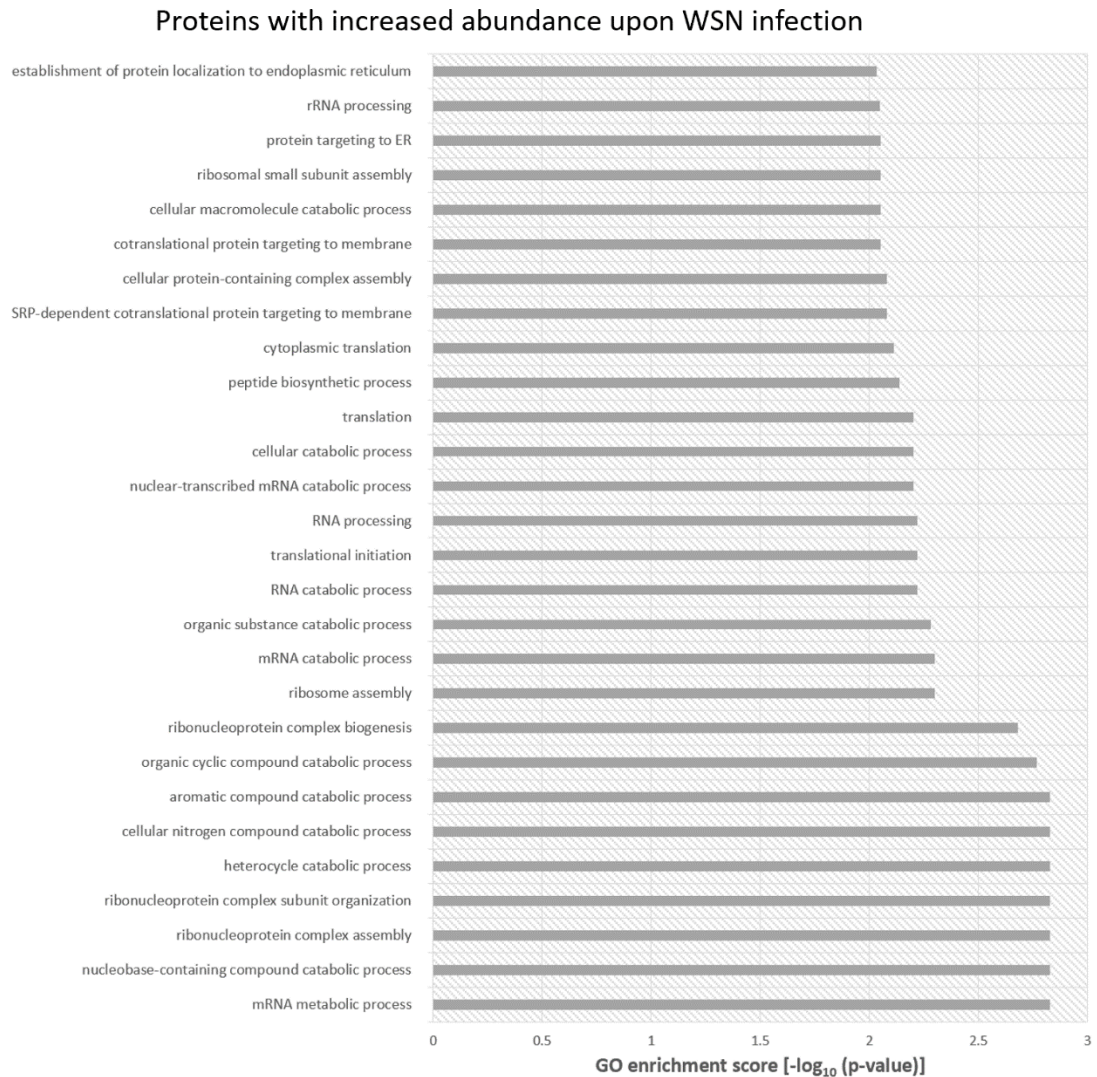


Figure 4.2: Gene ontology analysis for biological processes of the Rab11a interactome.

Host proteins which abundance increased upon WSN infection and were found to interact with Rab11a were subjected Manteia gene list analysis for biological processes (Tassy and Pourquié, 2014). Note that a single protein can be associated with several GO terms. GO analysis was performed by Dr Marjorie Fournier.

4.2.3 Switch I region of Rab11a is important for IAV PB2 binding

In line with the previously reported observations (Bhagwat *et al.*, 2020; Vale-Costa *et al.*, 2016), my findings show that IAV alters Rab11a binding partners possibly by outcompeting Rab11-FIP2 for its binding to Rab11a. The crystal structure of Rab11-FIP2 in complex with Rab11a is known, and shows the binding interface is formed by both protomers of Rab11-FIP2 which interact with switch 1 and switch 2 regions of Rab11a (Jagoe *et al.*, 2006) (**Figure 4.3A**). To test whether PB2-C interacts with the same region of Rab11a as Rab11-FIP2, I mutated residues 44-46 in switch I region of Rab11a, which are buried between the two helices of Rab11-FIP2 (**Figure 4.3B**), by PCR site-directed mutagenesis from IGV to AAA. This Rab11CA switch I mutant (hereafter referred to as Rab11CA A44-46) was expressed and purified as a fusion protein with an N-terminal GST tag to enable its immobilisation on glutathione sepharose beads. In a pull-down assay with immobilised GST-Rab11CA A44-46, the majority of purified NT60 PB2-C was in the flow through (**Figure 4.3C, FT**). In contrast, NT60 PB2-C bound to immobilised wild type GST-Rab11CA (**Figure 4.3D, bound**), as well as to wild type Rab11CA released from the beads (**Figure 4.3D, cleaved**). Hence, mutation in switch I of Rab11CA significantly reduces the affinity of interaction of Rab11CA with NT60 PB2-C (**Figure 4.3E**).

These data provide evidence that PB2 of the IAV RdRP potentially binds to the same site of Rab11a as Rab11-FIP2. Moreover, these data support the findings that IAV modulates host recycling by outcompeting Rab11-FIPs for interaction with Rab11a (Bhagwat *et al.*, 2020; Vale-Costa *et al.*, 2016).

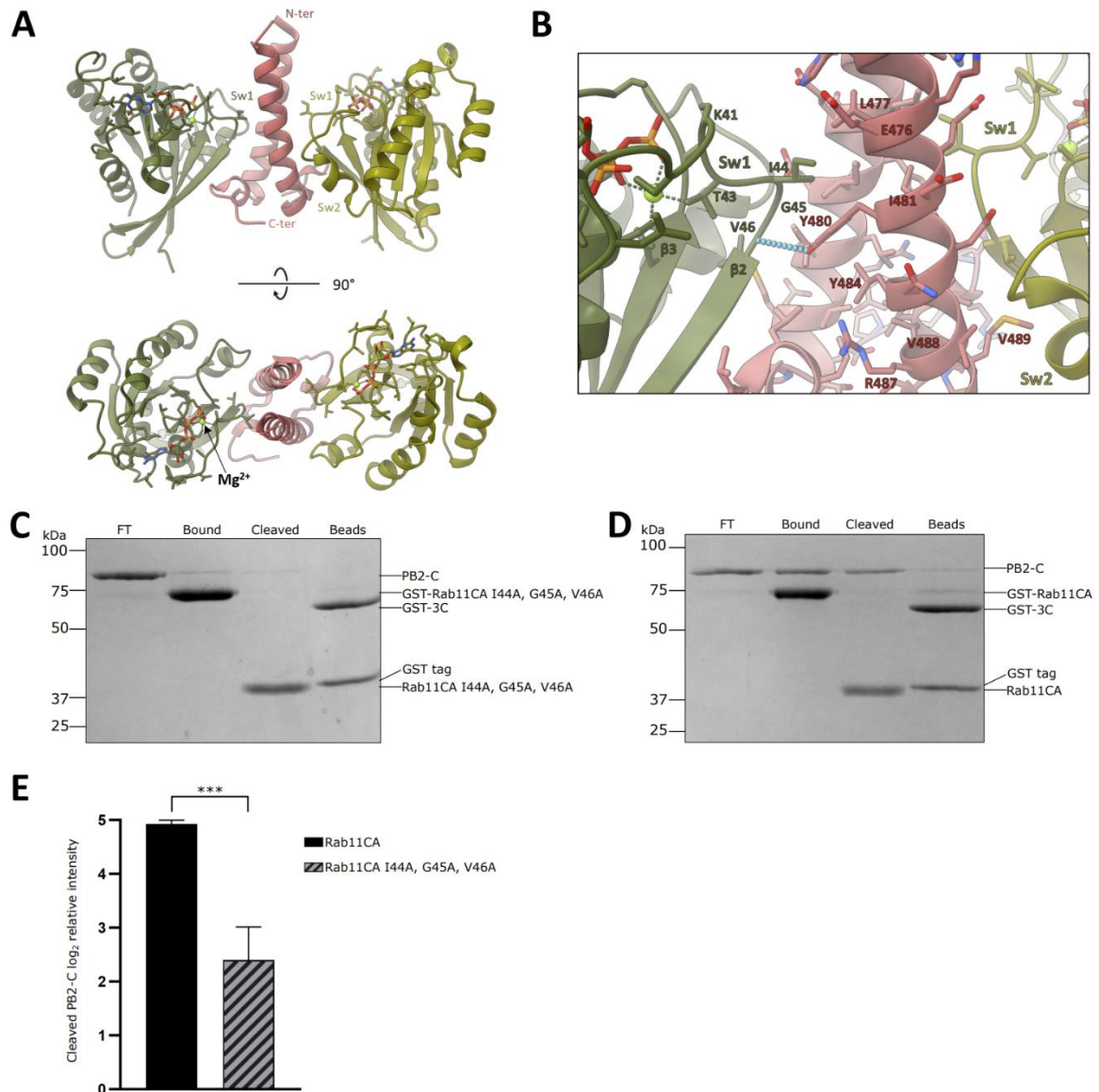


Figure 4.3: Switch I region of Rab11a is essential for IAV PB2 binding.

(A) Ribbon model of the heterotetrameric Rab11-(Rab11-FIP2)₂-Rab11 complex (top) and its 90° rotation (bottom). Rab11 molecules are shown in green, while Rab11-FIP2 molecules are coloured in red. Switch 1 and switch 2 are indicated (Sw1 and Sw2, respectively), GTP is represented as a stick model, and the conserved Mg²⁺ ion is drawn as a green sphere. PDB: 2GZD. **(B)** Detail of the Rab11a binding interface with Rab11-FIP2 showing the key residues in switch 1 which are buried between the two helices of Rab11-FIP2, packing against leucine 477 (L477), tyrosine 480 (Y480), and isoleucine 481 (I481). In addition to packing, the phenolic side chain of Rab11-FIP2 (tyrosine at position 480, Y480) makes a

hydrogen bond with the backbone of valine 46 (V46) of Rab11a (blue dots). Purified **(C)** GST-Rab11CA Ala44-46 or **(D)** wild type GST-Rab11CA was immobilised onto glutathione sepharose beads before being incubated with purified NT60 PB2-C. Complex formed was cleaved from the beads using 3C protease. Samples were analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after binding of the NT60 PB2-C; *Lane Bound*, bound sample; *Lane Cleaved*, cleaved complex; *Lane Beads*, washed beads after cleavage. **(E)** The graph shows the mean intensity signal of the NT60 PB2-C in cleaved fraction normalised to the mean intensity signal of cleaved Rab11CA relative to that of a Rab11CA switch I mutant from three independent biological repeats ($n = 3$). Data are shown as mean $\pm$ SD ($n = 3$ independent experiments/group); ns $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ (two-tailed unpaired student's t-tests). $P \leq 0.05$ is considered significant to reject the null hypothesis.

4.3 Discussion

In this study, the interaction between the influenza virus vRNP and cellular GTPase Rab11a was investigated using IAV infected cell lysate in a GST pull-down assay. Analysing RNAs present in co-precipitated sample, the study found that Rab11a differentially binds to vRNP and not cRNP. This is in contrast to the results obtained using purified vRNA- and cRNA-bound RdRP (**Chapter 3, Figure 5**), but in agreement that Rab11a is involved in vRNP trafficking and genome assembly. A previous study has observed cRNP complexes entering the cytoplasm after the RNA promoter sequence was mutated to match the one of the vRNP (Tchatalbachev *et al.*, 2001). Considering this, differential binding might occur even earlier during the vRNPs nuclear export via M1/NEP. Therefore, one could speculate that if Rab11a indeed differentially binds to vRNPs, its differential binding is determined by the same factor that causes differential binding of M1/NEP to vRNPs. This factor may be a difference in vRNA and cRNA sequences which exert a different conformational stress on the RdRP, thereby affecting the RdRP structure (Fan *et al.*, 2019; Hengrungs *et al.*, 2015; Pflug *et al.*, 2014; Reich *et al.*, 2014). These results also indicate that short RNAs used in the experiment presented in Chapter 3 did not affect the conformation of the RdRP, thus leading to a discrepancy in the data presented in this chapter.

In this study, the effect of IAV infection on the Rab11a interactome was analysed using mass spectrometry analyses. All three viral RdRP subunits PA, PB1, and PB2, as well as NP were detected in Rab11a interactome of WSN infected lysates. This demonstrated that vRNPs interact with Rab11a, in agreement with the findings described above. However, it is important to note that after a robust statistical analysis using a PLGEM (Pavelka *et al.*,

2008), these proteins were not found significantly abundant. This could be due to their abundance in the Rab11a interactome being comparable to their abundance in GST tag interactome, which is likely a result of non-specific binding of free RdRP present in a whole cell lysate to the beads. To avoid this in the future, the whole cell lysate should be pre-incubated with the beads before commencing with the precipitation with the GST-tagged Rab11a to clear of any proteins that are binding non-specifically to the beads.

Intriguingly, NS1 and NEP were the only viral proteins which were significantly abundant in the Rab11a interactome of infected lysates at a p-value cut-off of 0.05; NS1 being more abundant than NEP. One study unambiguously identified NS1 protein in purified virions by LC-MS/MS, pointing to its potential role in virion assembly (Hutchinson *et al.*, 2014). Supporting this, another study found that NS1 directly interacts with NP in the vRNP (Robb *et al.*, 2011). It would be, therefore, tempting to speculate that the presence of NS1 bound to vRNP and Rab11a complex has a role during vRNP trafficking and a subsequent genome assembly. However, the data presented in this chapter do not necessarily mean that NS1 directly interacts with vRNP as NS1 could also interact with Rab11a independently of the vRNP, either through direct interaction or via another factor such as a host RNA-binding protein. Alternatively, Rab11a could also interact with NS1 indirectly via the vRNP. To address, whether NS1 interacts with Rab11a, a GST pull-down assay should be repeated in the future using NS1 transfected HEK 293T cells and co-precipitant should be then analysed by Western blot or mass spectrometry.

Furthermore, it is also important to highlight that Rab11b was equivalently abundant in the Rab11a interactome before and after infection as Rab11a. Rab11a and Rab11b members

of the mammalian Rab11 family share 90.4% sequence homology (identity) with most of the amino acid differences found between their C-terminal hypervariable disordered regions, which is not involved in binding to the influenza virus RdRP as demonstrated in Chapter 5 (**Figure 5.2**). Strikingly equivalent high abundance of these two proteins might be, therefore, a result of assigning detected peptides identified in Rab11a to Rab11b due to their less distinguishable protein sequences.

Moreover, by comparing the Rab11a interactomes of uninfected and infected cells, it was also shown here that IAV infection induces changes in host proteins that associate with Rab11a, which is in line with earlier mass spectrometry analyses (Bhagwat *et al.*, 2020). Independent studies have previously observed a modulated host recycling pathway upon IAV infection (Alenquer *et al.*, 2019; de Castro Martin *et al.*, 2017; Vale-Costa *et al.*, 2016). Supporting this, a recent mass spectrometry analysis showed that IAV infection induces changes in cytoskeletal motor proteins bound by Rab11a; however, the precise mechanism remained obscure (Bhagwat *et al.*, 2020). Our mass spectrometry analysis showed no changes in abundance of either cytoplasmic dynein 1 or kinesin motor protein in Rab11a interactome after the infection, which is in contrast to previous observations showing that IAV infection decreases association of cytoplasmic dynein 1 with Rab11a (Bhagwat *et al.*, 2020). However, our mass spectrometry analysis showed that Rab11-FIPs are less abundant in Rab11a interactome after the infection, suggesting that IAV infection alters the microtubule based cytoskeletal motor stoichiometry of Rab11a by decreasing the association of Rab11-FIPs with motor proteins. This is in agreement with previous studies using mitochondrial targeting Rab11-FIP proteins to show that IAV vRNP segments alter the stoichiometry of interacting cytoskeletal motor proteins, potentially by outcompeting

Rab11-FIPs for their binding to Rab11a (Vale-Costa *et al.*, 2016). Interestingly though, dynein effector protein Rab11-FIP3 was not detectable in our samples generated from HEK 293T cells, which is consistent with a preceding mass spectrometry study which could not detect Rab11-FIP3 in adenocarcinomic human alveolar basal epithelial (A549) cells (Bhagwat *et al.*, 2020).

From structural studies it is known that Rab11-FIPs contain a highly conserved Rab11 binding domain (RBD) at their C termini, which forms a parallel coiled-coil homodimer, creating two symmetrical interfaces for binding the switch I and switch II regions of two Rab11a molecules (Eathiraj *et al.*, 2006; Jagoe *et al.*, 2006; Shiba *et al.*, 2006). Moreover, crystal structures of Rab11a in complex with Rab11-FIP2 (Jagoe *et al.*, 2006) and Rab11-FIP3 (Shiba *et al.*, 2006) revealed that residues 44-46 of Rab11a switch I region are involved in binding to RBD of both Rab11-FIP2 and Rab11-FIP3. Interestingly, using purified Rab11CA with mutations in residues 44-46 in a GST pull-down assay, this study demonstrated for the first time that these three residues are also involved in binding to the IAV PB2-C. Given that Rab11-FIP2 and Rab11-FIP3 bind to the same residues of Rab11a as IAV PB2-C, the results of this study present the first evidence supporting the hypothesis that IAV vRNP segments outcompete Rab11-FIPs for binding to Rab11a. Moreover, since residues 476-498 of the RBD are the most conserved residues in the Rab11-FIP family (Jagoe *et al.*, 2006), residues 44-46 of Rab11a switch I region are also likely to be involved in binding to RBD of Rab11-FIP5. Indeed, our mass spectrometry data showed that kinesin effector protein Rab11-FIP5 is less abundant in the Rab11a interactome after the infection while the abundance of kinesin motor protein itself is unchanged. Therefore, it is tempting to speculate that by outcompeting Rab11-FIPs for binding to Rab11a, IAV vRNPs associate with the plus-end

microtubule directed motors (cell membrane oriented), thereby promoting its transport in a direction towards the plasma membrane. This is indeed in line with the role of Rab11a in trafficking of vRNPs towards the cell membrane. In support of this is the fact that although both kinesin and dynein motor proteins are microtubule-based transport motors, they are structurally distinct (Ross *et al.*, 2008). However, further studies are needed to clarify whether IAV vRNP indeed binds to kinesin motor protein, and if so, if the interaction is direct or via another factor which works as an effector protein instead of Rab11-FIP5.

In summary, our study demonstrated for the first time that Rab11a has a differential specificity for vRNP and cRNP only binding to vRNP. Analysis of the Rab11a interactome further revealed that IAV infection reduces Rab11a binding to Rab11-FIPs, thereby altering the stoichiometry of cytoplasmic motor proteins bound by Rab11a. Furthermore, using mutant Rab11a in a GST pull-down assay, this study provides the first evidence that IAV RdRP potentially binds to the same site on Rab11a as Rab11-FIPs.

5 Rab11a does not determine the host specificity of influenza A virus

5.1 Introduction

Influenza viruses rely on numerous host factors to support their replication, therefore, most of the zoonotic transfer events are unproductive. Aquatic birds are a natural reservoir of influenza viruses able to infect specific mammalian species, albeit not very efficiently. In such rare cases avian influenza viruses (AIVs) must accumulate mutations that support efficient replication and transmission in the new host species (Long *et al.*, 2019). AIV requires as little as five adaptive mutations for efficient transmission between mammalian hosts, four of them in HA and one of them in PB2 (Herfst *et al.*, 2012; Imai *et al.*, 2012). Studies have shown that these mutations change the AIV receptor binding specificity from $\alpha 2,3$ - to $\alpha 2,6$ -linked sialic acid (Herfst *et al.*, 2012), and enable efficient replication by an avian RdRP in the mammalian upper respiratory tract epithelium which has lower temperatures compared to the gastrointestinal tracts of birds (Subbarao *et al.*, 1993). These mutations appear to be the bare minimum required for efficient airborne transmission of AIV between mammalian hosts, however, there are also host adaptive mutations found in other viral proteins apart from HA and PB2 that contribute to the efficiency of AIV adaptation to mammalian hosts (Cauldwell *et al.*, 2014; Long *et al.*, 2019). The AIV RdRPs do not function efficiently in mammalian cells without host-adaptive mutations (Nilsson *et al.*, 2017). Mutation of position 627 in the PB2 subunit is critical for AIV RdRP activity within mammalian cells (Subbarao *et al.*, 1993). It displays a clear host-associated genetic signature of lysine (K) in mammalian isolates and glutamic acid (E) in avian isolates. The role of the 627 residue and other host-adaptation residues in the PB2

subunit in viral replication have been extensively studied (reviewed in Long *et al.*, 2019). The host protein ANP32A restricts AIV replication in mammalian cells through differential regulation of the RdRP activity carrying avian-type PB2-627E over human-type PB2-627K (Long *et al.*, 2016). However, PB2 host-adaptive mutations do not solely have an effect on the enhancement of the RdRP activity. PB2-701 residue undergoes host-adaptive mutation to enhance nuclear translocation in mammalian cells by switching specificity from importin- α 3 to importin- α 7 upon mammalian adaptation (Gabriel *et al.*, 2011). It remains unclear whether any host-adaptation mutations are relevant in late stages of the IAV life cycle. Interestingly, it has been recently shown that avian-type PB2-627E but not human-type PB2-627K causes vRNPs to aggregate near the microtubule organising centre (MTOC) of mammalian cells, resulting in aberrant vRNPs trafficking (Liu *et al.*, 2021). To the best of our knowledge, this is the first study to implicate adaptive strategies of the influenza virus PB2 protein after nuclear export of the newly synthesised viral genome.

In this study, I aimed to investigate whether adaptive mutations in PB2 affect its interaction with GTPase Rab11a by using recombinantly expressed PB2 proteins in a GST pull-down assay. Using heterotrimeric RdRP from an AIV, I demonstrate that Rab11a does not determine the host specificity of IAV. An improved understanding of possible adaptive changes required during late stages of the IAV life cycle will address many of the remaining questions concerning the molecular mechanisms of influenza virus host range restriction.

5.2 Results

5.2.1 Avian-type PB2-C does not interact with Rab11a

Having shown that human-adapted IAV infection causes alterations in Rab11a cycle in mammalian cells by competing with Rab11 interacting partners, I next questioned whether avian-adapted IAV also interacts with Rab11a. To address this question, I generated and purified PB2-C protein from the influenza A/Duck/Fujian/01/2002 (H5N1) virus (hereafter referred to as Fj PB2-C) as described in Chapter 3.2.2 and incubated it with immobilised GST-Rab11CA. SDS-PAGE analysis of pull-down fractions showed that Fj PB2-C fails to bind to GST-Rab11CA (**Figure 5.1A, bound**) in contrast to NT60 PB2-C which binds to GST-Rab11CA (**Figure 5.1B, bound**), and was used as a positive control. This novel discovery represents an interesting starting point towards further understanding host specificity during IAV infection.

To determine, whether Fj PB2-C failed to bind to GST-Rab11CA because it specifically recognises Rab11a expressed in birds, I first compared protein sequences of Rab11a from *Homo sapiens* (human) and *Aythya fuligula* (duck) using Clustal Omega programme (**Figure 5.2A**). Interestingly, Rab11a protein sequences are identical apart from a single amino acid residue at position 210: valine (V) in human Rab11a and methionine (M) in duck Rab11a (**Figure 5.2A, red square**). Since the C-terminal region of Rab11a, containing amino acid 210, is known to reversibly anchor to lipid membranes (Goto-Ito *et al.*, 2019), I hypothesised that a change in this amino acid would not affect PB2-C binding to GST-Rab11CA. In order to test this, I used site-directed PCR mutagenesis to introduce a C-terminal truncation of 43 hypervariable residues of Rab11CA (hereafter referred to as

Rab11CA 1-173), which has been shown previously to have no impact on protein folding (Jagoe *et al.*, 2006; Shiba *et al.*, 2006). I confirmed the purification of a GST-Rab11CA 1-173 fusion protein (~40 kDa) by SDS-PAGE (**Figure 5.2B**) and purified it further by SEC (**Figure 5.2C**). SEC peak of GST-Rab11CA 1-173 was collected and analysed by SDS-PAGE side by side with the collected SEC peak of full length Rab11a (**Figure 5.2D**). As expected, SDS-PAGE analysis of GST pull-down fractions showed that NT60 RdRP binds to immobilised GST-Rab11CA 1-173 (**Figure 5.2E, bound**) and stays bound to Rab11CA 1-173 after 3C cleavage (**Figure 5.2E, cleaved**). Together, these data show that the hypervariable 43 C-terminal residues of Rab11a are not required for the interaction between IAV RdRP and Rab11a. Hence, these data support my hypothesis that failure of Fj PB2-C to bind to human Rab11a is not due to a difference in the amino acid sequence of mammalian and avian Rab11a.

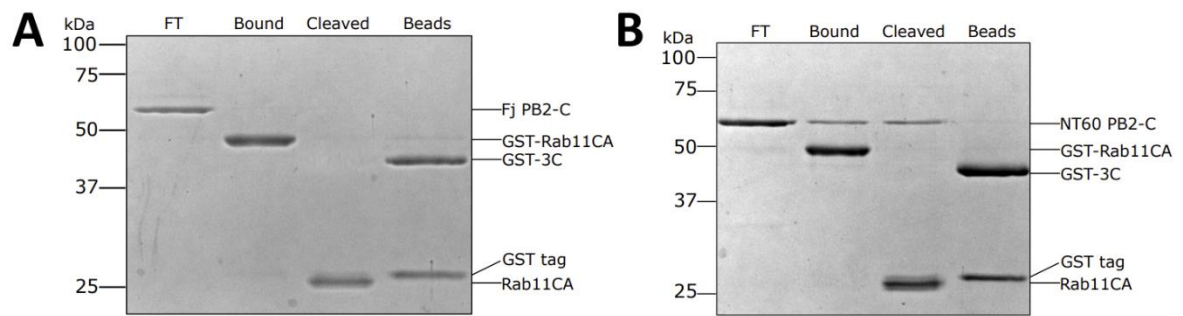


Figure 5.1: PB2-C from the influenza A/Duck/Fujian/01/2002 (H5N1) virus does not bind to Rab11a.

Purified GST-Rab11CA was immobilised onto glutathione sepharose beads before being incubated with a purified **(A)** Fj PB2-C or **(B)** NT60 PB2-C. GST-Rab11CA was cleaved from the beads using 3C protease. Samples were analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after binding of the PB2-C; *Lane Bound*, bound sample; *Lane Cleaved*, cleaved complex; *Lane Beads*, washed beads after cleavage.

A	Rab11a human	MGTRDDEYDYLKVVVLIGDSGVGKSNLLSRFTRNEFNLESKSTIGVEFATRISIQVDGKTI	60
	Rab11a duck	MGTRDDEYDYLKVVVLIGDSGVGKSNLLSRFTRNEFNLESKSTIGVEFATRISIQVDGKTI	60

	Rab11a human	KAQIWDTAGQERYRAITSAYYRGAVGALLVYDIAKHLTYENVERWLKELRDHADSNIIVIM	120
	Rab11a duck	KAQIWDTAGQERYRAITSAYYRGAVGALLVYDIAKHLTYENVERWLKELRDHADSNIIVIM	120

	Rab11a human	LVGNKSDLRHLRAVPTDEARAFAEKNGLSFIETSAKDSTNVEAAFQTILTEIYRIVSQKQ	180
	Rab11a duck	LVGNKSDLRHLRAVPTDEARAFAEKNGLSFIETSAKDSTNVEAAFQTILTEIYRIVSQKQ	180

	Rab11a human	MSDRRENDMSPSNVPIHVPPTTENKPKVQCCQNI	216
	Rab11a duck	MSDRRENDMSPSNVPIHVPPTTENKPKMQCCQNI	216

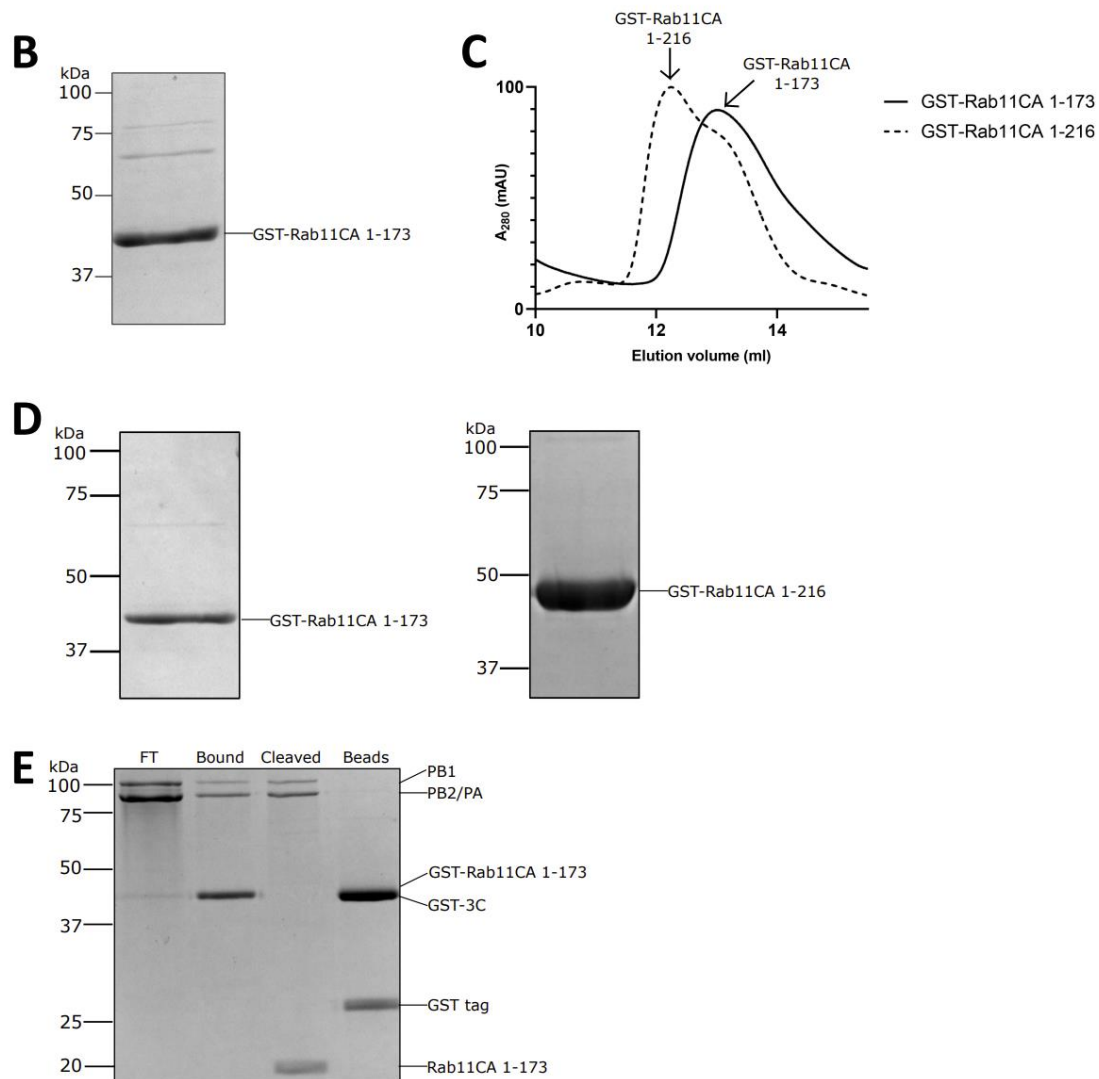


Figure 5.2: The C-terminal 43 residues of Rab11a are not involved in the interaction between the IAV RdRP and Rab11a.

(A) Multiple Sequence Alignment of *Homo sapiens* Rab11a (UniProt: P62491) and *Aythya fuligula* Rab11a (UniProt: A0A6J3DN50) using Clustal Omega. * indicates positions which have a single, fully conserved residue. : indicates conservation between groups of strongly similar properties. Red square indicates residue at position 210 which is valine (V) in human Rab11a and methionine (M) in duck Rab11a. **(B)** Recombinant Rab11CA 1-173 was expressed in *E. coli* cells and purified by affinity chromatography as a fusion protein with a GST tag on the N-terminus. GST-Rab11CA 1-173 fusion protein was then eluted under mild, non-denaturing conditions using reduced glutathione, and analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. **(C)** Elution profile of size exclusion chromatography (SEC) on a Superdex® 200 increase 10/300 column of eluted recombinant GST-Rab11CA 1-173 protein compared to the gel filtration profile of a full-length GST-Rab11CA 1-216. **(D)** SEC peak of GST-Rab11CA 1-173 was collected and analysed by SDS-PAGE reducing gel (left) side by side with the collected SEC peak of full length GST-Rab11CA 1-216 (right). Bands were visualised using Coomassie Brilliant Blue R-250 dye. **(E)** Purified GST-Rab11CA 1-173 was immobilised onto glutathione sepharose beads before being incubated with a purified NT60 RdRP. Complex formed was cleaved from the beads using 3C protease. Samples were analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after binding of the NT60 RdRP; *Lane Bound*, bound sample; *Lane Cleaved*, cleaved complex; *Lane Beads*, washed beads after cleavage.

5.2.2 Host determinant PB2-627 residue alone is not a determinant of the PB2-C–Rab11a interaction

I hypothesised that avian RdRP does not directly interact with avian Rab11a, or that if such interaction does occur it is not mediated by the PB2-C. This is supported by the observation that avian PB2-C does not interact with mammalian Rab11a, and by demonstration that the differences between mammalian and avian Rab11a are unlikely to affect the affinity of the interaction with IAV polymerase. Based on these findings, I sought to determine whether a particular residue in the avian-type PB2 prevents direct interaction between avian RdRP and Rab11a. My data (Chapter 3) show that the PB2⁶²⁷ domain is required for a direct interaction between IAV RdRP with a human-type PB2 and Rab11a. Furthermore, a single mutation at position 627 in the PB2⁶²⁷ domain of human adapted IAV has been shown to induce aggregation of vRNPs in mammalian host cells (Liu *et al.*, 2021). I hypothesised that the 627-point mutation prevents interaction between RdRP and Rab11a, in turn preventing vRNP trafficking and hence leading to their aggregation. Furthermore, I hypothesised that Fj PB2-C fails to bind to either avian or mammalian Rab11a due to the presence of glutamic acid (E) at position 627, which is lysine (K) in NT60 PB2-C. To test this hypothesis, I used site-directed PCR mutagenesis to introduce the K627E and E627K mutation in NT60 PB2-C and Fj PB2-C, respectively. I then purified these two mutant PB2-C proteins and used them in a GST pull-down assay to test their binding to GST-Rab11CA. Interestingly, SDS-PAGE analysis of pull-down fractions showed that NT60 PB2-C K627E binds to immobilised GST-Rab11CA (**Figure 5.3A, bound**) as did NT60 PB2-C K627 (**Figure 5.3B, bound**) which was used as a positive control. Furthermore, SDS-PAGE analysis of pull-down fractions showed Fj PB2-C E627K did not bind to immobilised GST-Rab11CA (**Figure**

5.3C, bound) as was the case for Fj PB2-C E627 (**Figure 5.3D, bound**), which was used as a negative control. While there were no significant changes in binding of either NT60 PB2-C K627E or Fj PB2-C E627K to GST-Rab11CA compared to their corresponding wild type proteins, there was significantly lower binding of Fj PB2-C E627 to GST-Rab11CA compared to NT60 PB2-C K627 (**Figure 5.3E**). Together these data show that the interaction between PB2-C and Rab11a may be host specific; however, the mutation of host determinant PB2 residue 627 alone does not prevent the direct interaction between NT60 PB2-C protein and Rab11a. Furthermore, a single mutation of the 627 residue of avian PB2-C does not enable interaction with mammalian Rab11a.

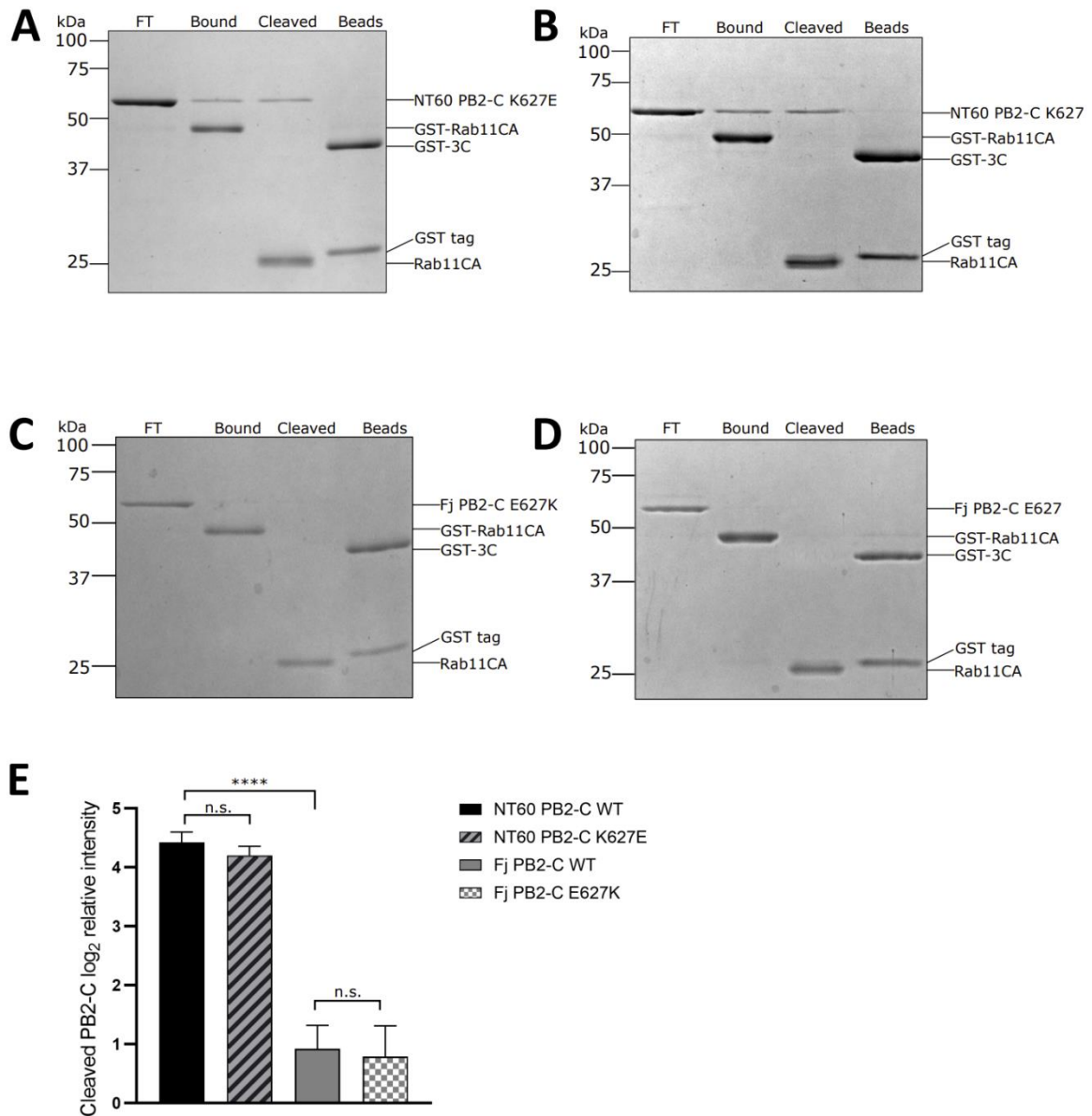


Figure 5.3: Host determinant PB2 residue 627 alone is not a determinant of interaction between human-type PB2-C and Rab11a.

Purified GST-Rab11CA was immobilised onto glutathione sepharose beads before being incubated with a purified **(A)** NT60 PB2-C K627E, **(B)** NT60 PB2-C K627, **(C)** Fj PB2-C E627K, or **(D)** Fj PB2-C E627. GST-Rab11CA was cleaved from the beads using 3C protease. Samples were analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after binding of the PB2-C; *Lane Bound*, bound sample; *Lane Cleaved*, cleaved complex; *Lane Beads*, washed beads after cleavage. **(E)** The graph shows the mean intensity signal of the PB2-C mutant in cleaved fraction

normalised to the mean intensity signal of cleaved Rab11CA relative to that of a corresponding wild type PB2-C from three independent biological repeats ($n = 3$). Data are shown as mean $\pm$ SD ($n = 3$ independent experiments/group); ns $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ (ordinary one-way ANOVA with Dunnett's post hoc test for multiple comparisons). $P \leq 0.05$ is considered significant to reject the null hypothesis.

5.2.3 PB2-C–Rab11a interaction is not determined by a single PB2 host-adaptive mutation

In order to determine whether there are other host-adaptive mutations present in the PB2-C which might be important for binding to Rab11a, I purified PB2-C protein from the influenza A/Viet Nam/1203/2004 (H5N1) (hereafter referred to as Viet PB2-C), which is a human isolate of IAV of the same subtype as Fj IAV but with the K627 residue. As Viet PB2-C and Fj PB2-C are both derived from H5N1 viruses, their protein sequences are more similar to each other (98.2% identity) than to the NT60 PB2-C, which is derived from an H3N2 virus (**Figure 5.4**). I ordered a synthetic Viet PB2-C gene and cloned into the pET151 plasmid (**Figure 5.5A**). I purified Viet PB2-C as a fusion protein with 6xHis and V5 tags on the N-terminus and confirmed the presence of a protein which size corresponds to 6xHis-V5-Viet PB2-C (~60 kDa) by SDS-PAGE (**Figure 5.5B**). 6xHis-V5-Viet PB2-C was then purified further by SEC which showed a distinct elution peak (**Figure 5.5C**). The SEC peak was collected, and the correct molecular weight of the 6xHis-V5-Viet PB2-C protein was confirmed by SDS-PAGE (**Figure 5.5D**). For simplicity, I will hereafter refer to 6xHis-V5-Viet PB2-C as Viet PB2-C even though 6xHis and V5 tags were not removed during the purification due to technical difficulties. Furthermore, I generated a Viet PB2-C protein with the K627E mutation. SDS-PAGE analysis of pull-down fractions showed Viet PB2-C binds to immobilised GST-Rab11CA (**Figure 5.5D, bound**) and remains bound to Rab11CA cleaved from the beads using HRV 3C protease (**Figure 5.5D, cleaved**). Furthermore, SDS-PAGE analysis of pull-down fractions showed that Viet PB2-C K627E protein also binds to GST-Rab11CA (**Figure 5.5E, bound**). This is reminiscent of NT60 PB2-C K627E.

Viet PB2-C differs in seven amino acid residues from Fj PB2-C (**Figure 5.6A**). To address the relevance of these residues in binding to Rab11a, I used site-directed PCR mutagenesis to individually mutate each of the three surface exposed residues in Viet PB2-C to equivalent amino acid residue in Fj PB2-C: A274T, K526R, and L607V. I then purified these three mutant Viet-PB2-C proteins and analysed their binding to GST-Rab11CA in a GST pull-down assay alongside wild type Viet-PB2-C as a positive control. SDS-PAGE analysis of pull-down fractions showed that regardless of which individual surface exposed residue was mutated Viet PB2-C binds to immobilised GST-Rab11CA (**Figure 5.6B, bound**), and remains bound to Rab11CA cleaved from the beads (**Figure 5.6B, cleaved**). Further mutants with either a double mutation (A274T + K526R or A274T + L607V) and the triple mutation (A274T + K526R + L607V) of surface exposed residues showed similar results (**Figure 5.6C, cleaved**). These data suggest that surface exposed residues alone do not affect the binding of Viet PB2-C to GST-Rab11CA.

Next, I used site-directed PCR mutagenesis to individually mutate non-exposed residues in Viet PB2-C to equivalent amino acid residue in Fj PB2-C: I553V, V584I, and A622T. Unfortunately, I failed to purify a A622T Viet-PB2-C mutant protein due low expression levels. However, I have successfully purified two of these mutant Viet-PB2-C proteins, I553V and V584I, and analysed their binding to GST-Rab11CA in a GST pull-down assay alongside wild type Viet-PB2-C as a positive control. SDS-PAGE analysis of pull-down fractions showed that regardless of which non-exposed residue was mutated Viet PB2-C binds to immobilised GST-Rab11CA (**Figure 5.6D, bound**), and remains bound to Rab11CA after cleavage (**Figure 5.6D, cleaved**). These results show that residue at neither position 553 nor at position 584 alone affects the binding of Viet PB2-C to Rab11a.

Overall, these data show that the host specific binding observed needs a combination of amino acid changes that was not tested in this study. Therefore, it would be highly informative to repeat the GST pull-down experiment using a purified heterotrimeric RdRP from Fj IAV.

NT60 PB2	MERIKELRNLMSSQSRTEILTKTTVDHMAIIKKYTSGRQEKNPRLMKWMMAMKYPITAD	60
Viet PB2	MERIKELR[REDACTED]LMSQSRTEILTKTTVDH[REDACTED]AIIKKYTSGRQEKNP[REDACTED]RLMKWMMAMKYPITAD	60
Fj PB2	MERIKELR[REDACTED]LMSQSRTEILTKTTVDH[REDACTED]AIIKKYTSGRQEKNP[REDACTED]RLMKWMMAMKYPITAD	60
	*****:*****:*****:*****	
NT60 PB2	KRITEMVPERNEQGQTLWSKMSDAGSDRVMVSPLAVTWNNRNGPMTSTVHYPKVYKTYFE	120
Viet PB2	KRI[REDACTED]EM[REDACTED]PERNEQGQTLWSK[REDACTED]DAGSDRVMVS[REDACTED]LAVTWNNRNGP[REDACTED]TS[REDACTED]VHYPKVYKTYFE	120
Fj PB2	KRI[REDACTED]EM[REDACTED]PERNEQGQTLWSK[REDACTED]DAGSDRVMVS[REDACTED]LAVTWNNRNGP[REDACTED]TS[REDACTED]VHYPKVYKTYFE	120
	*** *:*****.***** ***** **.******	
NT60 PB2	KVERLKHGTFGPVHFRNQVKIRRRVDINPGHADLSAKEAQDVIMEVVPNEVGARILTSE	180
Viet PB2	KVERLKHGTFGPVHFRNQVKIRRRVD[REDACTED]NPGHADLSAKEAQDVIMEVVPNEVGARILTSE	180
Fj PB2	KVERLKHGTFGPVHFRNQVKIRRRVD[REDACTED]NPGHADLSAKEAQDVIMEVVPNEVGARILTSE	180
	*****:*****	
NT60 PB2	SQLTITKEKKEELQDCKISPLMVAYMLERELVRKTRFLPVAGGTSSVYIEVLHLTQGTGW	240
Viet PB2	SQLTITKEKKEELQDCKI[REDACTED]PLMVAYMLERELVRKTRFLPVAGGTSSVYIEVLHLTQGTGW	240
Fj PB2	SQLTITKEKKEELQDCKI[REDACTED]PLMVAYMLERELVRKTRFLPVAGGTSSVYIEVLHLTQGTGW	240
	*****:*****	
NT60 PB2	EQMYTPGGEVRNDDVDQSLIIAARNIVRRAVSADPLASLLEMCHSTQIGGTRMVDILRQ	300
Viet PB2	EQMYTPGGEVRNDDVDQSLIIAARNIVRRA[REDACTED]VS[REDACTED]DPLASLLEMCHSTQIGG[REDACTED]RMVDILRQ	300
Fj PB2	EQMYTPGGEVRNDDVDQSLIIAARNIVRRA[REDACTED]VS[REDACTED]DPLASLLEMCHSTQIGG[REDACTED]RMVDILRQ	300
	*****:*.***** *****	
NT60 PB2	NPTEEQAVDICKAAMGLRISSSFSGGFTFKRTSGSSIKREEELLTGNLQTLKIRVHDGY	360
Viet PB2	NPTEEQAVDICKAAMGLRISSSFSGGFTFKRTSGSS[REDACTED]K[REDACTED]EEE[REDACTED]LTGNLQTLKIRV[REDACTED]EGY	360
Fj PB2	NPTEEQAVDICKAAMGLRISSSFSGGFTFKRTSGSS[REDACTED]K[REDACTED]EEE[REDACTED]LTGNLQTLKIRV[REDACTED]EGY	360
	*****:*.***:*****:*	
NT60 PB2	EEFTMVGKRATAILRKATRRLVQLIVSGRDEQSVAEAIIVAMVFSQEDCMIAVRGDLNF	420
Viet PB2	EEFTMVG[REDACTED]RATAILRKATRRL[REDACTED]QLIVSGRDQ[REDACTED]SAEAIIVAMVFSQEDCMIAVRGDLNF	420
Fj PB2	EEFTMVG[REDACTED]RATAILRKATRRL[REDACTED]QLIVSGRDEQ[REDACTED]SAEAIIVAMVFSQEDCMIAVRGDLNF	420
	*****:*****:*****:*.*****	
NT60 PB2	VNRRANQRLNPMHQLLRHFQKDAKVLQFNWNGIEHIDNVMGMIGVLPDMTPSTEMSMRGIRV	480
Viet PB2	VNRRANQRLNPMHQLLRHFQKDAKVLQFNWNGIE[REDACTED]IDNVMGMIG[REDACTED]LPDMTPSTEMS[REDACTED]RG[REDACTED]RV	480
Fj PB2	VNRRANQRLNPMHQLLRHFQKDAKVLQFNWNGIE[REDACTED]IDNVMGMIG[REDACTED]LPDMTPSTEMS[REDACTED]RG[REDACTED]RV	480
	***** *****:*****:*.***	
NT60 PB2	SKMGVDEYSSTERVVVSIDRFLVRDQRGNVLLSPEEVSETQGTEKLITYSSSMWEIN	540
Viet PB2	SKMGVDEYSSTERVVVSIDRFLVRDQRGNVLLSPEEVSETQGTE[REDACTED]LITYSSSMWEIN	540
Fj PB2	SKMGVDEYSSTERVVVSIDRFLVRDQRGNVLLSPEEVSETQGTE[REDACTED]LITYSSSMWEIN	540
	*****:*****	
NT60 PB2	GPESVLVNTYQWIIIRNWETVKIQWSQNPTMLYNKMEFEPFQSLVPKAIARGQYSGFVRTLF	600
Viet PB2	GPESVLVNTYQW[REDACTED]IRNWETVKIQWSQ[REDACTED]PTMLYNKMEFEPFQSL[REDACTED]PKA[REDACTED]ARGQYSGFVRTLF	600
Fj PB2	GPESVLVNTYQW[REDACTED]IRNWETVKIQWSQ[REDACTED]PTMLYNKMEFEPFQSL[REDACTED]PKA[REDACTED]ARGQYSGFVRTLF	600
	*****:*****:*****:*** *****	

NT60 PB2	QQMRDVLGTFDTTQIIKLLPFAAAPPKQSRMQFSSLTVNVRGSGMRILVRGNSPAIFYNK	660
Viet PB2	QQMRDVIGTFDTVQIIKLLPFAAAPKQSRMQFSSLTVNVRGSGMRILVRGNSPAIFYNK	660
Fj PB2	QQMRDVIGTFDTVQIIKLLPFAAAPKQSRMQFSSLTVNVRGSGMRILVRGNSPAIFYNK	660
	*****:*****.*****:*****:*****:*****.*****	
NT60 PB2	TTKRLTILGKDAGTLIEDPDEGTSGVESAVLRGFLILGKEDRRYGPALSINELSNLAKGE	720
Viet PB2	TTKRLTILGKDAGTLIEDPDEGTSGVESAVLRGFLILGKEDRRYGPALSINELSNLAKGE	720
Fj PB2	TTKRLTILGKDAGTLIEDPDEGTSGVESAVLRGFLILGKEDRRYGPALSINELSNLAKGE	720
	:*****:*****:* *****:*****:*****:*****:*****	
NT60 PB2	KANVLIGQGDVVLVMKRKRDSILTDSQTATKRIRMAIN	759
Viet PB2	KANVLIGQGDVVLVMKRKRDSILTDSQTATKRIRMAIN	759
Fj PB2	KANVLIGQGDVVLVMKRKRDSILTDSQTATKRIRMAIN	759

Figure 5.4: Influenza virus PB2 CLUSTAL O(1.2.4) multiple sequence alignment.

Sequence alignment of the PB2 protein from the influenza A/Northern Territory/60/1968 (H3N2), influenza A/Viet Nam/1203/2004 (H5N1), and influenza A/Duck/Fujian/01/2002 (H5N1) virus. Residues that are identical between Fj PB2 and Viet PB2 but different from NT60 PB2 are highlighted in green; those that are different between Fj PB2 and Viet PB2 are shown in red.

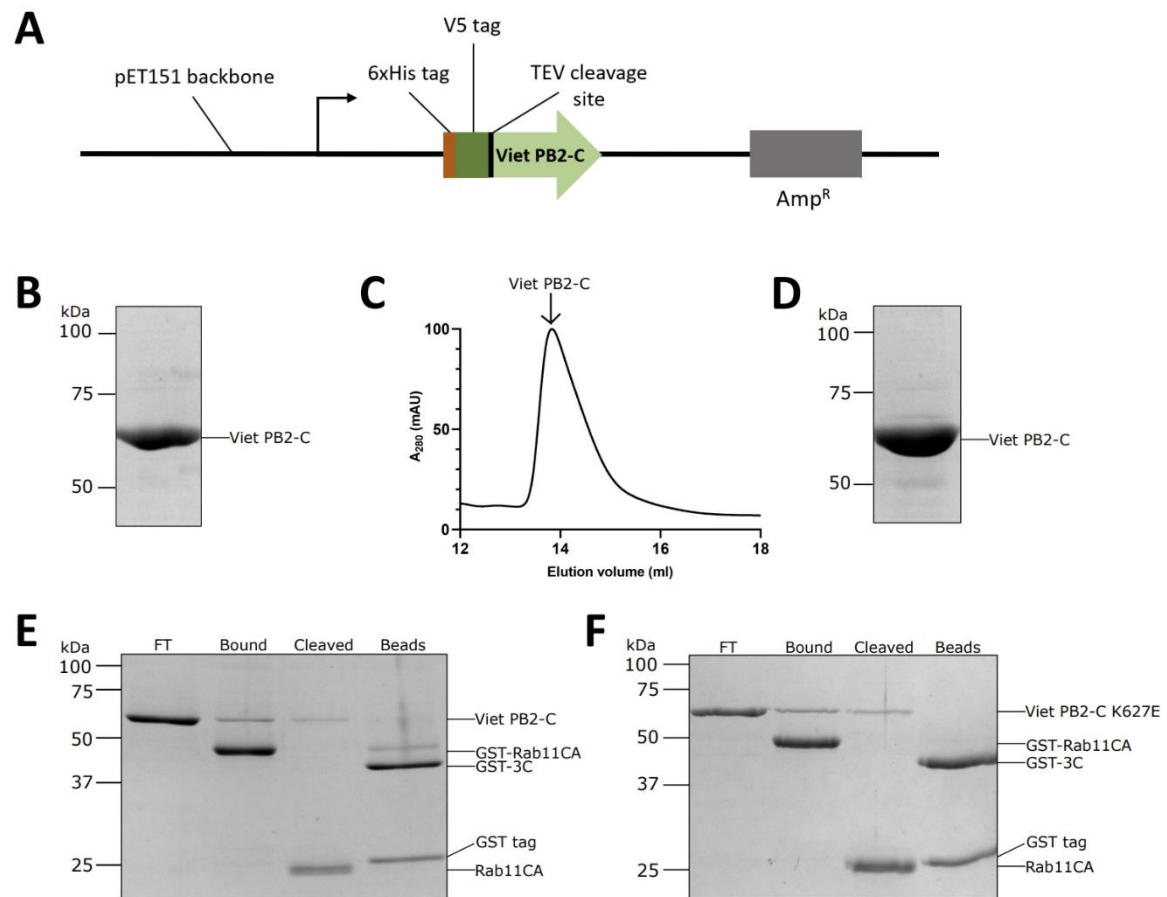


Figure 5.5: Viet PB2-C and Viet PB2-C K627E bind to Rab11a.

(A) A schematic of Viet PB2-C plasmid. Residues 246-736 (lacking the flexible NLS peptide, residues 737-760) of Viet PB2-C were cloned into the vector pET151 with N-terminal 6xHis and V5 tags separated by a tobacco etch virus (TEV) cleavage site. Direction of the open reading frame is indicated with the arrow. **(B)** Recombinant Viet PB2-C was expressed in *E. coli* cells and purified by affinity chromatography as a fusion protein with 6xHis and V5 tags on the N-terminus. 6xHis-V5-Viet PB2-C fusion protein was then eluted with 500 mM of imidazole and analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. **(C)** Elution profile of size exclusion chromatography (SEC) on a Superdex® 200 increase 10/300 column of purified 6xHis-V5-Viet PB2-C. Gel filtration chromatogram shows a distinct peak of purified 6xHis-V5-Viet PB2-C protein. **(D)** SEC peak of Viet PB2-C was collected and analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. **(E)** Purified GST-Rab11CA was

immobilised onto glutathione sepharose beads before being incubated with a purified 6xHis-V5-Viet PB2-C. GST-Rab11CA was cleaved from the beads using 3C protease. Samples were analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after binding of the 6xHis-V5-Viet PB2-C; *Lane Bound*, bound sample; *Lane Cleaved*, cleaved complex; *Lane Beads*, washed beads after cleavage. **(F)** Same as in **E** but incubating GST-Rab11CA with purified 6xHis-V5-Viet PB2-C K627E protein instead.

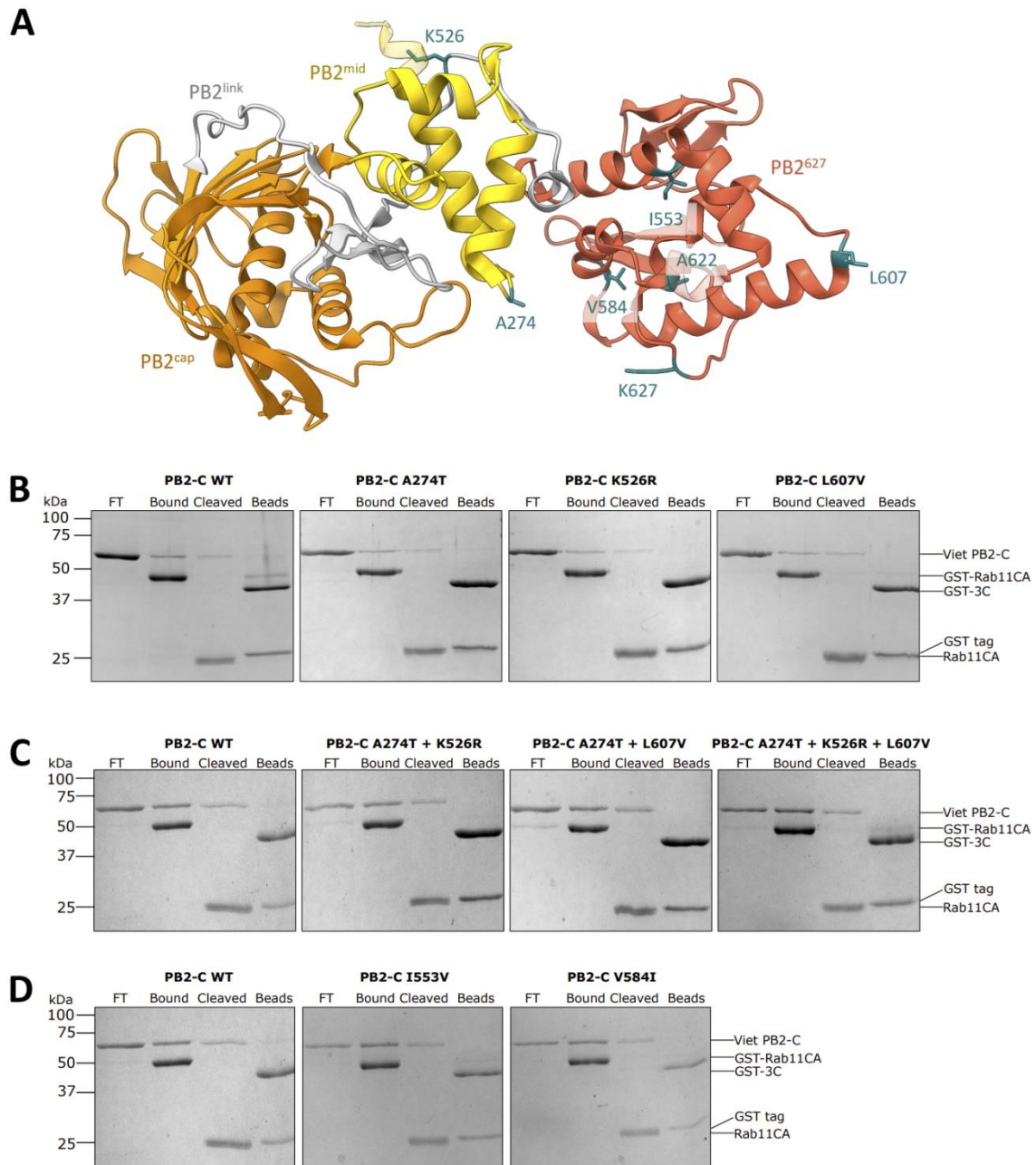


Figure 5.6: Investigating potential host-adaptive mutations in PB2-C protein from the influenza A/Viet Nam/1203/2004 (H5N1) involved in binding to Rab11a.

(A) Ribbon representation of the crystal structure of an isolated influenza A/Viet Nam/1203/2004 (H5N1) PB2-C with each PB2-C domain coloured in a different colour. Seven amino acid residues which differ from the influenza A/Duck/Fujian/01/2002 (H5N1) PB2-C are coloured in cyan. One of these residues is found in the PB2^{mid} domain (A274), one in the PB2^{link} domain (K526), and five of them are in the PB2⁶²⁷ domain (I553, V584,

L607, A622, K627). The entire NLS domain is missing from the electron density. PDB: 5FMM. Purified GST-Rab11CA was immobilised onto glutathione sepharose beads before being incubated with either purified 6xHis-V5-Viet PB2-C containing **(B)** a single amino acid substitution of a surface exposed residue, **(C)** a double or triple amino acid substitution of a surface exposed residue, or **(D)** a single amino acid substitution of a surface non-exposed residue. Complex formed was cleaved from the beads using 3C protease. Samples were analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after binding of the mutant 6xHis-V5-Viet PB2-C; *Lane Bound*, bound sample; *Lane Cleaved*, cleaved complex; *Lane Beads*, washed beads after cleavage.

5.2.4 Avian type RdRP binds to Rab11a

Finally, to test, whether avian-type RdRP binds to Rab11a, I repeated the GST pull-down experiment using a purified heterotrimeric RdRP from the influenza A/Duck/Fujian/01/2002 (H5N1) virus (hereafter referred to as Fj RdRP), cloned and expressed by Dr Haitian Fan. Surprisingly, SDS-PAGE analysis of pull-down fractions showed that Fj RdRP binds to immobilised GST-Rab11CA (**Figure 5.7A, bound**), and remains bound to Rab11CA after the cleavage (**Figure 5.7A, cleaved**). As a control, I incubated purified NT60 RdRP with immobilised GST-Rab11a which showed bound NT60 RdRP to immobilised GST-Rab11CA (**Figure 5.7B, bound**) as well as to Rab11CA released from the beads (**Figure 5.7B, cleaved**).

These data demonstrate that the loss of binding observed between Fj PB2-C and Rab11a is not due to PB2 host-adaptive mutations but may be a result of how different PB2-C proteins behave in solution.

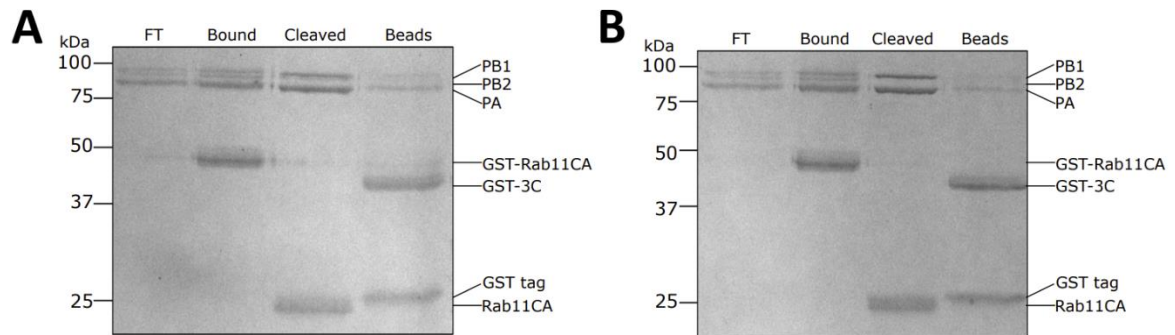


Figure 5.7: RdRP from influenza A/Duck/Fujian/01/2002 (H5N1) directly interacts with Rab11a.

Purified GST-Rab11CA was immobilised onto glutathione sepharose beads before being incubated with a purified **(A)** Fj RdRP or **(B)** NT60 RdRP. GST-Rab11CA was cleaved from the beads using 3C protease. Samples were analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after binding of the RdRP; *Lane Bound*, bound sample; *Lane Cleaved*, cleaved complex; *Lane Beads*, washed beads after cleavage.

5.3 Discussion

In this study, I investigated a role of Rab11a in determining the host specificity of IAVs during viral export with a GST pull-down assay. I showed that purified PB2-C from an avian adapted IAV RdRP fails to bind to Rab11a. This observation was shown to be independent of differences in Rab11a expressed in mammals and birds and instead most likely due to the conformation avian PB2-C adapted in solution outside the context of the heterotrimeric RdRP.

The 627 host-adaptation mutation is a significant factor determining the autophagy-related host restriction of avian infection during intracellular trafficking (Liu *et al.*, 2021). Namely, aberrant vRNP trafficking and aggregation occurs in A549 cells when infected with a WSN PB2 K627E but not with mammalian adapted WSN PB2 K627 (Liu *et al.*, 2021). Our study shows that the NT60 PB2-C K627 and NT60 PB2-C K627E directly interact with Rab11a. Furthermore, neither Fj PB2-C E627 nor Fj PB2-C E627K interact with Rab11a. Together, these data indicate that the mutation of PB2-C 627 residue conveys host adaptation during viral export independently of Rab11a. Furthermore, I showed that Viet PB2-C, from a human isolate of IAV of the same subtype as Fj PB2-C, binds to Rab11a both with and without the K627E mutation. Viet PB2-C differs in seven residues from Fj PB2-C, one of them being the 627 residue. Furthermore, Viet PB2-C retained direct interaction with Rab11a upon mutation of divergent residues regardless of whether these were surface exposed or non-exposed, and regardless of whether mutations were performed individually or in combination. Together, these data suggest that the Fj PB2-C fails to bind to Rab11a because of having a combination of amino acid changes that was not tested in

this study. Alternatively, lack of binding observed between Fj PB2-C and Rab11a may not be due to a particular host-adaptive mutation but rather a result of the order and nature of amino acids in the primary sequence of Fj PB2-C protein which determine its folding pattern in a solution that may be incompatible to bind to Rab11a. To test this hypothesis, I used purified heterotrimeric Fj RdRP and showed that it binds to Rab11a. In light of all these findings, we propose that the lack of avian-type PB2-C interaction with Rab11a observed in our *in vitro* assay is a result of differences in the conformation adopted in solution of PB2-C proteins isolated from different strains of IAV. Furthermore, as both avian and mammalian RdRP directly interact with Rab11a, we propose a requirement for Rab11a during IAV export may be shared across species. Given the high conservation of Rab11a, host adaptive mutations relevant during late stages of IAV infection are likely to be Rab11a independent. Consequently, a pharmacological intervention of the RdRP interaction with Rab11a may offer broad protection from influenza across livestock including swine and avian reservoirs.

However, it is important to note, that our assay has limitations as it was done *in vitro* using purified PB2-C and was therefore performed in the absence of other RdRP subunits and without vRNA. Avian RdRP restriction in mammalian host can be overcome by introducing mutations in the 3' vRNA promoter, independently of a PB2 E627K mutation (Crescenzo-Chaigne *et al.*, 2002; Paterson *et al.*, 2014). This occurs because within a vRNP the sequence of the bound vRNA specifically affects RdRP conformation; and critically, it is the RdRP conformation that conveys host adaptation. Furthermore, the RdRP conformation is also determined by host interacting factors and host-adaptive mutations. In agreement, structural studies show that PB2 domains adopt strikingly different conformations

depending on the type of the RNA promoter or host factor bound (Fan *et al.*, 2019; Pflug *et al.*, 2014; Thierry *et al.*, 2016), demonstrating the extremely flexible nature of the PB2-C peripheral domains. Unlike in previous structural studies, the PB2-C protein used in this study was present in a solution and in the absence of vRNA and other RdRP subunits, which could have resulted in yet another rearrangement of peripheral domains that was unable to bind host factors such as Rab11a. Additionally, host cargo receptor p62 was shown to be a critical host protein in facilitating the formation of vRNP aggregates in cells infected with IAV bearing avian-type PB2 (Liu *et al.*, 2021). This implies that studying the host restriction of AIV using an *in vitro* assay without the presence of mammalian host cell factors might have limitations. Thus, it would be highly informative to repeat the GST pull-down assay using whole cell lysates from AIV infected cells and analyse the co-precipitants by mass spectrometry.

In summary, this study did not identify adaptive mutations in PB2 that affect the interaction between RdRP and Rab11a. However, our results using PB2-C proteins from different strains of IAV shed further light on a high flexibility of the PB2 peripheral domains. Using these purified PB2-C proteins in further structural analyses would expand our understanding of IAV RdRP conformational flexibility.

6 Structural profile of the IAV RdRP interaction with Rab11a

6.1 Introduction

With determination of the first X-ray structure of myoglobin at 6 Å in 1958 (Kendrew *et al.*, 1958), X-ray crystallography became the anchoring tool of structural biology (Shi, 2014). The initial structure of the myoglobin at 6 Å was improved to 2 Å just two years later (Kendrew *et al.*, 1960), marking the first time that a protein's structure was determined at the atomic level. Since then, the toolbox of structural biology has been augmented by other physical techniques, including nuclear magnetic resonance spectroscopy, electron microscopy (EM), and solution scattering of X-rays and neutrons (Curry, 2015).

The last few decades have seen the remarkable impact of structural biology in particular X-ray crystallography and recently the developments in cryo-EM, on the study of viruses (Veesler *et al.*, 2016). Specifically, in the field of the influenza viruses, the past seven years have witnessed unprecedented progress in the structural understanding of the RNA-dependent RNA polymerase (RdRP) with the rise of high-resolution X-ray crystallography structures (Fan *et al.*, 2019; Hengrung *et al.*, 2015; Pflug *et al.*, 2014; Reich *et al.*, 2014; Thierry *et al.*, 2016) followed by high-resolution cryo-EM structures (Chang *et al.*, 2015; Fan *et al.*, 2019; Peng *et al.*, 2019; Wandzik *et al.*, 2020). Recently, Carrique *et al.* solved a structure of the IAV RdRP in complex with the host factor ANP32A (Carrique *et al.*, 2020), demonstrating the dependence of viral transcription and replication on host factors and providing new insights into the adaptation of the IAV to mammalian hosts. Similarly, the X-ray structure of Rab11a (1-173) was solved in the presence of either GDP or GTP (Pasqualato *et al.*, 2004) as well as in the presence of the Rab11-FIPs (Jagoe *et al.*, 2006;

Shiba *et al.*, 2006). However, despite the ongoing structural studies of the influenza virus RdRP, the structural basis for the IAV RdRP interaction with host factor Rab11a remains obscure.

The aim of this study was to obtain the structure of RdRP in complex with Rab11a. Initially, I tried to crystallise either the IAV RdRP or the PB2-C protein with either a full-length or truncated Rab11a (1-173). However, in the time given, I was unable to obtain protein crystals. Therefore, the work presented in this chapter includes my attempt to obtain the structure of the IAV RdRP in complex with Rab11a by cryo-EM. However, cryo-EM grids were most likely mixed up during the data collection; consequently, the structure presented in this chapter is a structure of the RdRP of the IBV and not of the IAV. Furthermore, using Rab11a lacking the disordered C-terminal region, and individually expressed PB2-C protein, I also validated binding and complex formation of PB2-C with Rab11a by performing a small-angle X-ray scattering (SAXS) analysis. The identification of amino acid residues involved in mediating RdRP–Rab11a interaction could facilitate the development of novel small-molecule inhibitors that disrupt the interaction interface between the IAV RdRP and Rab11a.

6.2 Results

6.2.1 Cryo-EM structure of RdRP–Rab11a complex

In order to determine the exact binding site between Rab11a and IAV RdRP, we aimed to perform a structural characterisation of the complex. Being a ~270 kDa protein complex, RdRP–Rab11a was suited for single particles cryo-EM analysis. To form this complex, I

expressed and purified proteins separately. Whole cell lysate of *E. coli* BL21(DE3) cells expressing GST-Rab11a was first incubated with Glutathione-Sepharose beads to which purified vRNA-bound RdRP was added. After extensive wash, the complex was released by cleaving of the GST tag using HRV 3C protease. SDS-PAGE analysis of Rab11a purification showed Rab11a as the major component of the fraction and, in lower proportion, bands with sizes that could correspond to the RdRP subunits (**Figure 6.1A**), suggesting that proteins were released together as a complex. Released complex was further purified by size exclusion chromatography (SEC). The gel filtration chromatogram showed a large void volume when both vRNA and Rab11a were added to the NT60 RdRP (**Figure 6.1B**). Interestingly, in the presence of Rab11a, the vRNA-bound RdRP eluted as a putative monomer with no obvious putative RdRP dimer peak, as suggested by previous data in the laboratory. The excess unbound Rab11a was detected at higher elution volume. To confirm the presence of NT60 RdRP and Rab11a in elution peaks, I analysed SEC fractions 2-13 by SDS-PAGE and blotted for Rab11a and the PB2 protein (**Figure 6.1C**). Both NT60 RdRP and Rab11a were detected in fractions 7 and 8, which correspond to RdRP monomer. These fractions were then pooled together and concentrated to 0.24 mg ml⁻¹ for cryo-EM experiment. To check the purity of the sample, I analysed combined fractions 7 and 8 by SDS-PAGE, which revealed the presence of multiple bands, indicating the sample's impurity (**Figure 6.1D**).

Regardless of the impurity, the sample was used to make cryo-EM grids. On a motion corrected micrograph, particles appeared homogenous with slight aggregation (**Figure 6.2A**). 2D classification showed that approximately 800,000 particles from the data set represent RdRP in a monomeric state (**Figure 6.2B**) while the rest, approximately 350,000

particles, represent RdRP in a dimeric state (**Figure 6.2C**). Intriguingly, a unique extra density was present in RdRP dimers only (**Figure 6.2C, red square**). Among the various previous characterisations of RdRP done by cryo-EM this extra density has never been observed in IAV RdRP dimers before, therefore, we hypothesised that it could either represent Rab11a or a change in the RdRP dimer conformation in its presence. To test our hypothesis, we generated ab-initio model using the 2D classes representing the RdRP dimer (335,747 particles) and refined them using heterogenous refinement. Each class was refined independently using non-uniform refinement. Particles from the best class were then imported into RELION(3.0.4) and further 3D classified after Bayesian polishing and CTF refinement. Unique extra densities were observed on the PA and PB2 subunits of the RdRP dimer in some of the 3D classes (**Figure 6.3A**). Approximately 37,000 particles from the selected 3D classes with an extra density around the PB2 subunit were then selected, refined to a resolution of 4.19 Å, and the structure was modelled by fitting an IAV RdRP dimer model (PDB: 6QNW) into the locally sharpened map (**Figure 6.3B**). The most obvious characteristic of the IAV RdRP dimer model fitting was that the unresolved extra density on one of the RdRP molecule in a dimer represents PB2 flexible domains rather than Rab11a, which is not present in the structure (**Figure 6.4A**). The endonuclease domain (PA-N) is not resolved while PB2 subunit is resolved up to residue 250 only. The dimer is symmetric, and, intriguingly, in an RNA-free conformation (Fan *et al.*, 2019; Pflug *et al.*, 2014; Reich *et al.*, 2014; Thierry *et al.*, 2016) (**Figure 6.4B**). The dimer interface of this dimer is mediated by the arch on the PA subunit (PA^{arch}) and the β-hairpin on the PB1 subunit from one RdRP which interact with residues 330-333 and 364-365 of the PA subunit of the other RdRP (**Figure 6.4C**).

To get a better insight of the 4.19 Å resolution NT60 RdRP structure solved in this study, we compared it to other known conformations of the RdRP: transcriptase, replicase, and encapsidase (**Figure 6.5**). Intriguingly, the PB2 domain arrangement in our structure is remarkably similar to the previously described ICV RdRP in the encapsidase conformation (Carrique *et al.*, 2020). Specifically, the position of the PB2⁶²⁷ domain is almost identical while the positions of the PB2^{mid} and PB2^{627-linker} domains are different. Simultaneously, our laboratory observed an identical RdRP dimer formed by the influenza B virus (IBV) RdRP in the absence of Rab11a (unpublished). This unexpected observation warranted further analysis of the NT60 RdRP structure presented in this study. By modelling the core of the RdRP, we noticed that the structure presented in this study represents the IBV RdRP not the NT60 IAV RdRP (**Figure 6.6**).

Taken together, the RdRP structure presented in this study is not the RdRP of an IAV but rather the RdRP of the IBV. Nevertheless, our results reveal a novel conformation of the RdRP dimer which is remarkably similar to the previously described ICV RdRP in the encapsidase conformation (Carrique *et al.*, 2020).

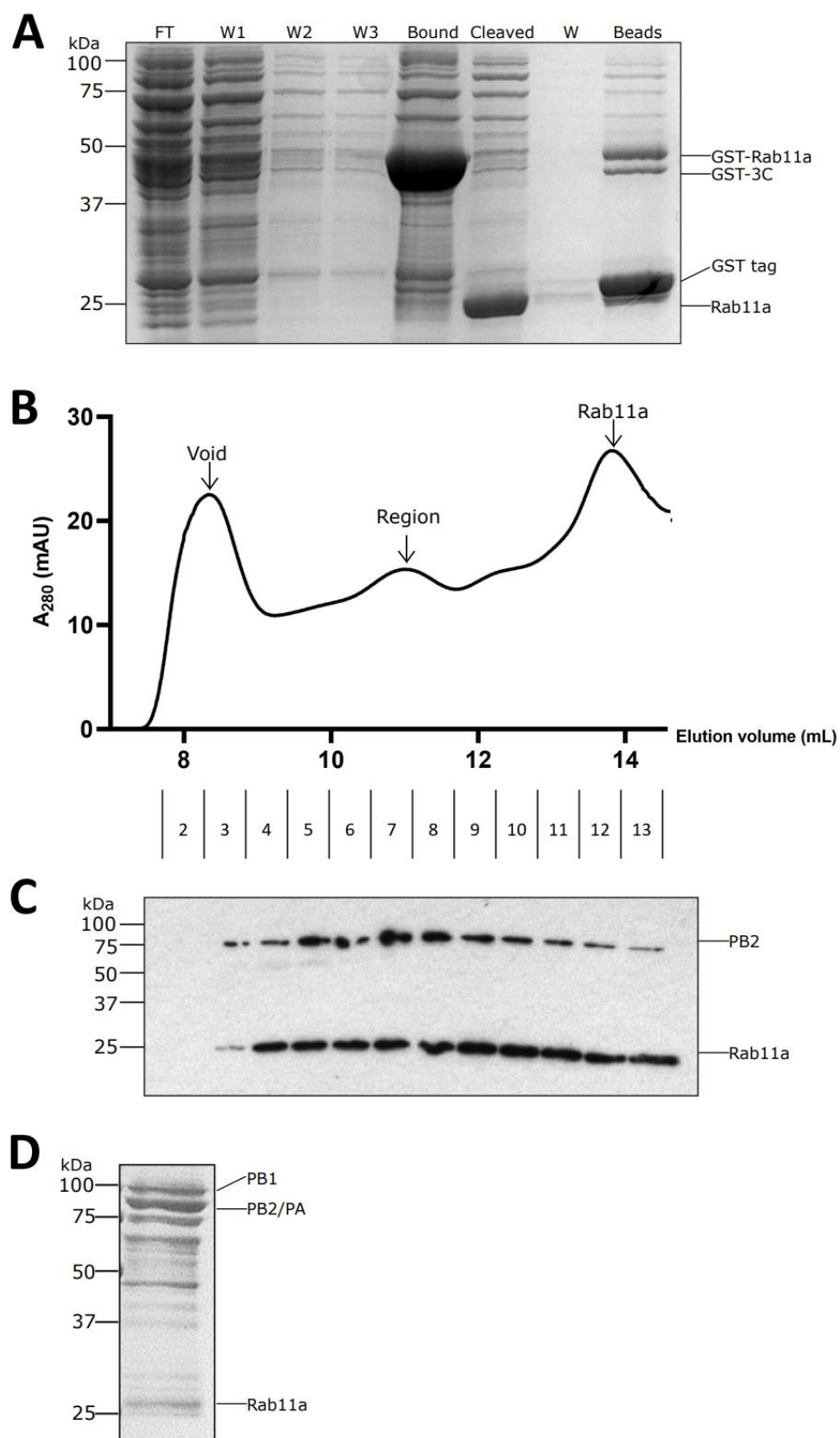


Figure 6.1: Cryo-EM sample preparation.

(A) The whole cell lysate of *E. coli* BL21(DE3) cells expressing GST-Rab11a was incubated with glutathione sepharose beads prior to the addition of purified vRNA-bound NT60 RdRP. Complex formed was cleaved from the beads using HRV 3C protease. Samples were analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye. *Lane FT*, flow through after the incubation of the whole cell lysate; *Lane W1*, wash 1; *Lane W2*, wash 2; *Lane W3*, wash 3; *Lane Bound*, after binding of the vRNA-bound NT60 RdRP; *Lane Cleaved*, cleaved complex; *Lane W*, flow through of washed beads after cleavage; *Lane Beads*, washed beads after cleavage. **(B)** Cleaved sample shown in **A** was concentrated and purified by size exclusion chromatography (SEC). Elution profile of SEC on a Superdex® 200 increase 10/300 column is shown. Gel filtration chromatogram shows a region which corresponds to the putative RdRP monomer (fractions 7 and 8) and an excess unbound Rab11a (fraction 13). **(C)** SEC fractions were analysed by 12% SDS-PAGE reducing gel. The presence of the Rab11a protein and the PB2 subunit of the IAV RdRP was confirmed by Western blotting using rabbit Rab11a protein antibody (GeneTex, GTX113301) and rabbit influenza A virus PB2 protein antibody (GeneTex, GTX125925), respectively. Fractions 7 and 8, which correspond to the putative IAV RdRP monomer, were pooled together, and concentrated to 0.24 mg ml⁻¹ for cryo-EM experiment. **(D)** Cryo-EM sample was analysed by 12% SDS-PAGE reducing gel. Bands were visualised using Coomassie Brilliant Blue R-250 dye.

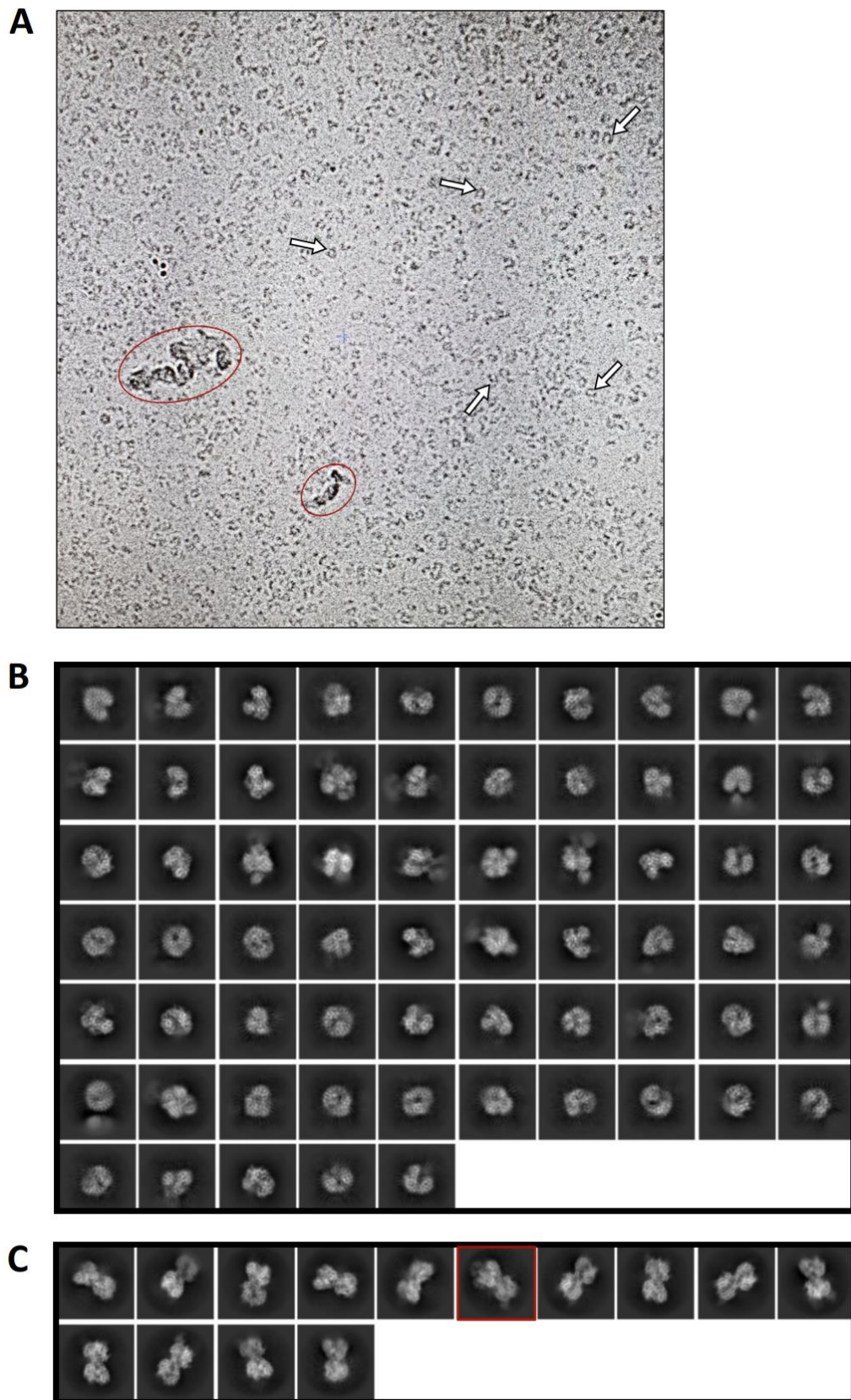


Figure 6.2: Cryo-EM analysis of vRNA-bound NT60 RdRP in complex with Rab11a.

(A) Representative motion corrected micrograph of vRNA-bound NT60 RdRP–Rab11a complex embedded in vitreous ice. White arrows point towards some of the individual particles while red circles indicate aggregated particles. **(B)** Representative 2D class averages of approximately 800,000 particles from the data set with monomeric NT60 RdRP. **(C)** Representative 2D class averages of approximately 350,000 particles from the data set with dimeric NT60 RdRP. Example of a 2D class with an extra density is highlighted in red.

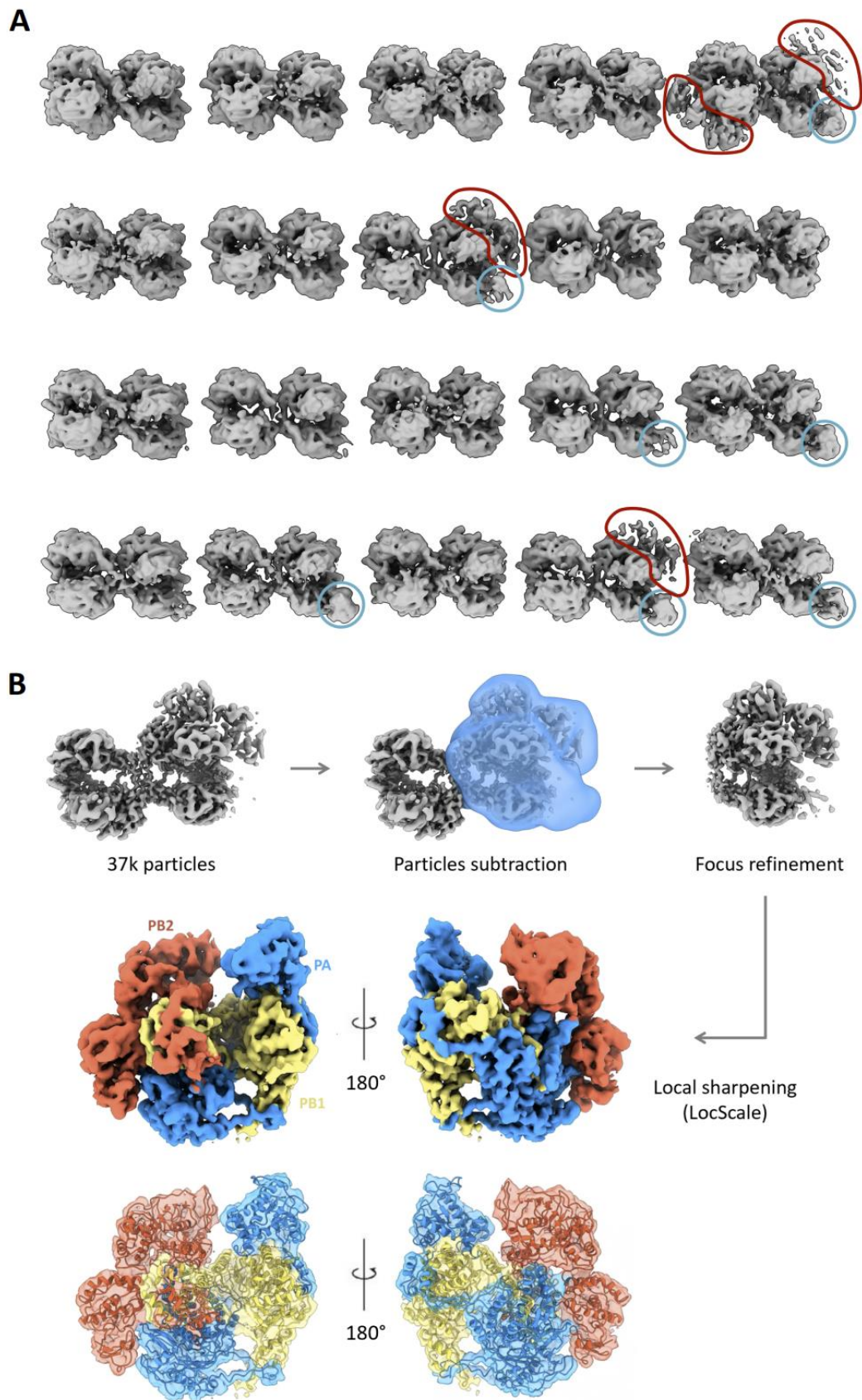


Figure 6.3: 3D classification and focus refinement of the NT60 RdRP dimer.

(A) 335,747 particles from the 2D classes representing the RdRP dimer (shown in Figure 6.2C) were refined using heterogenous refinement. Each class was refined independently using non-uniform refinement. Particles from the best class were then imported into RELION(3.0.4) and 3D classified. Extra densities around PB2 and PA subunits are circled in red and blue, respectively. **(B)** Approximately 37,000 particles from 3D classes containing extra density around the PB2 subunit were selected and refined to a resolution of 4.19 Å. The structure was modelled by fitting an IAV RdRP dimer model (PDB: 6QNW) into the locally sharpened map. Figure was made by Dr Loïc Carrique who has also done all data processing and modelling.

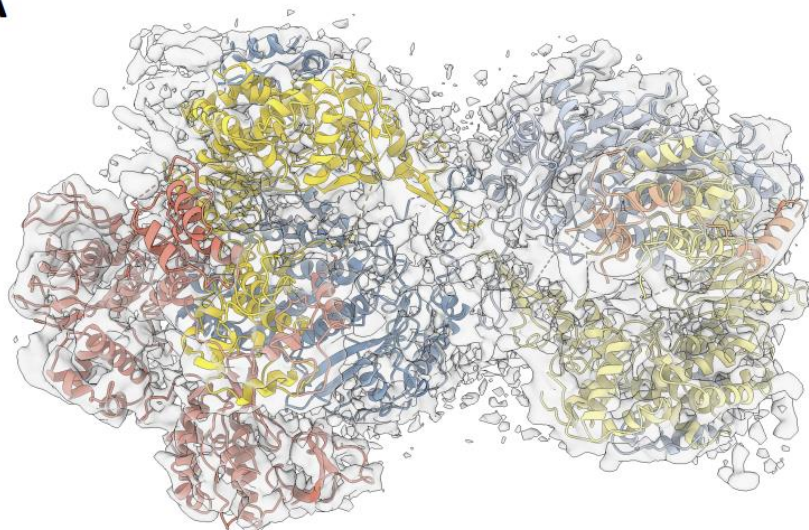
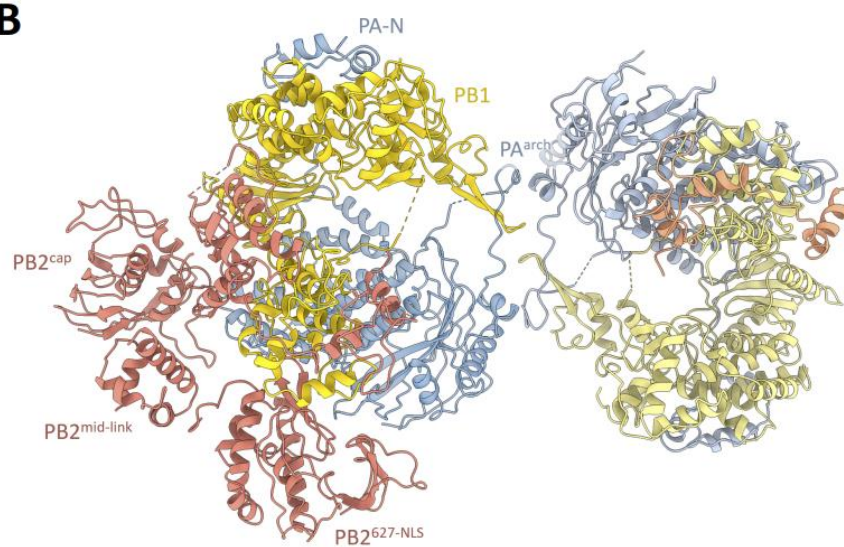
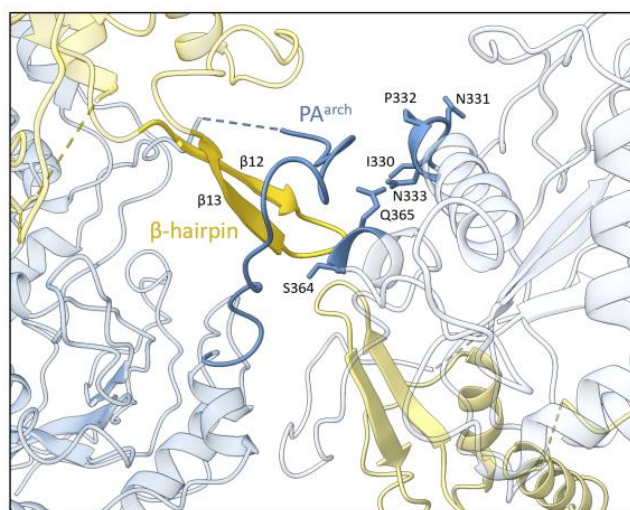
A**B****C**

Figure 6.4: Structure of the NT60 RdRP dimer obtained in this study.

(A) Modelled structure of the NT60 RdRP dimer was fitted into the local filter map. The unresolved extra density on one of the RdRP molecule in the dimer accommodates the PB2 flexible domains rather than Rab11a. PB1 subunit is coloured in yellow, PB2 in red, and PA in blue. **(B)** A ribbon representation of the NT60 RdRP dimer. PB2 domains, PA endonuclease (PA-N), and the arch on the PA subunit (PA^{arch}) are indicated. **(C)** A close-up view of the NT60 RdRP dimer interface. The PA^{arch} and the β -hairpin on the PB1 subunit from one RdRP interact with residues 330-333 and 364-365 of the PA subunit of the other RdRP. All data processing and modelling was done by Dr Loïc Carrique.

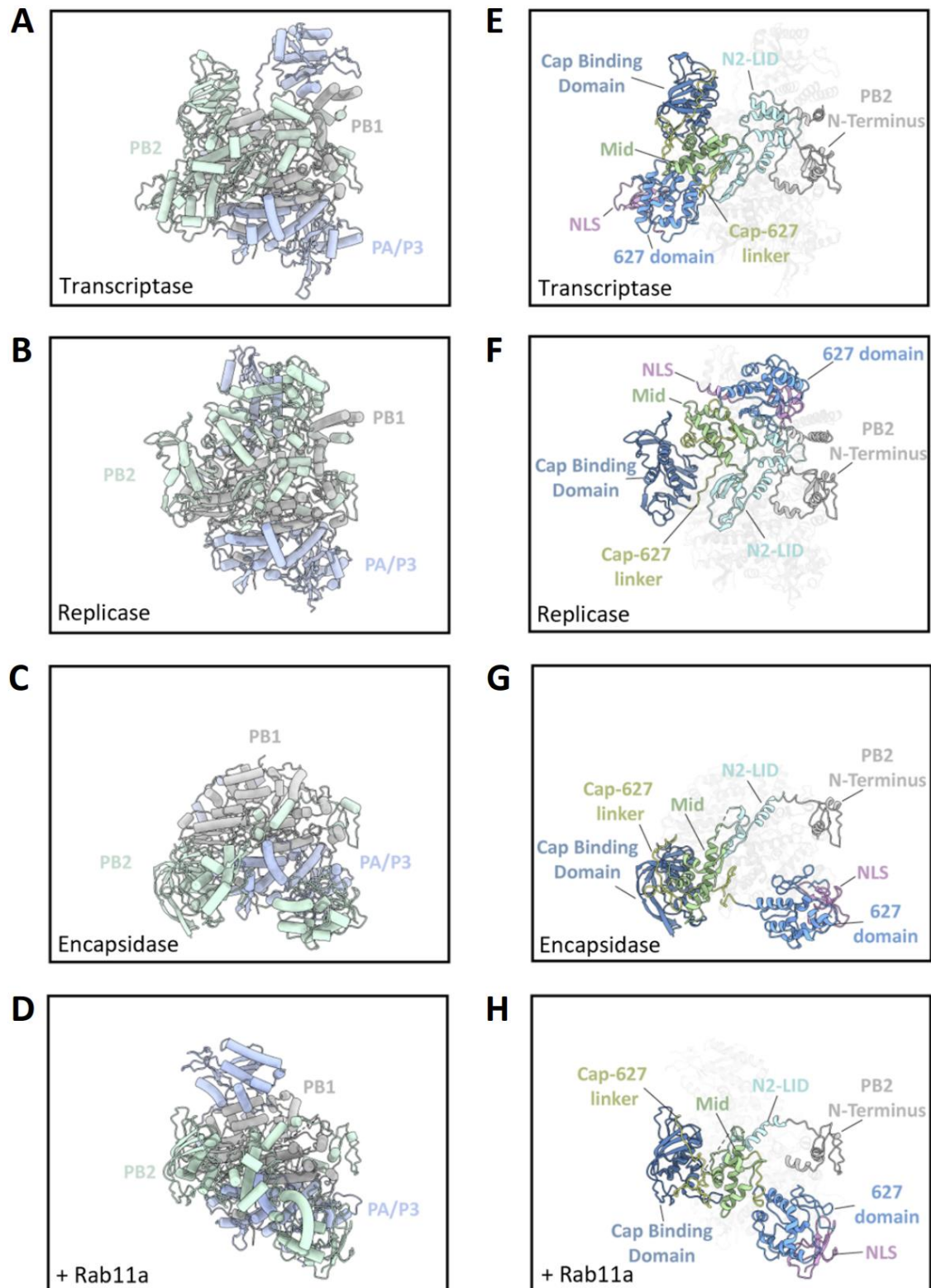


Figure 6.5: Comparison of NT60 RdRP structure in the presence of Rab11a with the transcriptase, replicase and encapsidase conformations of RdRP.

Cartoon models of the **(A)** NT60 RdRP bound to vRNA and capped RNA in the transcriptase conformation (PDB: 6RR7), **(B)** ICV RdRP in replicase conformation (PDB: 6XZR), **(C)** ICV RdRP in encapsidase conformation (PDB: 6XZR), and **(D)** NT60 RdRP in the presence of Rab11. **(E-H)** Comparison of the arrangement of the PB2 domains in the complexes shown in **A-D**. Figure was made by Dr Loïc Carrique.

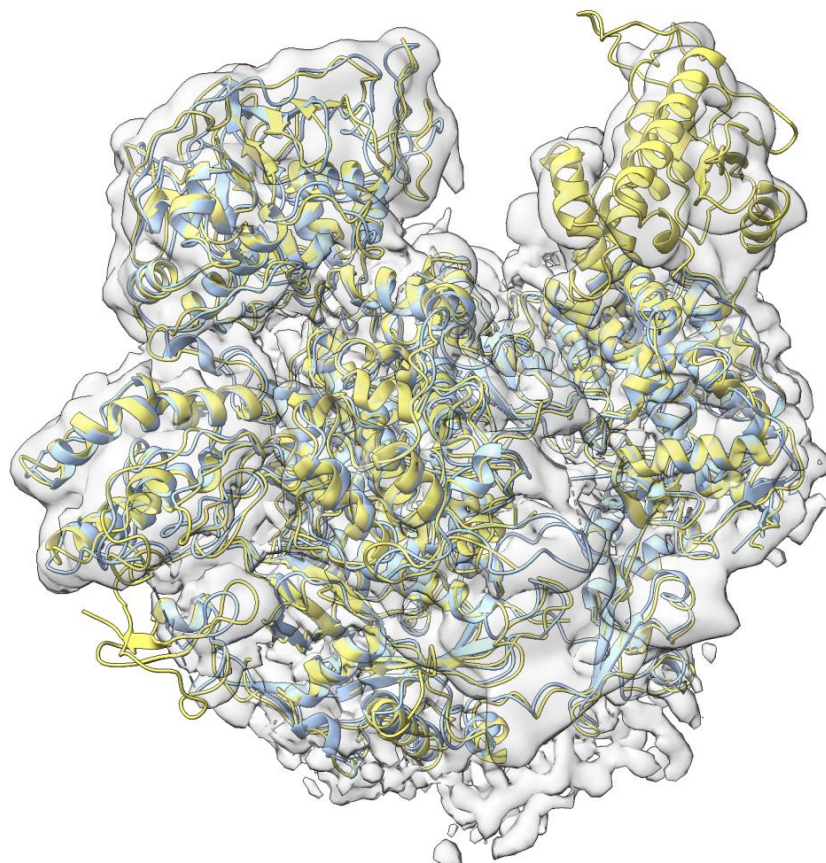
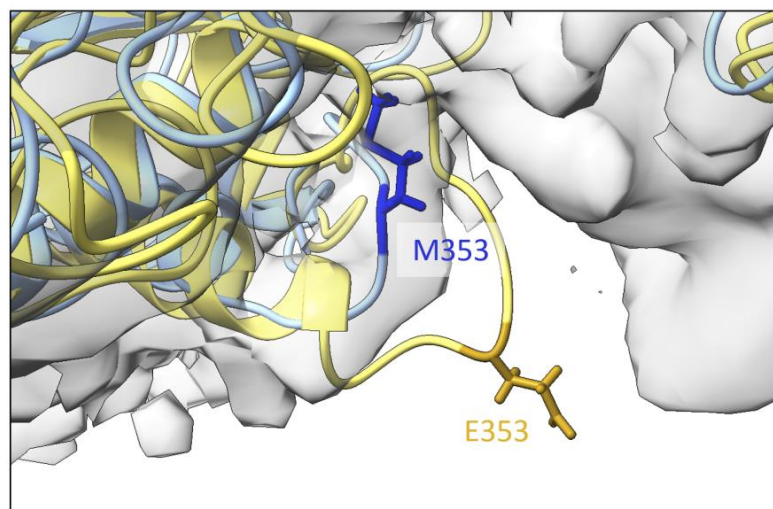
A**B**

Figure 6.6: Comparison of IAV RdRP and IBV RdRP models fitted into the local filter map.

(A) Modelled structure of the NT60 RdRP (PDB: 6RR7, yellow) and IBV RdRP (PDB: 6QWL, blue) was fitted into the local filter map. **(B)** A close-up view of the residue 353 of the PA

subunit showing that the IBV RdRP fits better to the density than the NT60 RdRP. Data modelling was done by Dr Loïc Carrique.

6.2.2 SAXS analysis of PB2-C–Rab11a complex

To validate our pull-down assay results a small-angle X-ray scattering (SAXS) experiment was performed. SAXS experiments require proteins at reasonably high concentrations to maximise the sample's scattering intensity, which is proportional to the concentration (Kikhney and Svergun, 2015). The aim was to characterise by SAXS the interaction of Rab11a with a PB2-C protein construct. Viet PB2-C and Rab11CA 1-173 proteins (hereafter referred to as PB2-C and Rab11a, respectively) were purified as described in Chapter 5. To confirm that these two proteins associated under size exclusion chromatography (SEC) conditions, PB2-C was mixed with a 1.5-fold molar excess of Rab11a, and their binding was assessed. The elution profile using a Superdex® 200 increase 10/300 column showed the main peak at an earlier elution volume of 13.67 ml compared to the elution peak of the PB2-C protein alone, which eluted at 13.83 ml (**Figure 6.7A, peak 1**). This suggests a complex is being formed between the two proteins. In addition to peak 1, the elution profile of the PB2-C–Rab11a complex also showed an additional later eluting peak at 16.46 ml, which likely is the excess unbound Rab11a (**Figure 6.7A, peak 2**). SDS-PAGE confirmed the presence of PB2-C and Rab11a in peak 1 (**Figure 6.7B**) with PB2-C and Rab11a detected in fractions 7-8, which correspond to the main elution peak (peak 1). This data confirmed that PB2-C eluted on a SEC as a complex with Rab11a. SDS-PAGE analysis showed that Rab11a also eluted in fraction 10, which corresponds to a minor peak (peak 2). These data reflect that there was an excess Rab11a, which eluted as an unbound protein.

To determine the binding site of Rab11a to the PB2-C, PB2-C and Rab11a were then subject to SEC coupled to SAXS (SEC-SAXS) analysis of the individual proteins. SEC-SAXS analysis showed PB2-C and Rab11a proteins to elute as symmetric peaks in a frame number 353

(**Figure 6.8A**) and 398 (**Figure 6.8B**), respectively. To determine the point of PB2-C saturation, SAXS data of PB2-C–Rab11a complex were collected across a range of molar ratios (PB2-C:Rab11a): 1:0.5, 1:1, 1:1.5, and 1:2. There was a shift in the elution peak of PB2-C with a 0.5-fold molar excess of Rab11a, which eluted in frame number 351 (**Figure 6.8C**), confirming that PB2-C eluted as a complex with Rab11a. However, when at least equimolar excess of Rab11a was added to PB2-C, PB2-C elution peak occurred at an even earlier frame number 348 (**Figure 6.8D-F**), suggesting a concentration dependence in elution position. The data showed that at above 1-fold molar excess of Rab11a the PB2-C protein was saturated, resulting in no longer shifted position of the PB2-C–Rab11a peak. In agreement, at frame number 398, which corresponds to the elution peak of Rab11a, the average intensity increased from 9.20 to 10.85 and 13.49 when equimolar ratio was increased to either 1.5- or 2-fold molar excess of Rab11a, respectively (**Figure 6.8D-F**). Based upon these results, we determined model-free parameters from SAXS of PB2-C, Rab11a, and PB2-C–Rab11a complex when in an equimolar ratio only.

The scattering profile of PB2-C (**Figure 6.9A**) and Rab11a (**Figure 6.9B**) showed an upper curvature at low q values, a result of the presence of radiation-induced aggregates in solution. In contrast, the scattering profile of the PB2-C–Rab11a complex showed a downturn at low q values (**Figure 6.9C**), indicating interparticle repulsion. At high q values, however, the signal of all scattering profiles started to fade into the noise level. An estimated radius-of-gyration (R_g) and the intensity at zero angle ($I(0)$) were obtained from a Guinier region of $I(q)$ at low q values (**Figure 6.9A-C, inset plots**). To ensure that the Guinier fit is good, I looked at qR_g limits: the minimum q ($q_{\min}$) times the R_g ($q_{\min}R_g$), and the maximum q ($q_{\max}$) times the R_g ($q_{\max}R_g$) of Guinier fits (**Table 6.1**). For all three proteins,

the $q_{\min}R_g$ value equals to 0.65 or less, indicating that the selected q range was sufficient to calculate R_g and $I(0)$ values. Moreover, $q_{\max}R_g$ of all Guinier fits equals to 1.30, which, in case of globular proteins, demonstrates that the Guinier fit is indeed in the linear range. To qualitatively assess the flexibility and degree of unfolding, Kratky plots of PB2-C, Rab11a, and PB2-C–Rab11a complex were generated (**Figure 6.9D**). All protein samples show bell-shaped peaks with a peak at low q values, a characteristic of well-folded proteins (Putnam *et al.*, 2007; Tuukkanen and Svergun, 2014).

To confirm that PB2-C, Rab11a, and PB2-C–Rab11a complex are globular proteins, pair-distribution functions ($P(r)$) were generated. The $P(r)$ of PB2-C (**Figure 6.10A**) and PB2-C–Rab11a complex (**Figure 6.10B**) are slightly skewed, which suggest that PB2-C and PB2-C–Rab11a complex are slightly elongated globular proteins. Interestingly, the $D_{\max}$ of PB2-C–Rab11a complex is 110 Å while $D_{\max}$ of PB2-C alone is 140 Å. These data suggest that the complex is a compact folded protein. In contrast to PB2-C and PB2-C–Rab11a complex, the $P(r)$ of Rab11a does not reach zero smoothly and shows an extended tail with $D_{\max}$ of 110 (**Figure 6.10C**).

Finally, to determine the oligomeric state of proteins in solution, molecular weights of PB2-C, Rab11a, and PB2-C–Rab11a complex were derived from five independent methods (Hajizadeh *et al.*, 2018), as implemented in PRIMUS (Konarev *et al.*, 2003) (**Table 6.2**). Molecular weight estimations for PB2-C and Rab11a proteins are close to the expected molecular weight of their monomeric species (59.2 kDa and 19.4 kDa, respectively) while molecular weight estimations for PB2-C–Rab11a complex suggest that PB2-C and Rab11a proteins form a heterodimer in solution.

Taken together, SAXS analysis show that PB2-C and Rab11a form a compact folded protein complex in solution. Moreover, these results further demonstrate that the interaction between PB2-C and Rab11a is direct, which is in agreement with our results from the GST pull-down assay.

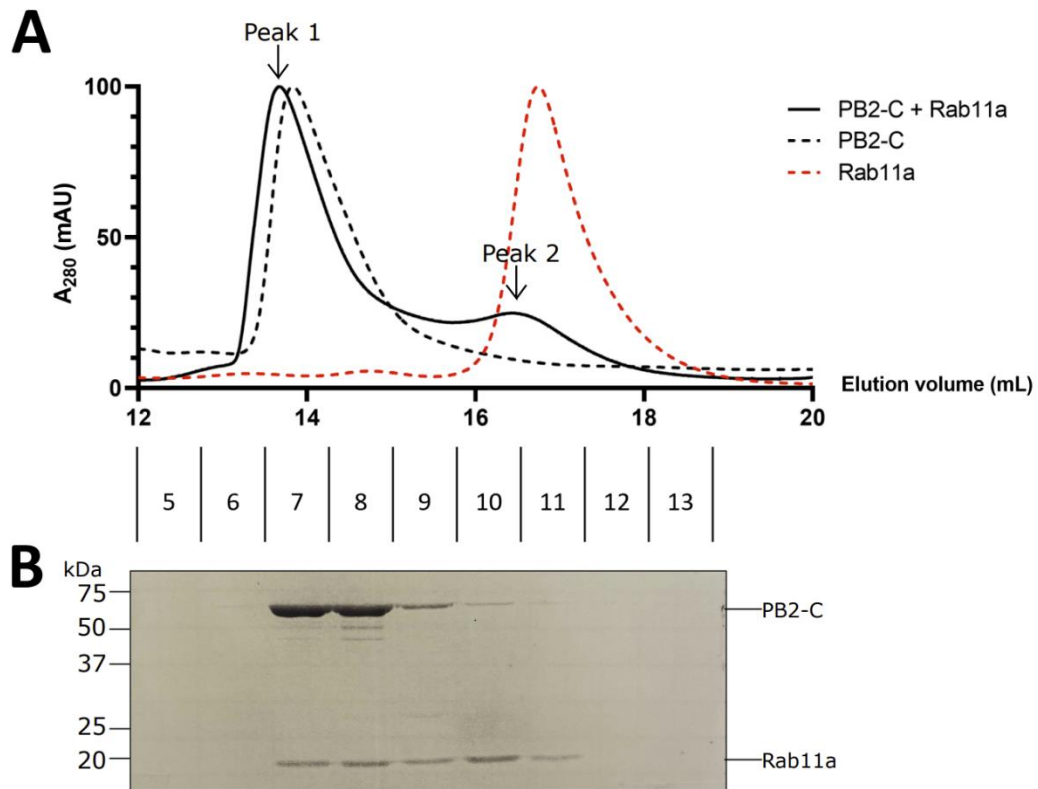


Figure 6.7: PB2-C forms a complex with Rab11a.

(A) Elution profile of size exclusion chromatography on a Superdex® 200 increase 10/300 column of purified PB2-C in complex with equimolar ratio of Rab11a. Gel filtration chromatogram shows a main peak (peak 1), which occurs at an earlier elution volume compared to the elution peak of the PB2-C alone, as well as a minor peak (peak 2), which elutes at a similar volume as Rab11a protein alone. **(B)** SEC fractions 5-13 were collected and analysed by 12% SDS-PAGE reducing gel to confirm the presence of PB2-C and Rab11a in the main peak. Fractions 7-8 correspond to the complex of PB2-C and Rab11a (peak 1) while fraction 10 corresponds to an excess unbound Rab11a (peak 2). Bands were visualised using Coomassie Brilliant Blue R-250 dye.

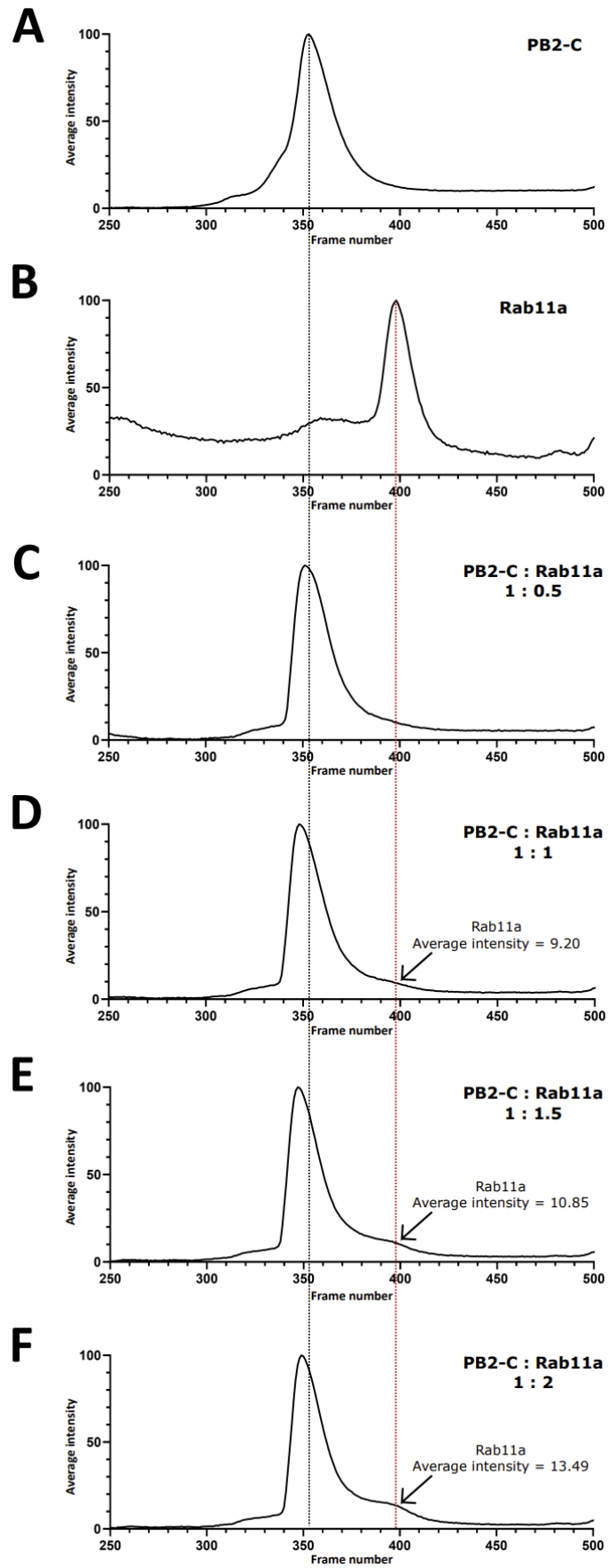


Figure 6.8: SEC–SAXS analysis confirms that PB2-C forms a complex with Rab11a.

Chromatograms from the differential refractive index detector are shown for **(A)** PB2-C, **(B)** Rab11a, and PB2-C–Rab11a complex in either **(C)** 1:0.5, **(D)** 1:1, **(E)** 1:1.5, or **(F)** 1:2 molar ratio. Black and red vertical dotted lines indicate PB2-C and Rab11a elution peaks, respectively.

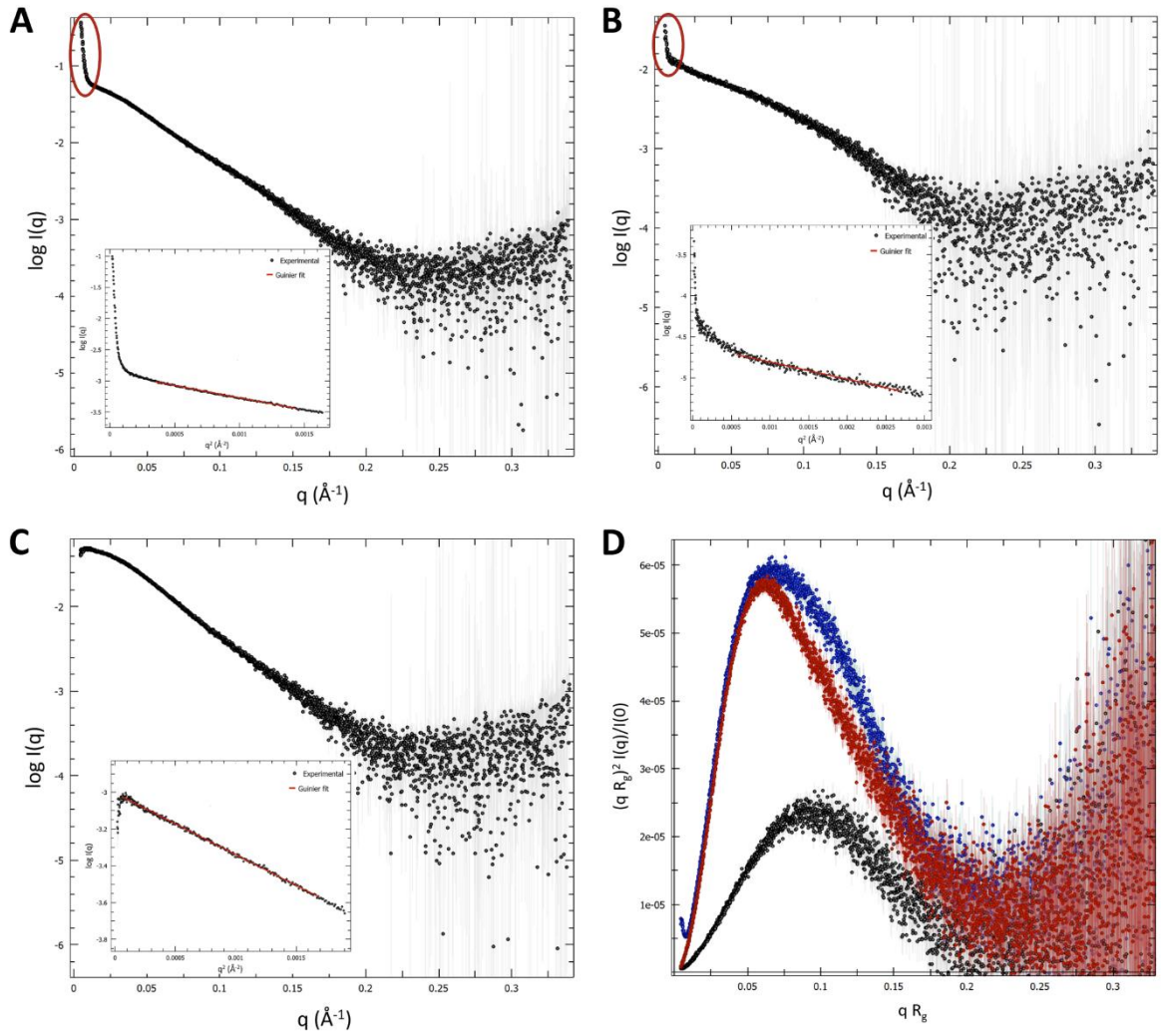


Figure 6.9: PB2-C–Rab11a complex is a well-folded protein in solution.

The SAXS signal with insert Guinier analysis of **(A)** PB2-C, **(B)** Rab11a, and **(C)** PB2-C–Rab11a complex. Red circles indicate a radiation induced aggregation of the sample. **(D)** Dimensionless Kratky plot of PB2-C (blue), Rab11a (black) and PB2-C–Rab11a complex (red) shows bell-shaped peaks, a characteristic of well-folded proteins. Data were analysed and plotted in PRIMUS (Konarev *et al.*, 2003).

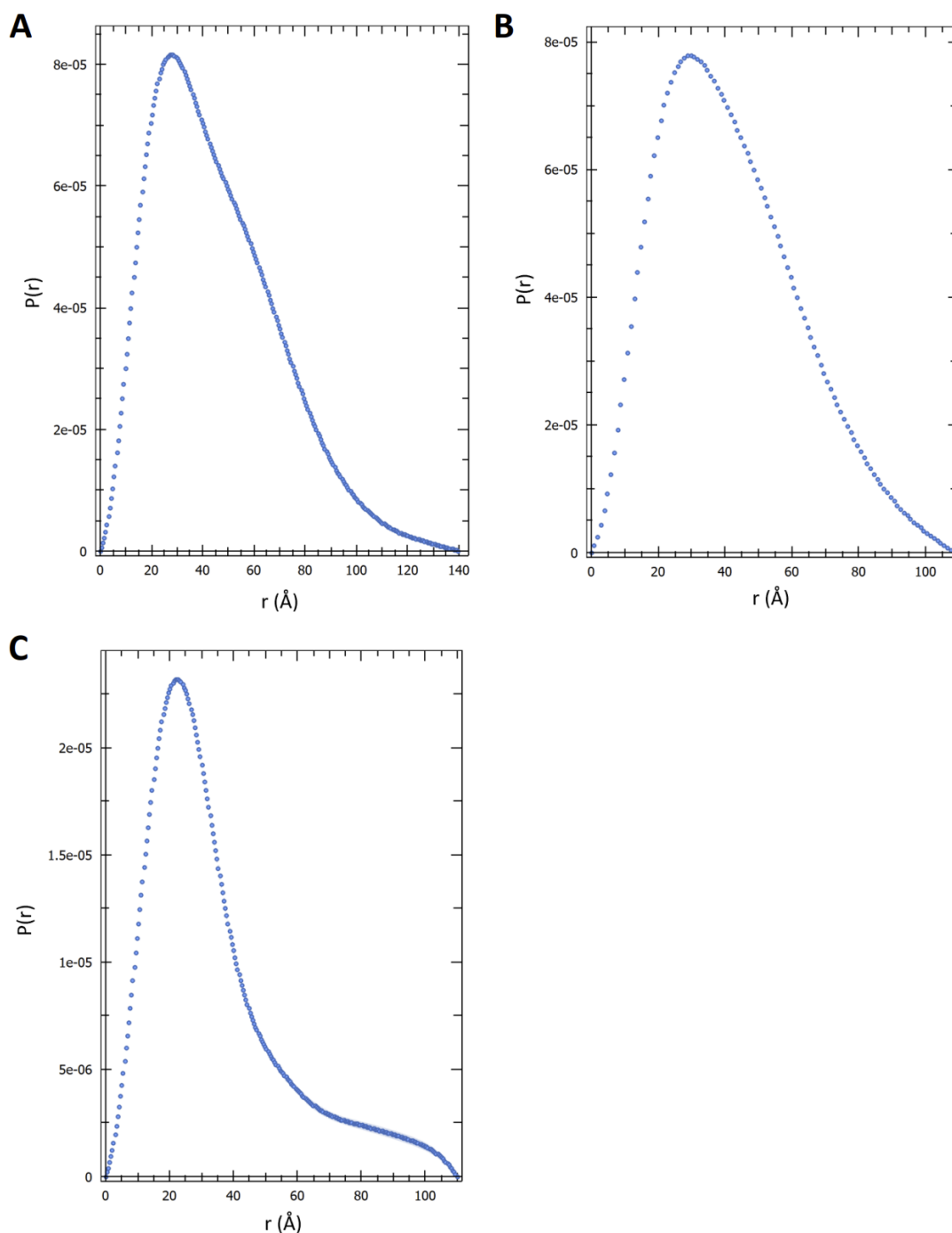


Figure 6.10: Pair-distribution ($P(r)$) plots confirm PB2-C, Rab11a, and PB2-C-Rab11a complex are globular proteins.

The $P(r)$ distribution of **(A)** PB2-C, and **(B)** PB2-C-Rab11a complex shows a slightly skewed $P(r)$ distribution with a well-defined $D_{\max}$, suggesting a slightly elongated globular protein. **(C)** The $P(r)$ distribution of Rab11a is rather extended with a $D_{\max}$ of 110, which does not reach zero smoothly.

Table 6.1: SAXS parameters for PB2-C, Rab11a, and PB2-C–Rab11a.

	R_g^a (Å)	$I(0)^b$	$q_{\min}R_g^c$	$q_{\max}R_g^c$	$D_{\max}^d$ (Å)
PB2-C	34.33	0.056	0.65	1.30	140
Rab11a	24.99	0.01	0.60	1.30	110
Complex	31.67	0.049	0.26	1.30	110

^aRadius-of-gyration (R_g) estimated from Guinier analysis.

^bIntensity at zero scattering angle extrapolated from the linear fit to the low q region.

^cThe upper and the lower limit of the Guinier region where $q_{\min}$ and $q_{\max}$ refer to the lowest and highest resolutions collected in the experiment, respectively.

^dMaximum dimension of particle (d-max) estimated from pair distance distribution ($P(r)$) function.

Table 6.2: Predicted molecular weights of PB2-C, Rab11a, and PB2-C–Rab11a.

Molecular weights were derived from five independent methods (Hajizadeh *et al.*, 2018), as implemented in PRIMUS (Konarev *et al.*, 2003). *Qp*, molecular weight from porod invariant; *MoW*, molecular weight from data on a relative scale; *Vc*, molecular weight from volume of correlation; *Size and shape*, molecular weight by comparison to known structures; *Bayesian Inference*, molecular weight calculated using Bayesian inference with the molecular weight calculations from the Porod volume, volume of correlation, and comparison to known structures methods as the evidence.

	Qp (kDa)	MoW (kDa)	Vc (kDa)	Size and shape (kDa)	Bayesian inference (kDa)	Expected (kDa)
PB2-C	65.64	69.95	59.31	71.70	65.45	59.2
Rab11a	24.38	30.33	25.03	25.84	26.23	19.4
Complex	65.66	69.05	59.67	65.00	65.45	78.6

6.3 Discussion

In this study, the structural basis of the IAV RdRP–Rab11a interaction was investigated. Being a ~270 kDa protein complex, RdRP–Rab11a is suited for single particle cryo-EM analysis. Whilst the structure determination of the complex of IAV RdRP bound to Rab11a has proven intractable for the time being, I present data showing a novel conformation of an IBV RdRP dimer which is remarkably similar to that previously described ICV RdRP of the encapsidase structure in the RdRP-ANP32A dimer (Carrique *et al.*, 2020). Specifically, the position of the PB2⁶²⁷ domain is almost identical while the positions of the PB2^{cap} and PB2^{mid-link} domains are different. However, these differences could be due to the intrinsic flexibility of these two domains, which has been observed previously in the ICV RdRP in the encapsidase conformation in which approximately 52% of particles lacked density for these domains (Carrique *et al.*, 2020). As Rab11a was not resolved in this complex, a further attempt was made to prepare more samples for cryo-EM. Unfortunately, in the course of this study, I was unable to reproduce the experiment. Namely, all samples suffered from severe aggregation during the sample preparation resulting in poor sample quality which was not suitable for cryo-EM sample collection. Moreover, it turned out that the only successful cryo-EM data collection, which results are presented in this thesis, in fact represents the IBV RdRP not the IAV RdRP. This was evident from modelling the core of the RdRP structure by fitting both the NT60 and the IBV RdRP into the local filter map. It is, therefore, not surprising that Rab11a was not resolved, and that I was never able to reproduce the experiment. Whether cryo-EM grids were mixed up during the data collection or a mistake occurred earlier during the sample preparation is impossible to determine; however, this unfortunate event nevertheless opens an interesting avenue for

future investigation where further optimisations will be required. These include protein crosslinking (Drulyte *et al.*, 2018) and using RdRPs from different IAV subtypes.

In this chapter it was also demonstrated that the PB2-C protein associates with Rab11a under SEC conditions. This is demonstrated by SEC experiments alone as well as by the SEC-SAXS analysis. Since the SAXS analysis is difficult for intrinsically disordered proteins (Kikhney and Svergun, 2015), I used Rab11CA mutant which lacks its C-terminal disordered region consisting of 43 hypervariable residues (Jagoe *et al.*, 2006; Shiba *et al.*, 2006) (for simplicity referred to as Rab11a in this chapter). To determine the point of PB2-C saturation, SAXS data of PB2-C–Rab11a complex were collected across a range of molar ratios. While there was a shift in the elution peak of PB2-C with either 0.5- or 1-fold molar excess of Rab11a, the PB2-C–Rab11a peak no longer shifted its position when either 1.5- or 2-fold molar excess of Rab11a was added to PB2-C protein. These data demonstrate that at above 1-fold molar excess of Rab11a the PB2-C protein is saturated, with the PB2-C–Rab11a complex peak having Rab11a at 100% occupancy. These results warrant further SEC-SAXS analyses as using the scattering peak of the PB2-C–Rab11a complex at a 100% occupancy therefore indeed represents the complex and not a partially occupied assembly. Moreover, these data also suggest that there is a concentration dependence in elution position. To support these data, I compared the symmetry of SEC-SAXS elution profiles. Unlike the PB2-C protein on its own, the SEC-SAXS elution profiles of PB2-C–Rab11a peaks were asymmetric. This behaviour is characteristic of a protein undergoing a concentration dependent dissociation (Keown *et al.*, 2016). As the protein complex dilutes, an excess unbound Rab11a is retarded in the column resulting in a trailing edge of the peak.

Next, I determined model-free parameters from SAXS of PB2-C, Rab11a, and PB2-C–Rab11a complex when in an equimolar ratio. The scattering profile of PB2-C and Rab11a showed an upper curvature at low q values, which is a consequence of radiation-induced aggregates in solution. An alternative explanation could be that while there is no radiation damage, the upper curvature at low q values present in the scattering profile of PB2-C and Rab11a is a result of a non-flat buffer profile present in these two samples, which may affect the analysis. The appropriate approach to address these two possibilities would be to repeat the SEC-SAXS experiment making sure that the column is properly equilibrated in the SEC running buffer. If radiation damage is indeed present, using a free radical absorber, such as DTT, in the solvent could lessen the effects of the radiation damage (Kuwamoto *et al.*, 2004). Additionally, as radiation damage is dose-dependent, collecting several exposures of PB2-C and Rab11a proteins would allow to compare their scattering profiles and select for the least radiation damage affected profile (Brosey and Tainer, 2019; Jacques and Trewhella, 2010). In contrast to PB2-C and Rab11a proteins on their own, the scattering profile of the PB2-C–Rab11a complex showed a downturn at low q values. This is typically caused by electrostatic interactions (Jacques and Trewhella, 2010), which have been shown to have an important role in protein binding (Hu *et al.*, 2000).

Further analysis of the SAXS curves showed that PB2-C, Rab11a, and PB2-C–Rab11a complex are globular proteins in solution, as evident from their q -range over which the Guinier approximation is valid ($q_{\max}R_g$), which is 1.3 as is the case for globular proteins (Putnam *et al.*, 2007). Furthermore, the Kratky plots and the $P(r)$ function indicate that all proteins are well-folded as evident from their bell-shaped peaks (Putnam *et al.*, 2007; Tuukkanen and Svergun, 2014). However, it is important to note that the $P(r)$ function of

Rab11a protein does not reach zero smoothly and shows an extended tail. This could be a consequence of a non-flat buffer profile of this sample that has most likely affected SAXS data analysis. Interestingly, $D_{\max}$ of PB2-C–Rab11a complex is 110 Å while $D_{\max}$ of PB2-C alone is 140 Å. As $D_{\max}$ represents the maximum distance present in the scattering particle (Putnam *et al.*, 2007), these data support previous observations of this study that the PB2-C–Rab11a complex adopts a compact folded structure in solution. Finally, based on the calculated molecular weight estimations, PB2-C and Rab11a proteins seem to form a heterodimer in solution. In the course of this study, we were unable to determine the binding site of Rab11a to the PB2-C by *ab initio* and rigid body modelling of the PB2-C–Rab11a complex due to too low-resolution information. A future study should, therefore, aim to address this question by using X-ray crystallography. Alternatively, I have tested single-chain antibodies available in our laboratory, known as nanobodies, which bind to PB2-C and do not disturb the PB2-C–Rab11a interaction. Cloning these nanobodies into a megabody backbone (Uchański *et al.*, 2021) could enable overcoming the major obstacle that limit the resolution of single-particle cryo-EM reconstruction, i.e., particle size, and allow one to determine the binding site of Rab11a to the PB2-C by cryo-EM.

In summary, while this study was unsuccessful in solving the structure of the IAV RdRP–Rab11a complex and revealing how Rab11a binds to PB2-C, it represents an active area of investigation and could be explored further using RdRPs from different IAV subtypes. It remains to be seen if Rab11a binds to a previously observed conformation of PB2 seen in the structures of the influenza virus RdRP, or whether PB2 takes up a new conformation necessary for binding to Rab11a. The results of this study also demonstrate that PB2-C and Rab11a proteins form a compact heterodimer in solution. These results further

demonstrate that the interaction between PB2-C and Rab11a is direct, which is in agreement with results from the GST pull-down assays in previous chapters.

7 Establishing a workflow to study vRNP trafficking in the context of live infected cells

7.1 Introduction

Since the time of Galileo, imaging has been the eye of science. In recent years, the imaging field in the life science has seen an explosion of electron microscope technologies, which can deliver high-resolution 3D information about cells and tissues in relation to their surrounding ultrastructural environment at nanometre resolution (Carzaniga *et al.*, 2014). To distinguish features of interest from the resin, it is fundamental to generate a contrast within the specimen (Carzaniga *et al.*, 2014). This can be indeed achieved by embedding samples in vitreous ice and imaging them using cryo-electron tomography, but electron microscopy (EM) methods nonetheless suffer from limited penetration depth. Transmission electron microscopy, for instance, can penetrate to $<1\ \mu\text{m}$ while some mammalian cells can grow beyond $3\ \mu\text{m}$ (Carzaniga *et al.*, 2014; Kounatidis *et al.*, 2020). Sample sectioning can be employed to overcome this limitation and to control sample thickness; however, this either involves chemical treatment or complex cryogenic workflows (Kounatidis *et al.*, 2020; Rigort and Plitzko, 2015). Cryo-soft X-ray tomography (cryo-SXT) addresses this need for imaging cellular ultrastructure in thicker vitrified specimens, such as whole cells, having sufficient energy that can achieve penetration depth in the order of $10\ \mu\text{m}$ (Carzaniga *et al.*, 2014; Harkiolaki *et al.*, 2018; Kounatidis *et al.*, 2020). The ultrastructural information of biological samples can become particularly valuable when distribution of specific molecules is included (Harkiolaki *et al.*, 2018; Kounatidis *et al.*, 2020). This can be achieved by imaging the same sample in fluorescence and then

correlating the ultrastructural information with a fluorescent signal. Correlating different imaging modalities, such as EM and fluorescence microscopy (FM), known as correlative light-electron microscopy (CLEM), has been used extensively to date, allowing one to find areas of interest by using fluorescent proteins as markers within biological samples (Chang *et al.*, 2014; Kounatidis *et al.*, 2020; Paez-Segala *et al.*, 2015; Schwartz *et al.*, 2007). The advent of FM has been a major step forward in the study of living cells (Kherlopian *et al.*, 2008). Its correlation to other higher resolution imaging modalities can be done as imaging at either cryogenic or room temperature (Kounatidis *et al.*, 2020). When imaging in cryo-FM stage, however, photobleaching is slower and emission spectra is sharper, resulting in a better signal to noise ratio (Kounatidis *et al.*, 2020; Schwartz *et al.*, 2007). Unfortunately, most of cryo-fluorescent imaging is done using air objectives with a limited optical resolution of ~400 nm, which severely limits its usefulness in the correlative approach (Kaufmann *et al.*, 2014; Kounatidis *et al.*, 2020). Moreover, given the limit imposed by the lack of cryo-immersion objectives, it also remains technically challenging to prevent ice crystals formation and sample damage while imaging at cryogenic temperatures (Kounatidis *et al.*, 2020). Therefore, implementing high resolution FM at cryogenic temperatures remains a major challenge in correlative imaging.

Fortuitously, cryo-3D-structured illumination microscopy (cryo-3D-SIM), a super resolution FM method for cryogenically preserved biological samples, can overcome this challenge. This imaging modality is particularly well suited for biological imaging as it uses light-dose exposure to laser light, it has an excellent contrast, and a large field of view (Abrahamsson *et al.*, 2017; Gustafsson, 2005; Gustafsson *et al.*, 2008). Furthermore, it enables 3D imaging of biological samples at resolutions beyond those achievable by confocal imaging, making

it an excellent complement to either cryo-SXT or cryo-EM (Phillips *et al.*, 2020). Moreover, cryo-3D-SIM can achieve penetration depth of volumes over 10 μm in thickness, which is very similar to the penetration depth of cryo-SXT, and can detect fluorescent molecules at resolution of up to 200 nm (Kounatidis *et al.*, 2020). Thus, cryo-3D-SIM can provide close correlation to the ultra-structure imaged by cryo-SXT microscope.

The aim of this study is to establish a workflow to study vRNP trafficking in the context of live infected cells using a novel correlative imaging scheme for biological samples at cryogenic temperatures available at the Diamond Light Source beamline B24. This new correlative imaging scheme uses a combination of a purpose-built cryo-3D-SIM microscope (Demmerle *et al.*, 2017; Gustafsson *et al.*, 2008) and a synchrotron full field transmission X-ray microscope (TXM) capable of tomographic imaging (Harkiolaki *et al.*, 2018). The schematic of the proposed workflow is shown in **Figure 7.1**.

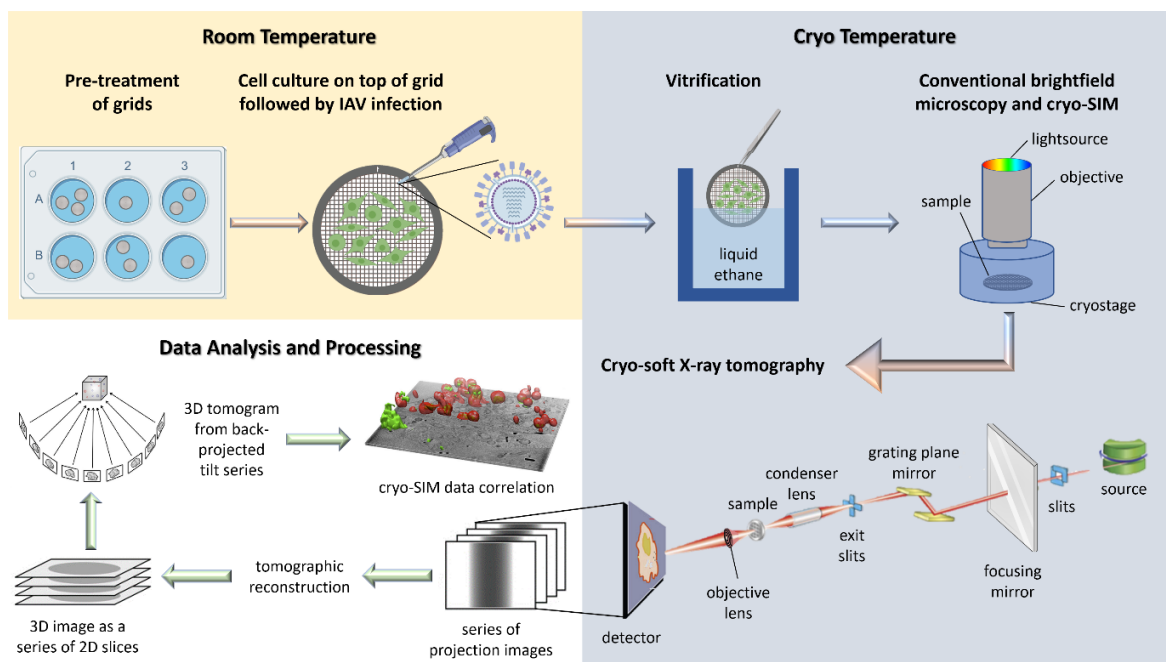


Figure 7.1: Proposed workflow for the full field transmission X-ray microscope (TXM).

Step by step workflow at the Diamond Light Source beamline B24. Cells are grown on top of the transmission electron microscopy (TEM) grid which has been soaked in serum beforehand. Once grown to a desired confluency, cells are infected with a recombinant influenza A/WSN/1933 (H1N1) PA-GFP virus or mock infected. Fiducial markers are added onto the TEM grid, and any excess media is removed by blotting. Grids are then vitrified in liquid nitrogen-cooled liquid ethane. Once frozen, grids are checked using cryo-light microscopy to examine cell morphology and to confirm absence of non-vitreous contaminants. Using cryo-3D-SIM, potential areas of interest are selected, and their fluorescent data is collected in 3D. After fluorescent imaging, the sample is transferred into TXM holder. Once in the TXM holder, grids are examined and mapped with visible light to produce a mosaic. Mosaic is then aligned and areas of interest from SIM data collection are identified to allow soft X-ray data collection in the same region. Initially, the sample is evaluated for susceptibility to heat damage (heating due to X-ray absorption) and its uniformity of vitrification using low-dose X-rays, and 2D mosaic images are collected in the areas of interest covering a single grid square. Next, 3D X-ray data are collected as tilt series by exposing the chosen TEM grid square to high-dose X-rays. Finally, data are relayed in real-time to an automated pipeline by using IMOD Batchruntomomo (Mastronarde and Held,

2017), and, provided the sample has good contrast and fiducials are present, the data are reconstructed to tomograms by IMOD's back-projection. Finally, the tomogram can be used to map in 3D SIM fluorescent data. Parts of the figure were made using BioRender. Cryo-SIM fluorescent correlated data was taken from Kounatidis *et al.* (2020).

7.2 Results

7.2.1 Recombinant influenza A/WSN/33(H1N1) PA-GFP virus is viable and expresses PA-GFP in infected cells

To study the interaction between IAV vRNP and cellular GTPase Rab11a during a productive infection, I generated a recombinant influenza A/WSN/1933 (H1N1) virus with a full length green fluorescent protein GFP (26.9 kDa) fused to the C-terminal region of the viral PA protein, which has been described previously (Lakdawala *et al.*, 2014). For simplicity, I will refer to the influenza A/WSN/1933 (H1N1) PA-GFP virus as WSN PA-GFP.

Human embryonic kidney (HEK) 293T cells were co-transfected with eight plasmids containing the IAV segment genomic sequences. Co-transfection with all plasmids except the plasmid expressing PA was used as a negative control. First, the viability of the reporter WSN PA-GFP virus was analysed by observing cytopathic effects in HEK 293T. As expected, 48 hours post transfection (hpt), virus-induced cytopathic effects were observed in cells co-transfected with eight plasmids containing genes encoding for viral proteins of either WSN PA-GFP or wild type WSN virus, which was generated as a positive control. In contrast, no cytopathic effects were observed in cells co-transfected with seven plasmids. Interestingly, stronger virus induced cytopathic effect was observed in the cells co-transfected with eight plasmids containing genes encoding for viral proteins of wild type WSN virus compared to the WSN PA-GFP virus. These observations confirmed the viability of the WSN PA-GFP reporter virus.

Having established that the WSN PA-GFP reporter virus was viable, I then evaluated the titre of virus released from co-transfected HEK 293T cells. Viruses were harvested at 24 and

72 hpt and the virus stocks were titrated on Madin-Darby Canine Kidney (MDCK) epithelial cells. At 24 hours post infection (hpi) the cell supernatant contained 130 plaque forming units (PFU) of infectious WSN virus per millilitre of the medium from transfected cells (PFU ml⁻¹) while no plaques were formed by WSN PA-GFP virus (**Figure 7.2A**). However, at 72 hpi, the virus titres for WSN and WSN PA-GFP viruses were 4.95·10⁶ PFU ml⁻¹ and 1.55·10⁵ PFU ml⁻¹, respectively (**Figure 7.2A**). These data show that at 72 hpi the WSN PA-GFP virus produced titres approximately 1 log unit lower than wild type WSN virus. Taken together, these results show that a co-transfection of the eight expression plasmids allows the recovery of the fluorescently tagged IAV.

To determine whether WSN PA-GFP expresses the fluorescent reporter gene in infected cells, I directly visualised fixed MDCK cells at 24 hpi using confocal microscopy. This type of visualisation allows a rapid identification of GFP-labelled virus particles as well as determination of infection time points at which virus can be detected in the cytoplasm after its successful replication in the cell nucleus. First, cells seeded on glass coverslips were infected with WSN PA-GFP at multiplicity of infection (MOI) of 0.1. At 24 hpi cells were fixed and stained for the viral nucleoprotein (NP), a viral protein critical for replication, and with blue fluorescent DAPI dye, specific for DNA, to visualize cell nuclei (**Figure 7.2B**). Confocal microscopy revealed a predominantly nuclear localisation of the GFP fluorescent signal at 24 hpi, as expected due to the nuclear replication of the IAV genome (**Figure 7.2B, PA-GFP**). However, both the NP immunofluorescent signal and the GFP fluorescent signal were also detected in the cytoplasm (**Figure 7.2B, Merge + DAPI**), which is in agreement with vRNPs undergoing nucleocytoplasmic export at late stages of the viral cycle (Eisfeld *et al.*, 2015). Unexpectedly, the PA-GFP expression was not detected in all infected cells

expressing the NP (**Figure 7.2C**). These data show that the supernatant of co-transfected cells contains a heterogeneous population of GFP-positive and GFP-negative viral particles. As most viral particles express the PA-GFP, this recombinant WSN PA-GFP virus was used in subsequent studies. Taken together, these results show that a recombinant virus expressing PA-GFP was successfully generated. However, as not all infected cells express the PA-GFP, the recombinant virus is likely to be unstable.

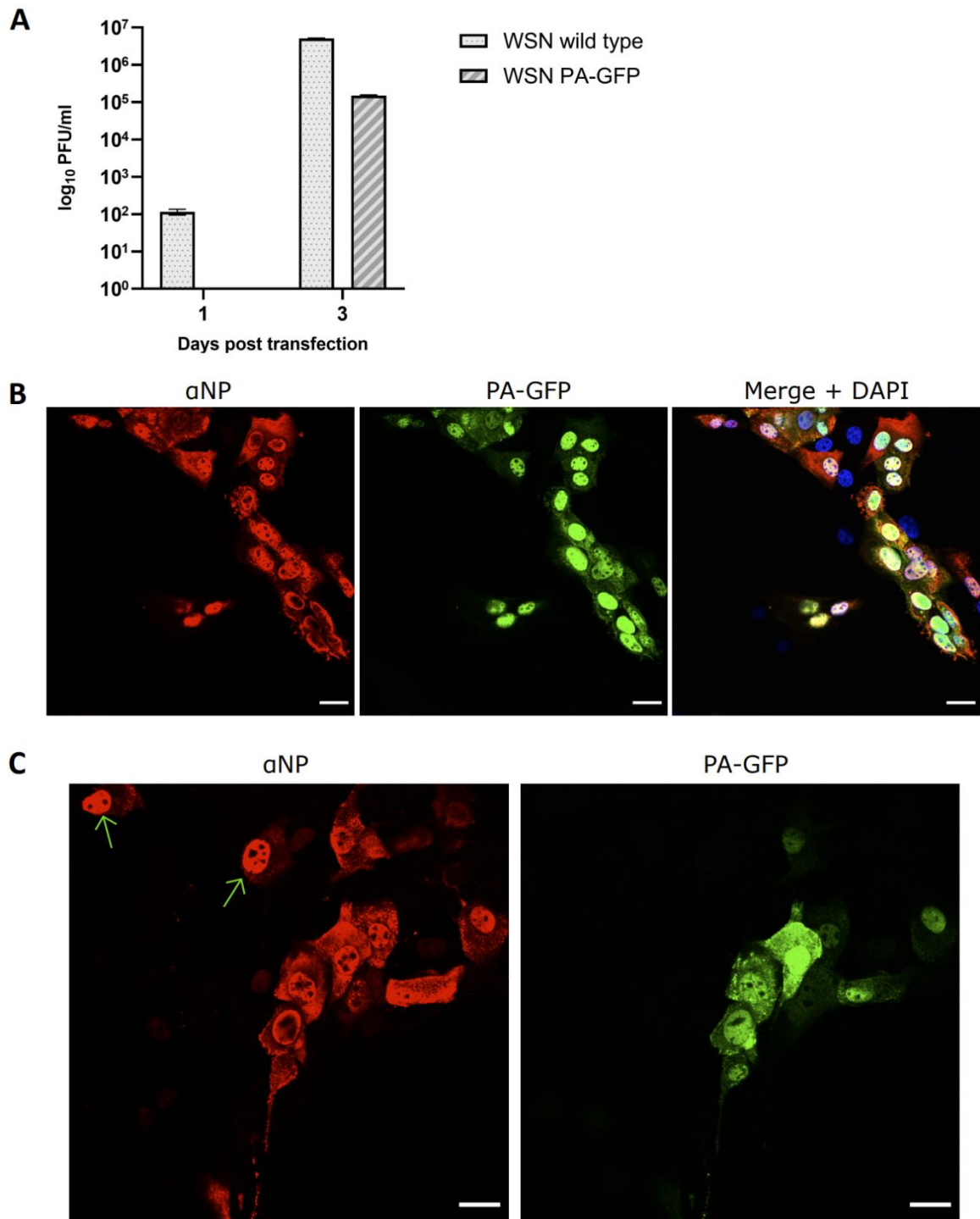


Figure 7.2: Recombinant WSN PA-GFP virus expresses PA-GFP in most of the infected cells.

(A) Titres of recombinant influenza A/WSN/1933 (H1N1) and influenza A/WSN/1933 (H1N1) PA-GFP viruses. MDCK cells in 6-well plates (approximately 10⁶ cells per well) were

infected with 100 μ l of serial ten-fold dilutions of each virus for 1 h at room temperature. After infection, the cells were overlaid with 2 ml of a 1:1 mixture of 2%(w/v) agarose in PBS and MEM, 0.5%(v/v) FCS. The cells were incubated for 3 days at 37°C and plaques were counted. Data are shown as mean $\pm$ SD (n = 2 independent experiments/group). **(B)** MDCK cells seeded on coverslips were infected with WSN PA-GFP virus at an MOI of 0.1, and fixed at 24 hpi. The WSN PA-GFP fluorescent signal is shown in green, NP gene is visualized in red (Cy3) and nuclei in blue (DAPI). Pictures were acquired on Olympus FluoView FV1000 using a 60X magnification objective lens. **(C)** As in **B** but using a 100X magnification objective lens. Infected cells expressing NP but lacking detectable PA-GFP expression are indicated with green arrows. Scale bar for **B** and **C** = 20 μ m.

7.2.2 A549 cell line is not suitable for cryo-soft X-ray tomography

The adenocarcinoma human alveolar basal epithelial cell line (A549) has been widely used for *in vitro* studies of IAV replication (Ujie *et al.*, 2019). In order to use A549 cells at the beamline, its suitability for cryo-SXT had to be determined. First, I assessed the ability to culture A549 cells on gold TEM grids. A recent report suggested that prior to cell culture, the surface of TEM grids must be treated by glow discharge followed by adherence-promoting agents such as poly-D-lysine to promote cell adherence (Harkiolaki *et al.*, 2018). To reduce the number of handling steps and consequently improve the quality of the grids, I tested whether soaking TEM grids in fetal calf serum (FCS) containing the adhesion-promoting proteins is sufficient to promote cell attachment. Initial in-line brightfield imaging showed that treating TEM grids with FCS before cell culturing is sufficient to promote cell attachment and to grow A549 cells to a desired confluency of ~40% (**Figure 7.3A**).

Given the fact that heat damage of biological samples remains a limiting factor in high resolution X-ray microscopy (Gianoncelli *et al.*, 2015), I next assessed the effect of soft X-ray irradiation on A549 cells. A raster scanning of a whole grid square containing a cryo-preserved A549 cell using low-dose X-rays showed a nucleus with a defined membrane as well as an intact cell membrane (**Figure 7.3B**). No significant lateral cellular shrinkage was identified, as often reported to be a consequence of X-ray exposure (Gianoncelli *et al.*, 2015). However, the 2D X-ray mosaic of the cell at the end of tilt series data collection, shows white dots in the cytoplasm, concentrating at the cell membrane, which represent pits and bulges, a commonly observed feature of increased X-ray dose (**Figure 7.3C**). This

observation revealed that A549 cells suffered from heat damage after being exposed to high-dose X-rays to collect tilt series.

A general goal for cryo-SXT is to grow cells on a TEM grid to a ~40% confluency due to the limitation of soft X-rays which can only effectively travel the width of a single cell without substantial dissipation (Carzaniga *et al.*, 2014; Harkiolaki *et al.*, 2018; Kounatidis *et al.*, 2020). However, since higher cell confluency generally increases the thickness of a monolayer culture, an area with multiple cells should be thicker and potentially less sensitive to heat damage. Therefore, to prevent substantial heat damage, I collected tilt series from an area on the grid with two cells next to each other (**Figure 7.4A**). Even though this area still suffered from heat damage, I could nevertheless process one dataset to obtain a reconstructed 3D volume of a mock infected A549 cell (**Figure 7.4B**). The feature characteristics of A549 cell were identified using previously published cells' TEM cytoplasmic ultrastructure as a reference (de Castro Martin *et al.*, 2017; Jiang *et al.*, 2010). The microtubule-organizing centre (MTOC) area was analysed where mitochondria surround the centrioles near the nucleus. Mitochondria of ~1 μm were characterised by cristae and their irregular and polymorphic shape (Jiang *et al.*, 2010). Unfortunately, I could not collect tilt series of WSN PA-GFP infected A549 cells due to their severe heat damage upon exposure to high-dose X-rays. This indicates that IAV infection-induced stress resulted in higher sensitivity of A549 cells to heat damage. Together, these results show that A549 cells, which are otherwise widely used for studying the IAV replication *in vitro*, are not an optimal choice for cryo-SXT experiment as they suffer from heat damage when exposed to an increased X-ray dose.

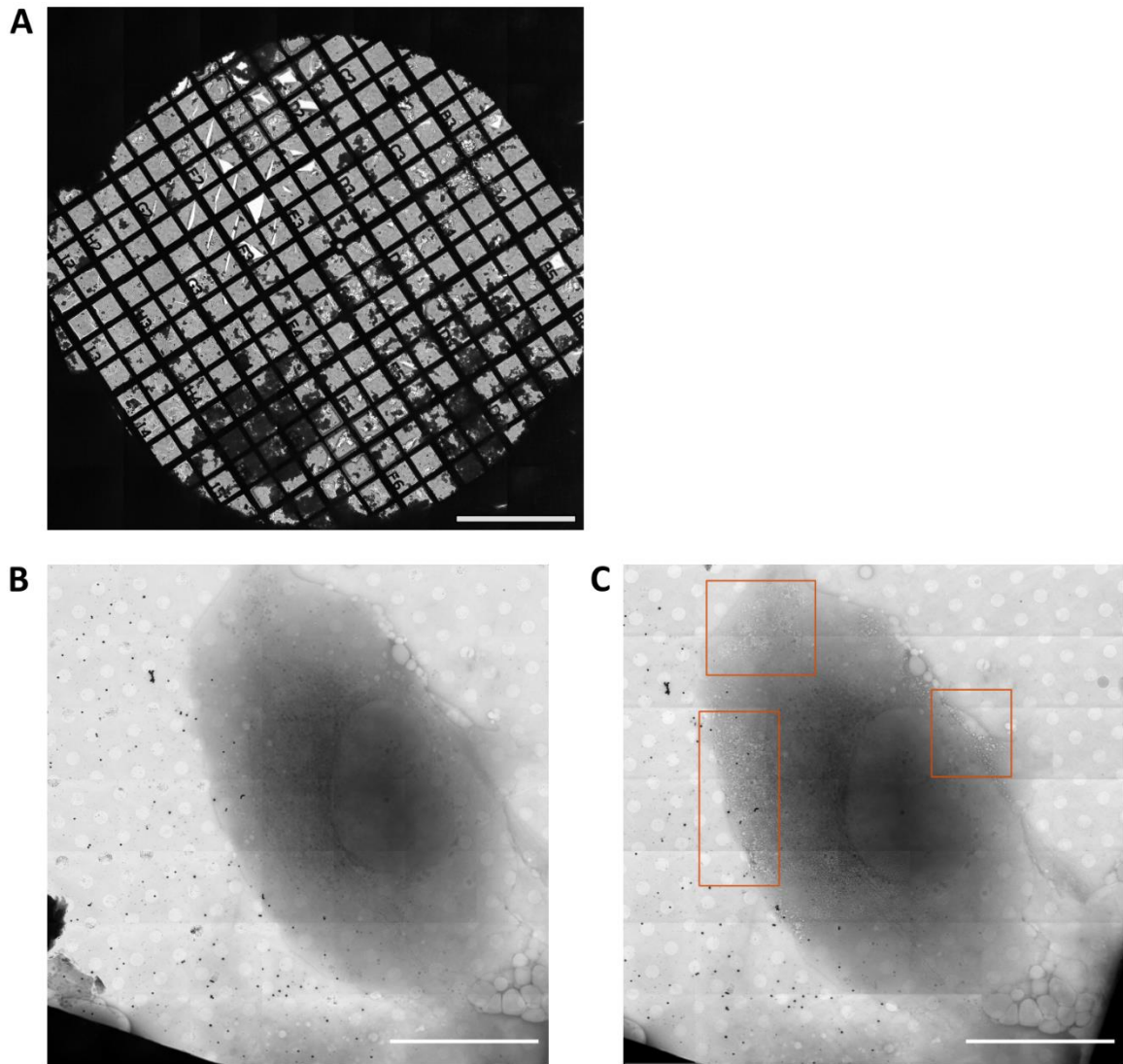


Figure 7.3: A549 cells are susceptible to heat damage caused by X-ray absorption.

(A) Brightfield overview of cryogenically preserved A549 cells on a TEM grid. A549 cells were grown on a pre-treated TEM grid to a confluency of ~40% at which point the grid was cryogenically preserved. A cryo-stage-equipped light microscope was used to examine the grid to confirm that the cells have retained their gross morphology, and that the grid does not have ice contamination or large breaks in the support film. Scale bar = 5 μm . **(B)** 2D X-ray mosaic image of an individual A549 cell of interest before its exposure to high-dose X-rays to collect tilt series. Whole grid squares were examined using low-dose X-ray exposures to collect mosaic images. **(C)** As in **B** but after exposing A549 cell to high-dose X-rays to collect tilt series. Red rectangles indicate areas with pits and bulges concentrating

at the cell membrane which are commonly observed upon an increased X-ray dose. Scale bar for **B** and **C** = 20 μm . Data were collected and processed at the Diamond Light Source beamline B24.

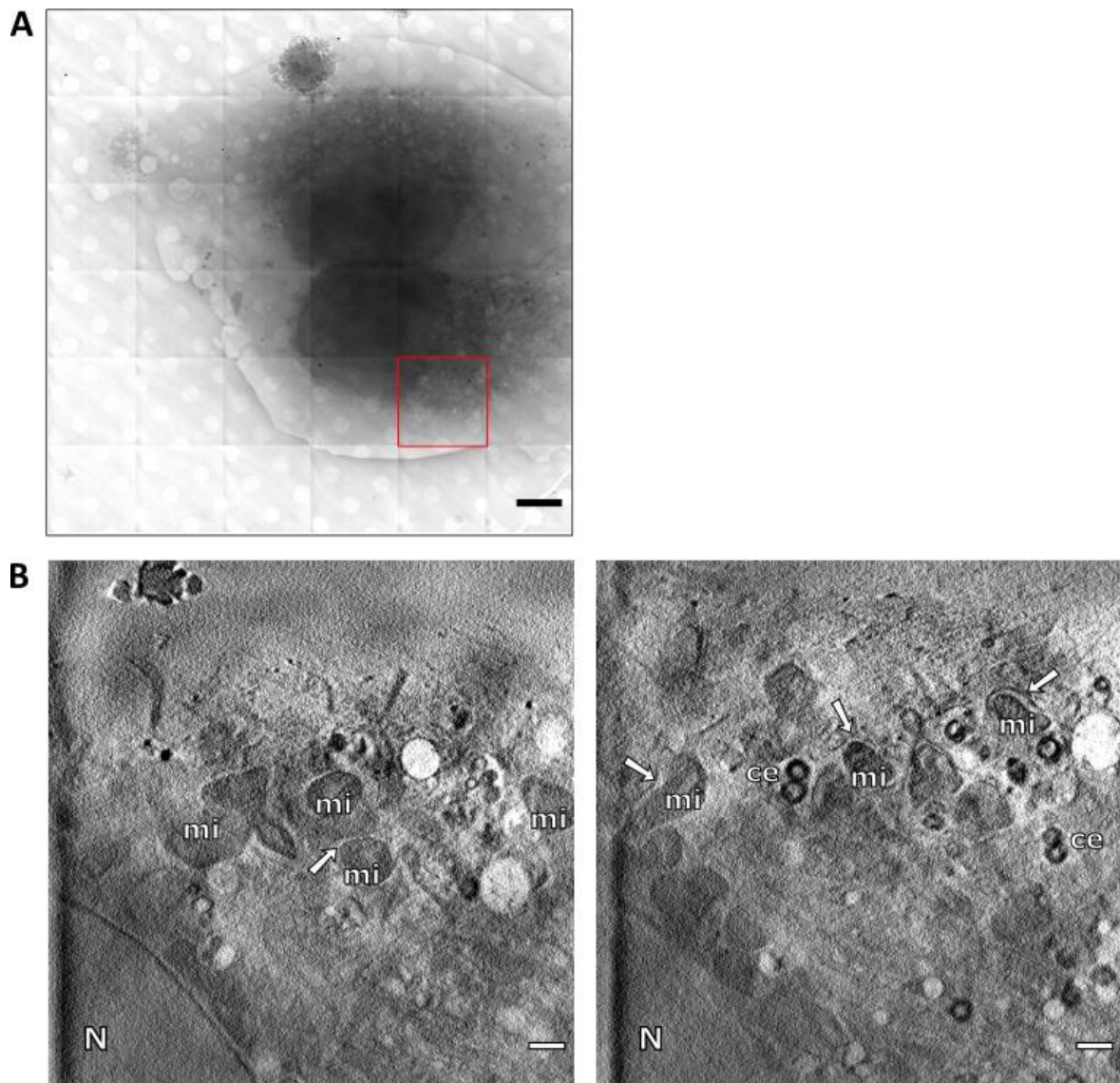


Figure 7.4: A549 cell line is susceptible to heat damage.

(A) 2D X-ray mosaic of a grid square showing two mock infected A549 cells. A549 cells were cultured on a TEM grid to a confluency of ~40% at which point they were cryogenically preserved. Whole grid square was examined using low-dose X-ray exposures to collect mosaic images. Red box indicates the field of view of camera; this area was exposed to high-dose X-rays to collect tilt series. Scale bar = 5 μm . **(B)** Two representative slices through a tomogram after reconstruction of tilt series collected from the area in the red box in **A**, showing the nucleus (N) and surrounding mitochondria (mi), centriole (ce), and endoplasmic reticulum (arrows) in cytoplasm. Scale bar = 1 μm . Data were collected and processed at the Diamond Light Source beamline B24 using the in-house pipeline that

employs Batchruntomo (Mastronarde and Held, 2017), which is a part of the IMOD package (Kremer *et al.*, 1996), via several scripts to allow parallel and near real-time processing. Specifically, both alignment and reconstruction were done in IMOD(4.9.10) (Kremer *et al.*, 1996).

7.2.3 U2OS cell line is susceptible to WSN PA-GFP infection and proves resistant to heat damage

Having shown that IAV infected A549 cells are too sensitive to heat damage, human bone osteosarcoma epithelial (U2OS) cell line was used to investigate whether similar effects can be observed during X-ray tilt series collection. Fortunately, U2OS cell line has been used at the beamline before and has proven to be resistant to heat damage (Kounatidis *et al.*, 2020). However, since this cell line is not commonly used to study influenza virus infection, it is important to monitor different time points post infection prior to the cryo-SXT experiment. To identify key time points corresponding to late stages of the IAV replication cycle in U2OS cells, I infected the cells with WSN virus at a high MOI of 5 or mock infected and at the indicated time point fixed for immunofluorescent staining. Staining of infected cells with an antibody targeting the viral NP protein showed that at 6 and 8 hpi signals of NP were mainly detected in the nuclei of infected cells; however, from 12 hpi NP was detected in most cells and was also recognised in the cytoplasm (**Figure 7.5**). This is in agreement with previous observations (Khaperskyy *et al.*, 2014). Together, these results show that U2OS cells are susceptible to WSN infection. Moreover, the results indicate that viral protein synthesis significantly increases between 6 and 8 hpi.

To analyse, whether the WSN PA-GFP virus can replicate and express PA-GFP in U2OS cells, I also infected the cells with WSN PA-GFP virus at an MOI of 5, and analysed the infection using confocal microscopy. Given that until 12 hpi the majority of NP signals were detected in the nuclei of cells infected with the wild type WSN, the U2OS cells infected with WSN PA-GFP virus were fixed only at 12 and 18 hpi, corresponding to late stages. Staining of infected cells with an antibody targeting the viral NP protein showed that at 12 and 18 hpi

signals of NP were detected in both the nuclei and the cytoplasm of infected cells (**Figure 7.6A, α NP**). In contrast, at 12 hpi signals of PA-GFP were only detected in the nuclei of infected cells while at 18 hpi PA-GFP was also recognised in the cytoplasm (**Figure 7.6A, PA-GFP**). This is in agreement with previous observations of this study that the recombinant WSN PA-GFP virus is a heterogeneous population of GFP-positive and GFP-negative viral particles. Moreover, these results indicate that GFP-positive virus has a slower replication cycle compared to the wild type virus. Importantly, while the fluorescent intensity of the GFP signal in the cell nuclei is comparable at 12 and 18 hpi, it is significantly higher than that of a mock infected sample which was used as a negative control (**Figure 7.6B**). Taken together, these data show that U2OS cell line can be used to study late stages of WSN PA-GFP virus life cycle; however, at least 18 h is needed to detect the PA-GFP signal in the cytoplasm.

Finally, to test whether U2OS cell line is resistant to heat damage upon IAV infection-induced stress, I infected cells grown on a TEM grid with WSN PA-GFP virus at a high MOI of 5 and at 18 hpi cryogenically preserved the grid. No lateral cellular shrinkage was identified after exposing the cells to high-dose X-rays to collect tilt series (**Figure 7.7A**). To analyse the morphology of the cells, I then aligned and reconstructed tilt series into 3D volume (**Figure 7.7B-C**). The morphology of WSN PA-GFP infected U2OS cells was determined by referring to a previously reported cytoplasmic ultrastructure of U2OS cell line obtained by TEM (Hu *et al.*, 2012) and EM (Kolb *et al.*, 2014) as a reference. The most specific characteristics of the cell morphology were mitochondria which size corresponded to the size reported previously (Hu *et al.*, 2012) (**Figure 7.7B**). Interestingly, a potential mitochondrial fragmentation was detected in one of the cells (**Figure 7.7C, arrow**).

However, the areas on the grid which fluorescent data were collected were damaged during the transfer between the microscopes. Therefore, I collected cryo-SXT data from a different area on the grid which lacks corresponding cryo-3D-SIM data. Therefore, the available data are insufficient to determine whether the observed mitochondrial fragmentation is a result of the IAV infection as there is no evidence that the cell was indeed infected.

Collectively, these data demonstrate that U2OS cells promote WSN PA-GFP virus replication. Furthermore, cryo-SXT results show that the cell line potentially remains resistant to heat damage after being infected with IAV.

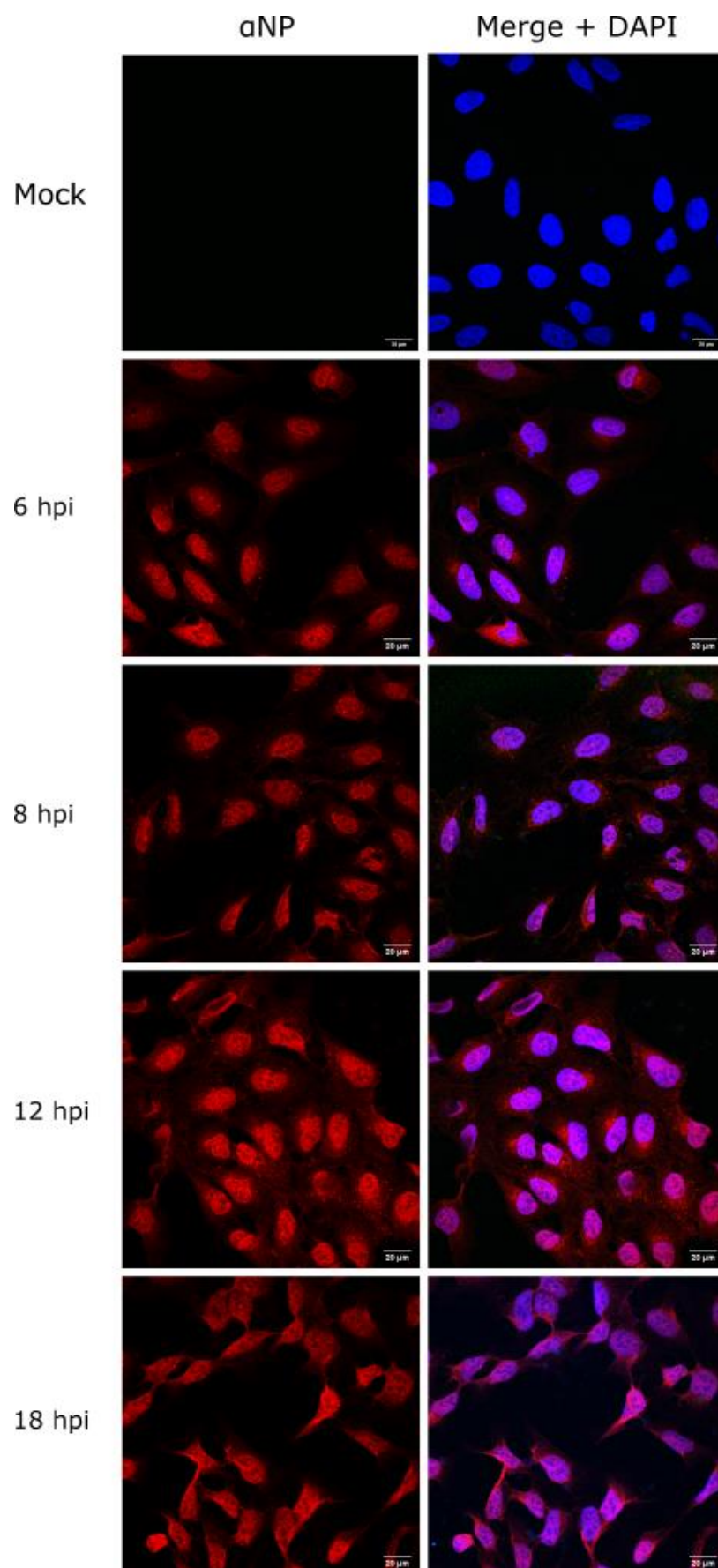


Figure 7.5: U2OS cell line is susceptible to WSN IAV infection.

U2OS cells seeded on coverslips were infected with WSN IAV at a high MOI of 5 or mock infected and subsequently fixed for immunofluorescent staining at either 6, 8, 12 or 18 hpi. Infected cells were stained with an antibody targeting the viral NP. NP protein was visualised with Cy3 (red) while the nuclei was visualised with DAPI (blue). Pictures were acquired on Olympus FluoView FV1000 using a 60X magnification objective lens. Scale bar = 20 μm .

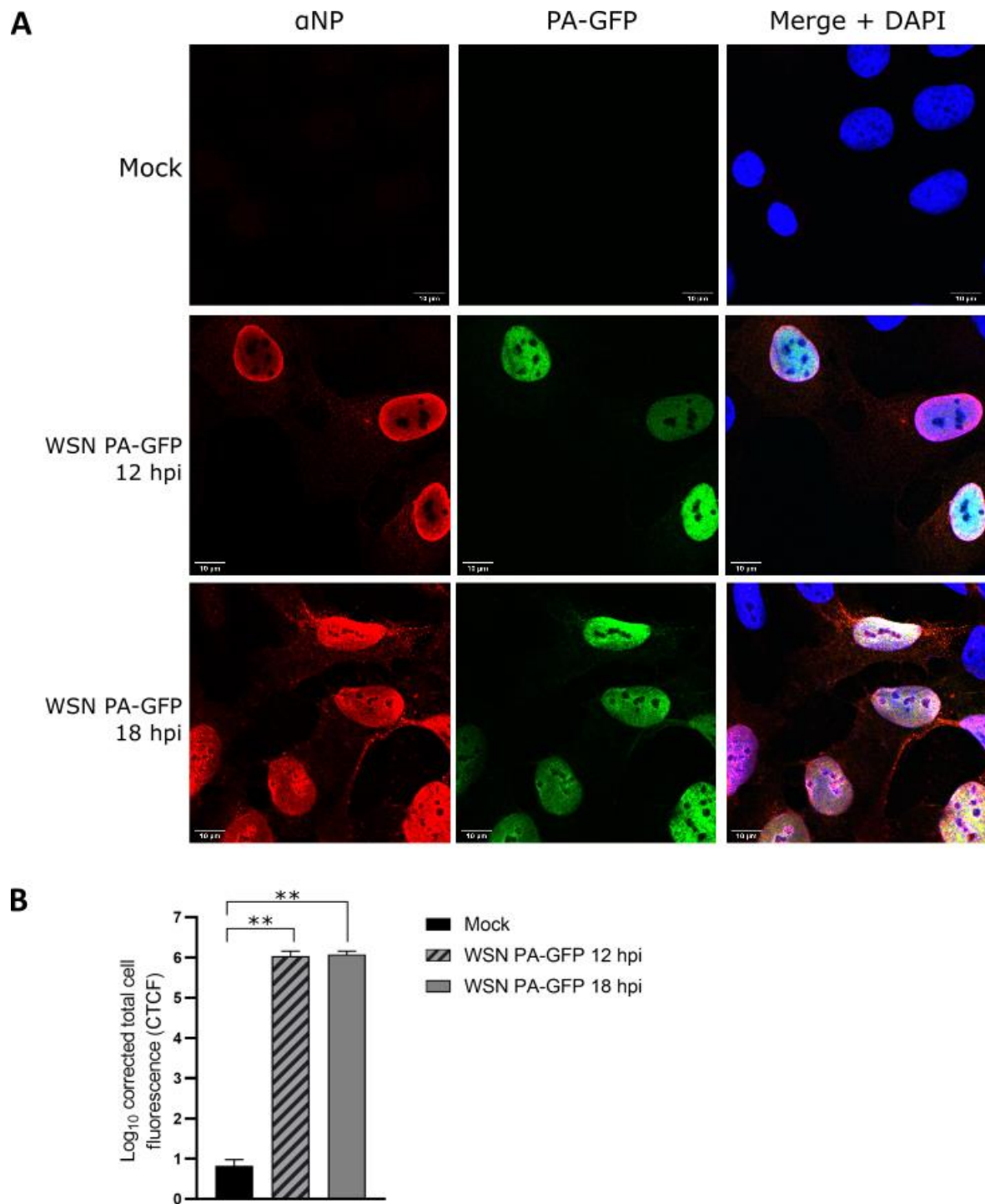


Figure 7.6: WSN PA-GFP virus replicates in U2OS cell line.

(A) U2OS cells seeded on coverslips were infected with WSN PA-GFP virus at a high MOI of 5 or mock infected and subsequently fixed for immunofluorescent staining at either 12 or 18 hpi. Infected cells were stained with an antibody targeting the viral NP. The PA-GFP fluorescent signal is shown in green, NP protein was visualised with Cy3 (red), and the

nuclei was visualised with DAPI (blue). Pictures were acquired on Olympus FluoView FV1000 using a 60X magnification objective lens. Scale bar = 10 μ m. **(B)** Images in panel A were analysed in ImageJ to determine corrected total cell fluorescence (CTCF) [Integrated density – (area of selected cell $\cdot$ mean fluorescence of background)] at different time points. Data are shown as mean $\pm$ SD (n = 3 independent experiments/group); ns $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ (one-way ANOVA test). $P \leq 0.05$ is considered significant to reject the null hypothesis.

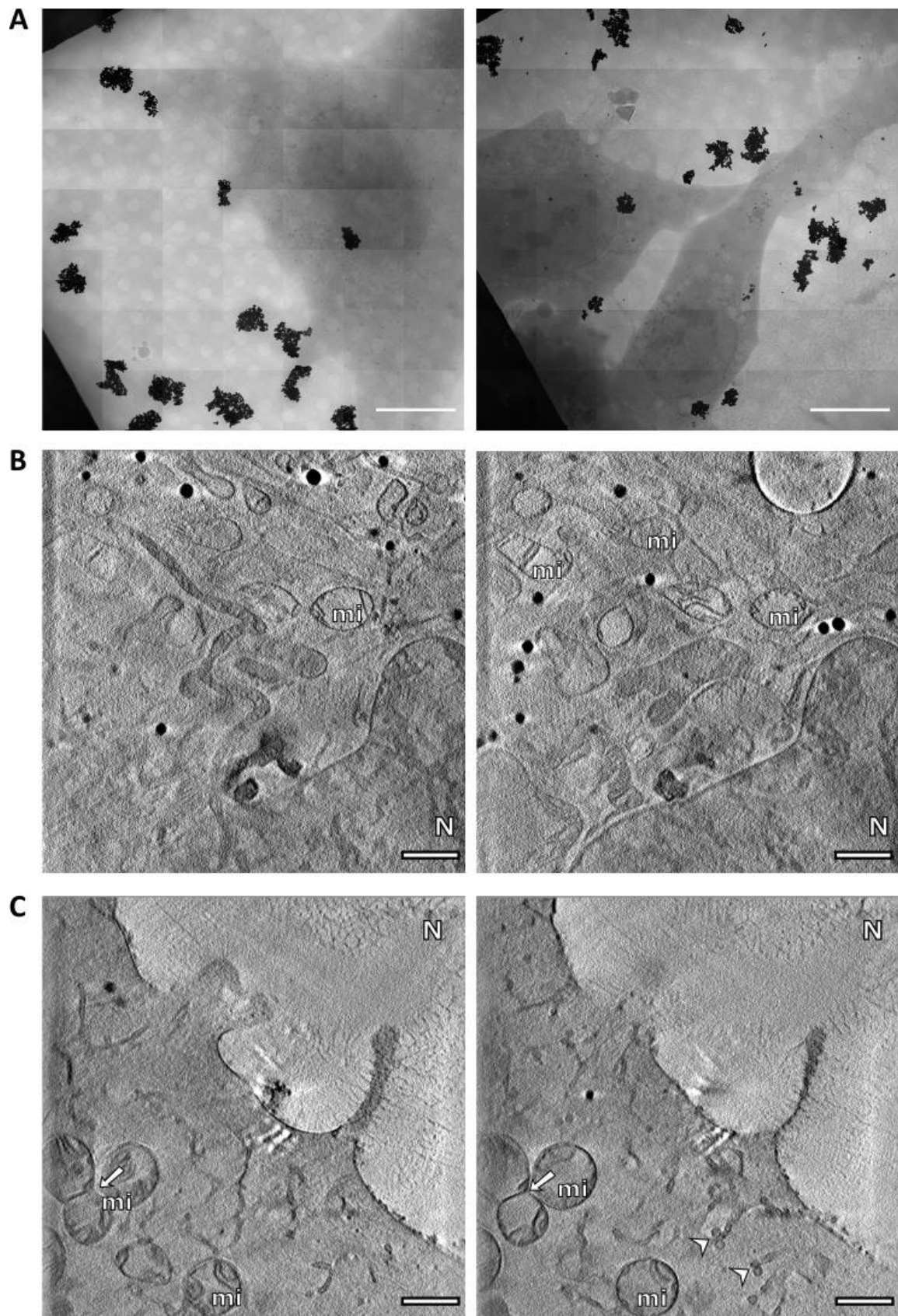


Figure 7.7: U2OS cells infected with WSN PA-GFP are resistant to heat damage.

(A) 2D X-ray mosaics of two different grid squares from the same TEM grid. U2OS cells were cultured on a TEM grid to a confluency of ~60% at which point they were infected with WSN PA-GFP virus at a high MOI of 5 and at 18 hpi cryogenically preserved. Whole grid squares were examined using low-dose X-ray exposures to collect mosaic images. Scale bar = 20 μm . **(B)** Two representative slices through a tomogram after reconstruction of tilt series collected from the cell presented in the left 2D X-ray mosaic in panel **A**, showing the nucleus (N) and surrounding mitochondria (mi) in cytoplasm. **(C)** Two representative slices through a tomogram after reconstruction of tilt series collected from the cell presented in the right 2D X-ray mosaic in panel **A**, showing a mitochondrial event (arrows) and small vesicles (arrowheads). Scale bar for **B** and **C** = 2 μm . Data were collected and processed at the Diamond Light Source beamline B24 using the in-house pipeline that employs Batchruntomo (Mastronarde and Held, 2017), which is a part of the IMOD package (Kremer *et al.*, 1996), via several scripts to allow parallel and near real-time processing. Specifically, both alignment and reconstruction were done in IMOD(4.9.10) (Kremer *et al.*, 1996).

7.2.4 Monoclonal U2OS stable cell line expressing mRuby-Rab11CA proves to be resistant to WSN PA-GFP infection and is morphologically damaged

Cryo-3D-SIM enables one to map in 3D the intracellular location of fluorophores with the ability to excite four different fluorophores (405 nm, 488 nm, 561 nm, and 647 nm for blue, green, red, and far-red fluorophore, respectively). To address the question, whether IAV vRNPs colocalise with Rab11a in the cytoplasm of infected cells, I aimed to generate a stable U2OS cell line expressing fluorescently labelled Rab11a. Since the recombinant WSN virus expresses GFP, and DAPI dye emits in the blue range, I cloned Rab11CA gene in a lentiviral transfer plasmid as a fusion protein with mRuby red fluorescent protein at its N-terminus. A polyclonal U2OS cell line stably expressing exogenous mRuby-Rab11CA gene was then generated using a lentiviral system as described previously (Elegheert *et al.*, 2018). Single cells were isolated by fluorescence activated cell sorting (FACS) using mRuby as a detectable marker to generate monoclonal mRuby-Rab11CA U2OS lineages. After single cell clone expansion, 10 clones were selected for further analysis. This enabled quantification of the mRuby-Rab11CA fusion protein by flow cytometry detection of mRuby. While some selected clones failed to express mRuby-Rab11CA (data not shown), others showed 3 orders of magnitude brighter signal of mRuby compared to the wild type U2OS cell line used as a control (clones 1, 2, 4, 5, and 9) (**Figure 7.8**). These data show that monoclonal cell lines expressing mRuby-Rab11CA contain a homogenous cell population. However, it is important to note that doubling time of monoclonal cell lines expressing mRuby-Rab11CA was slower compared to the wild type U2OS cell line.

To analyse, whether WSN PA-GFP virus can replicate in a monoclonal mRuby-Rab11CA U2OS cell line, clone 1 was infected with WSN PA-GFP virus at a high MOI of 5 or mock

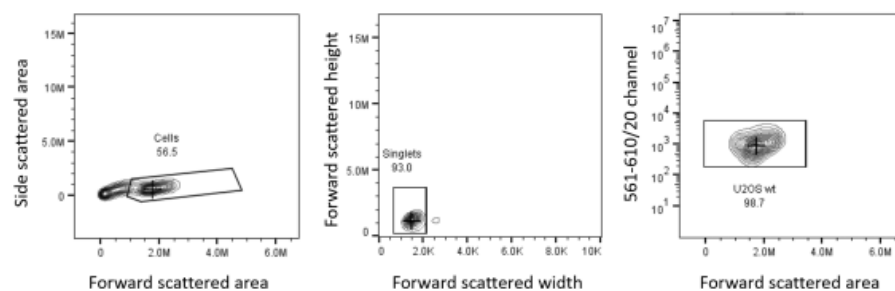
infected and at the indicated time point fixed for fluorescence microscopy analysis. Confocal microscopy revealed that at 18 hpi signals of mRuby-Rab11CA were detected not only in the cytoplasm but also in the nuclei of infected cells (**Figure 7.9A, mRuby-Rab11CA**). Moreover, at 18 hpi no PA-GFP was detected in cells (**Figure 7.9A, PA-GFP**). Similarly, no NP signal was detected in cells infected with wild type WSN virus at 18 hpi (data not shown). In contrast, at 18 hpi signals of PA-GFP were detected in wild type U2OS cells used as a positive control (**Figure 7.9B, PA-GFP**). Together, these results show that a monoclonal mRuby-Rab11CA U2OS cell line is resistant to WSN PA-GFP infection. Moreover, the observations of this study indicate a disruption in cellular localisation of exogenous mRuby-Rab11CA. To confirm that these observations are not specific to the selected clone 1 of monoclonal mRuby-Rab11CA cell line, I repeated the experiment using clones 2 and 5. Confocal microscopy revealed that at 18 hpi signals of mRuby-Rab11CA were detected in the nuclei of infected cells when using either monoclonal cells from clone 2 or clone 5 (**Figure 7.9C, mRuby-Rab11CA**). Similar to the observations in clone 1, at 18 hpi PA-GFP was detected in neither clone 2 nor clone 5 (**Figure 7.9C, PA-GFP**). These data show that different clones of monoclonal mRuby-Rab11CA U2OS cell lines are resistant to WSN PA-GFP infection and show mislocalisation of mRuby-Rab11CA, suggesting that U2OS cells suffered from lentiviral transduction.

While mRuby-Rab11CA mislocalisation could indeed be an artefact from either fixation or imaging step, it could also be induced by high levels of expression of tagged Rab11CA. To determine, whether high levels of expression of mRuby-Rab11CA caused changes in cell morphology, I seeded clone 1 of mRuby-Rab11CA U2OS cells on a TEM grid to analyse their morphology by cryo-SXT (**Figure 7.10A**). Cells were examined using high-dose X-ray

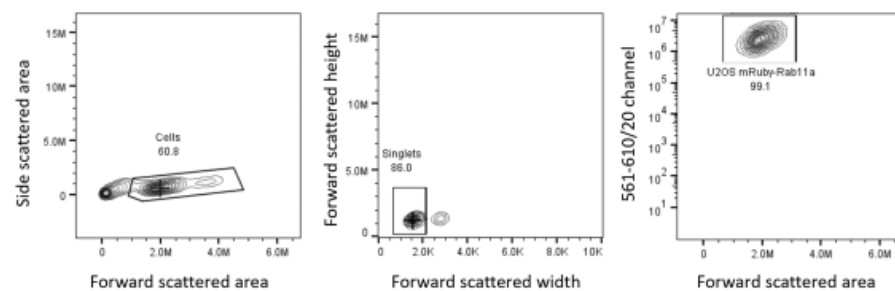
exposures to collect tilt series. The morphology of mRuby-Rab11CA U2OS cells was analysed by using a reconstructed tomogram of untreated U2OS cells as a reference (**Figure 7.10B**). The most striking morphological difference was seen in mitochondria, which lost their cristae arranging and seemed to have undergone mitophagy (**Figure 7.10C–D, mi**). Moreover, extracellular vesicles were observed, which were more abundant than in the untreated control cell line (**Figure 7.10C–D, arrowheads**). These vesicles could be an indicator of extreme stress conditions (O'Neill *et al.*, 2019).

Collectively, these data show that monoclonal mRuby-Rab11a U2OS cell line is not only resistant to the WSN PA-GFP infection but it has also suffered during the lentiviral transduction, resulting in a morphological damage. Consequently, the cell line is not suitable for further experiments.

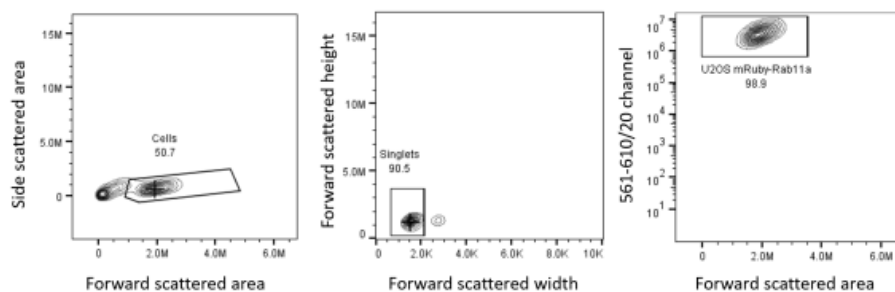
Wild type



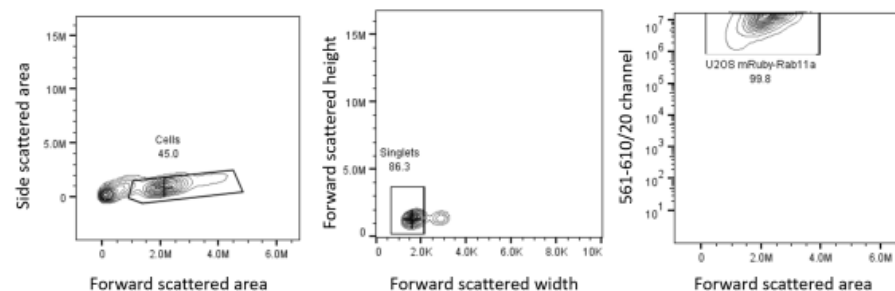
Clone 1



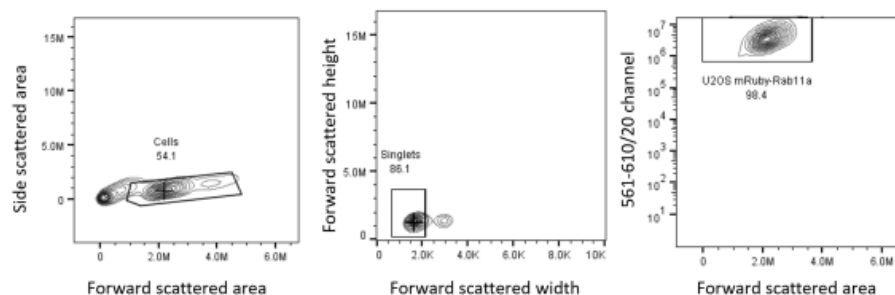
Clone 2



Clone 4



Clone 5



Clone 9

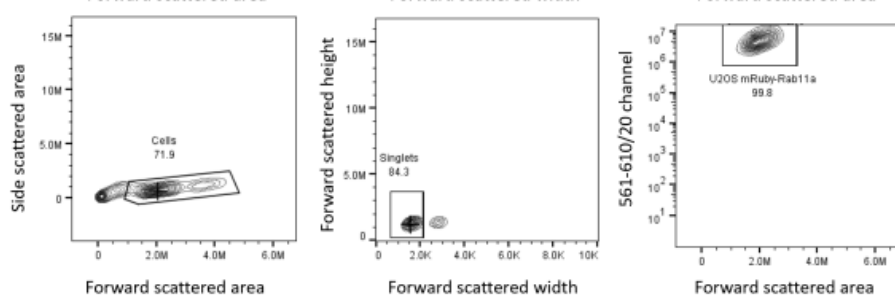


Figure 7.8: Monoclonal mRuby-Rab11a U2OS cell lines contain a homogenous cell population.

A gating strategy to examine selected clones of monoclonal mRuby-Rab11CA U2OS cell lines. Total cell culture was first gated on a forward scatter/side scatter plot to select for viable and singlets, and then gated on the 561-610/20 (yellow/green channel to excite mRuby) to select mRuby-positive population. Untreated U2OS cell line (wild type) was used as a control. All data was analysed in FlowJo.

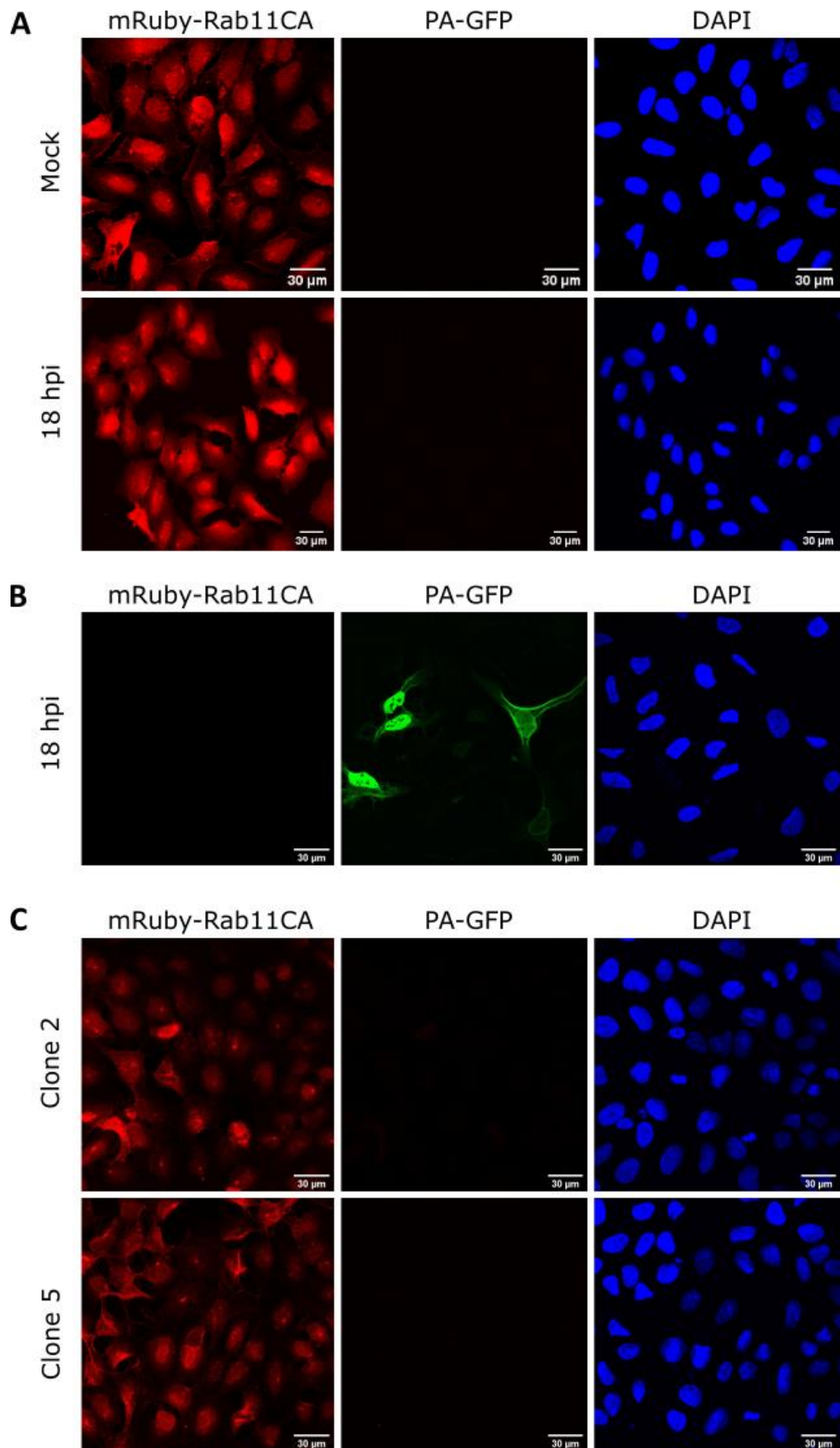


Figure 7.9: Monoclonal mRuby-Rab11CA U2OS cell line is resistant to WSN PA-GFP infection and is morphologically damaged.

(A) Clone 1 of monoclonal mRuby-Rab11CA U2OS cell line seeded on coverslips was infected with WSN PA-GFP virus at a high MOI of 5 or mock infected and subsequently fixed for fluorescent microscopy analysis at 18 hpi. Rab11CA fluorescent signal is shown in red (mRuby), PA-GFP fluorescent signal in green, and the nuclei was visualised with DAPI (blue). **(B)** Same as in **A** but using untreated, wild type U2OS cells as a positive control. **(C)** Same as in **A** but using clones 2 and 5 of monoclonal mRuby-Rab11CA U2OS cell lines instead. Pictures were acquired on Olympus FluoView FV1000 using a 60X magnification objective lens. Scale bar for **A**, **B**, and **C** = 30 μm .

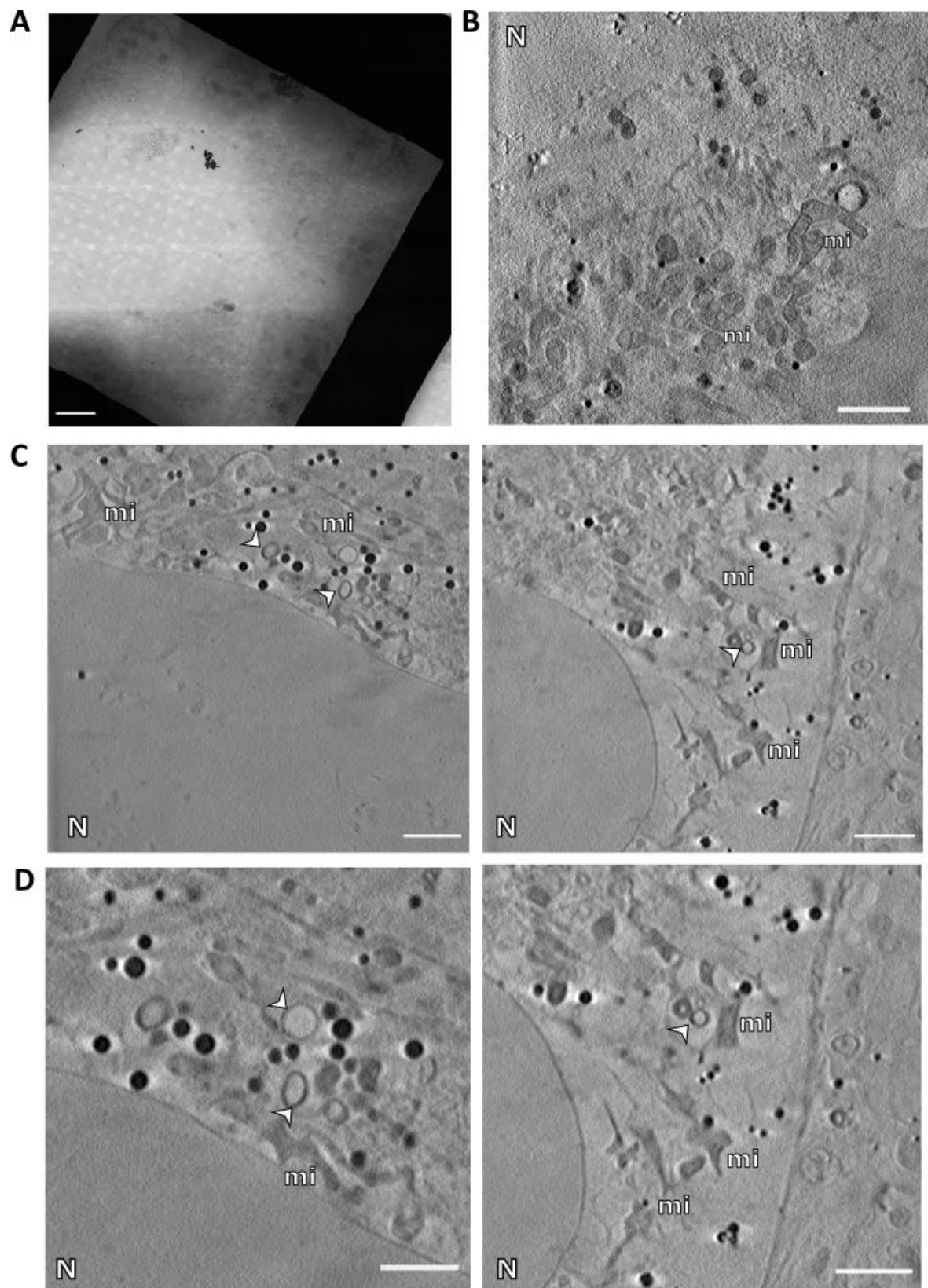


Figure 7.10: Monoclonal mRuby-Rab11CA U2OS cell line is morphologically damaged.

(A) 2D X-ray mosaic of a grid square showing WSN PA-GFP infected mRuby-Rab11CA U2OS cells (clone 1). mRuby-Rab11CA U2OS cells were cultured on a TEM grid to a confluency of ~60% at which point they were infected with WSN PA-GFP virus at a high MOI of 5 and at 18 hpi cryogenically preserved. Whole grid square was examined using low-dose X-ray exposures to collect mosaic image. Scale bar = 10 μm . **(B)** A representative slice through a tomogram after reconstruction of tilt series collected from an untreated U2OS cell line, showing the nucleus (N) and surrounding mitochondria (mi) in cytoplasm. Data were collected and processed by Chidinma A. Okolo at the Diamond Light Source beamline B24. **(C)** Two representative slices through a tomogram after reconstruction of tilt series collected from clone 1 of monoclonal mRuby-Rab11CA U2OS cell line, showing the nucleus (N), surrounding morphologically damaged mitochondria (mi), and extracellular vesicles (arrowheads) in a cytoplasm. **(D)** As in **C** but zoomed in to a region in cells with morphologically damaged mitochondria (mi), and extracellular vesicles (arrowheads). Scale bar for **C** and **D** = 2 μm . Data were collected and processed at the Diamond Light Source beamline B24 using the in-house pipeline that employs Batchruntomo (Mastronarde and Held, 2017), which is a part of the IMOD package (Kremer *et al.*, 1996), via several scripts to allow parallel and near real-time processing. Specifically, both alignment and reconstruction were done in IMOD(4.9.10) (Kremer *et al.*, 1996).

7.3 Discussion

The aim of this study was to establish a workflow for a novel correlative imaging scheme which would allow to study the interaction between IAV vRNPs and cellular GTPase Rab11a in the context of live infected cells. By using a combination of cryo-3D-SIM and synchrotron cryo-SXT microscopy techniques on a single specimen, fluorescently labelled vRNPs and Rab11a could be mapped in 3D to obtain a high-resolution ultrastructural information of a cell with a corresponding location of fluorophores during the infection. The study presented in this chapter showed that while this method indeed has great potential, it nevertheless comes with some major limitations that require further optimisation, which could not be addressed in the given course of this study.

To study the interaction between vRNPs and Rab11a in the context of live infected cells, I first generated a recombinant WSN PA-GFP virus. The results presented in this chapter show that a recombinant IAV expressing fluorescently tagged PA gene can be generated as a heterogeneous virus population, suggesting that some viruses lost their reporter activity after passage in cell culture. This is contradictory to previous observations suggesting that WSN PA-GFP reporter virus is stable (Lakdawala *et al.*, 2014); however, it is important to note that the WSN PA-GFP virus used in our study was not plaque purified and that its infections of MDCK and A549 cells were longer. A549 cells have been widely used for *in vitro* studies of IAV replication (Ujie *et al.*, 2019). Here, their sensitivity to an X-ray exposure was evaluated prior to cryo-SXT data collection. While A549 cells can be successfully grown on pre-treated TEM grids, they proved to be prone to heat damage when exposed to high-dose X-rays. This could be due to their flattened morphology and a thin cytoplasm which

have been observed previously (Swain *et al.*, 2010). Expectedly, heat damage increased after A549 cells were infected with IAV virus, which is likely due to an additional infection-induced stress. While severe heat damage can be indeed prevented by growing cells to a higher confluency, it is important to note that a cryo-SXT microscope possesses its own limitations and can penetrate samples of only a certain thickness (Groen *et al.*, 2019; Harkiolaki *et al.*, 2018; Schneider *et al.*, 2010). Therefore, the cells could not be grown to a higher confluency to prevent their susceptibility to a severe heat damage, thereby A549 cells being unsuitable for subsequent experiments in this study.

From earlier work at the beamline, the U2OS cell line is known for its robustness and resistance to heat damage. Moreover, the cell line has been used previously to study IAV infection (Khaperskyy *et al.*, 2014). In agreement, the results of this study show that recombinant WSN and WSN PA-GFP viruses can replicate in U2OS cell line; however, their replication cycle appears slower compared to their replication cycle in A549 cell line. Previous research at the beamline showed that U2OS cell line remains resistant to heat damage after being infected with the reovirus type 3 (strain Dearing) (Kounatidis *et al.*, 2020). In this study, however, I failed to determine, whether U2OS cells are also resistant to heat damage upon IAV infection-induced stress. While I collected cryo-SXT data successfully, I was unable to determine whether the cells were infected due to the lack of corresponding fluorescent data. Namely, the area on the grid which was used to collect cryo-3D-SIM fluorescent data was damaged during the grid's transfer to the TXM holder, thereby making it impossible to collect cryo-SXT data. Consequently, I collected cryo-SXT data from a different region on the same grid. Since the exposure to soft X-rays leads to atomic bond damage within fluorescent centres (Kounatidis *et al.*, 2020), this region could

no longer be used for cryo-3D-SIM data collection. Thus, if the U2OS cell shown in reconstructed tomogram is indeed infected with WSN PA-GFP virus, the observed mitochondrial event could represent an infection-induced mitochondrial fragmentation. Supporting this is the evidence that the IAV protein PB1-F2 can translocate to the mitochondria during infection where it attenuates the mitochondrial inner membrane potential, thereby accelerating mitochondrial fragmentation (Tiku *et al.*, 2020; Yoshizumi *et al.*, 2014). However, given that not all cells on the grid would be infected, the observed morphological difference of mitochondria could also represent a mitochondrial fusion and fission event in a healthy, uninfected cell. This ambiguousness highlights one of the main limitations of our proposed workflow, which involves multistep handling of the fragile TEM grids. A loss of a grid or its damage at any given step during the workflow can potentially prevent from obtaining the data needed for the interpretation of biological observations.

Ideally, the experiment described above should be repeated to ensure that both cryo-3D-SIM and cryo-SXT data are collected from the same region of interest. However, in the limited time available, I focused on generating a stable U2OS cell line that expresses fluorescently tagged Rab11a instead. This would allow me to tract vRNPs and Rab11a during the infection and to analyse their potential colocalization in infected cells. Recently, lentiviral transduction of mammalian cells was reported as a fast and high-level production method of soluble proteins (Elegheert *et al.*, 2018); however, the results of this study show that this might not be the best choice for generating a stable expression cell line. Although most live cell microscopy studies rely on cells transiently overexpressing a host protein fused to a fluorescent protein, a large body of evidence shows that transiently or constitutively overexpressed proteins can cause multiple artifacts including aberrant

organelle morphology (Harder *et al.*, 2004; Voeltz *et al.*, 2006), and protein aggregation and mislocalisation (Rizzo *et al.*, 2009). Intriguingly, using a lentiviral system, the results in this chapter show that overexpression of exogenous mRuby-Rab11CA resulted in not only aberrant organelle morphology but also in potential aggregation and mislocalisation of the mRuby-Rab11CA protein. To confirm that mRuby-Rab11CA localisation in the cell nuclei is not an artefact from confocal sample preparation or imaging, mRuby-Rab11CA U2OS cells should be grown on a TEM grid and subsequently analysed by cryo-3D-SIM; however, this was not undertaken since the cell line proved to have a strikingly aberrant morphology of mitochondria.

Given that the lentivirus was not titrated before transducing the expression U2OS cell line, it is plausible that the virus titre was too high, thereby resulting in an accumulation of unintegrated viral DNA. High concentrations of unintegrated viral DNA produced by experimental manipulations would be normally associated with high frequency of integration (Skalka and Katz, 2005); however, this can be also toxic to cells as unintegrated viral DNA can induce DNA damage signalling pathways, resulting in cell apoptosis. It has been recently shown that DNA damage also induces mitophagy (Dan *et al.*, 2020), a mitochondrial quality control mechanism, which could explain the aberrant morphology of mitochondria observed in our study. To avoid this problem, plasmid-driven overexpression should have been monitored in this study; for instance, chemically inducible promoter such as Tet-On could have been used instead of constitutively active spleen focus-forming virus (SFFV) promoter. Moreover, additional experiments would be needed to demonstrate that the exogenous expression of Rab11CA signalling element does not intervene with cell network dynamics (MacHacek *et al.*, 2009). Alternatively, these concerns could have been

avoided by genomic labelling of Rab11a using endogenous promoters instead of constitutively active promoter. Namely, instead of using a lentiviral transduction system, expression cell line could be generated by using the type II bacterial clustered, regularly interspaced short repeats (CRISPR)-associated (Cas) system. When using the CRISPR-Cas9 system, the genomic regulatory sequences in cells remain unaltered, thereby resulting in close to endogenous expression levels of the fusion protein (Ratz *et al.*, 2015). This would not only avoid problems otherwise associated to plasmid-driven overexpression, but also allow quantitative studies of protein dynamics at native expression levels (Dean and Palmer, 2014).

In summary, I have partially established a workflow to study vRNP trafficking in the context of live infected cells. While I have successfully generated a fluorescently tagged IAV and characterised its replication cycle in U2OS cell line, I was unsuccessful in correlating cryo-3D-SIM fluorescent data with its corresponding high resolution cryo-SXT data. The correlation of data collected by different microscopy techniques from a single specimen proved to be very challenging due to multistep handling of the TEM grids. The latter remains the main limitation of this study and seems to be unavoidable at this stage of methodology development.

8 Concluding remarks

The work presented in this thesis expands our understanding of the cytoplasmic transport of the influenza virus viral ribonucleoproteins (vRNPs). Newly assembled vRNP complexes are exported from the nucleus and transported across the cytoplasm to be packed into progeny virions in a Rab11a-dependent manner. Our pull-down assays exploring the binding between influenza virus RNA-dependent RNA polymerase (RdRP) and Rab11a have revealed that the RdRP of the influenza A virus (IAV) directly interacts with Rab11a, and that the 627-domain of the PB2 subunit (PB2⁶²⁷) of the IAV RdRP is required for this interaction. This is demonstrated in our study by a GST pull-down assay using purified heterotrimeric IAV RdRP that lacks the PB2⁶²⁷ domain which fails to bind to Rab11a. This is the first time that a particular domain of the RdRP has been found to be important for the interaction with Rab11a. While PB2⁶²⁷ domain has been recently reported to be involved in a direct interaction with a host protein ANP32A in the cell nucleus (Carrique *et al.*, 2020), the data presented in our study now shed light on its involvement in the vRNP cytoplasmic transport for the first time. To support these data, I generated and purified the 627-NLS domain as well as the PB2-C protein on their own to test their binding to Rab11a in a pull-down assay. While the isolated 627-NLS domain fails to bind to Rab11a, PB2-C protein binds directly to Rab11a. This was further supported by a small-angle X-ray scattering experiment, demonstrating that the PB2-C protein associates with Rab11a under size exclusion chromatography conditions. In light of all these findings, we propose here that the PB2⁶²⁷ domain is important for a direct interaction between IAV RdRP and Rab11a while other domains of the PB2 subunit are needed to stabilise the RdRP–Rab11a complex.

Although the RNP of the influenza virus has been shown to interact with Rab11a (Amorim *et al.*, 2011; Avilov *et al.*, 2012a; Einfeld *et al.*, 2011; Momose *et al.*, 2011), it remained unclear whether Rab11a has a selective specificity for vRNP but not complementary RNP (cRNP). If the RNP interaction with Rab11a were to facilitate trafficking towards the cell membrane for virion assembly, vRNPs but not cRNPs would bind to Rab11a. Our work using IAV infected cell lysates in a pull-down assay, together with functional assays, has allowed us to demonstrate for the first time that Rab11a has a differential specificity for vRNP and cRNP only binding to vRNP. While cRNA-containing RNPs are believed to remain in the nucleus (Tchatalbachev *et al.*, 2001), newly synthesised vRNA-containing RNPs are exported from the nucleus via cellular CRM1-dependent nuclear export. Current data support a daisy chain model of nuclear vRNP export in which the viral M1 and NEP proteins are essential for linking vRNP with the importin- β protein CRM1 (Paterson and Fodor, 2012). Interestingly though, it has been shown that cRNP complexes can enter the cytoplasm if their RNA promoter sequence is mutated to match the one of the vRNP (Tchatalbachev *et al.*, 2001). This suggest that a differential specificity for vRNP and cRNP only binding to vRNP might occur even earlier during the vRNPs nuclear export via M1/NEP. Together with the observation of our study, these data also suggest that the factor determining the selectivity for vRNP may be a difference in vRNA and cRNA sequences which exert a different conformational stress on the RdRP, thereby affecting the RdRP structure (Fan *et al.*, 2019; Hengrung *et al.*, 2015; Pflug *et al.*, 2014; Reich *et al.*, 2014). Further studies will be needed to elucidate this, which includes the reconstruction of recombinant RNP complexes in HEK 293T cells transfected with Rab11a followed by a pull-down assay. Our mass spectrometry data showing that NS1 is highly abundant in Rab11a

interactome shed light on its potential role in the vRNP cytoplasmic trafficking, and, potentially, in Rab11a differential binding to vRNPs. Including NS1 protein in a pull-down assay from transfected HEK 293T cells could, therefore, provide further insight into how Rab11a recognises vRNP exclusively.

A large body of evidence shows that influenza virus infection impairs the function of Rab11a by influencing its binding partners leading to its redistribution within the cytoplasm (Amorim *et al.*, 2011; Avilov *et al.*, 2012b; Bhagwat *et al.*, 2020; Eisfeld *et al.*, 2011; Momose *et al.*, 2011; Vale-Costa *et al.*, 2016). In corroboration, our mass spectrometry data suggest that IAV infection reduces the abundance of Rab11-FIPs, thereby potentially alters the stoichiometry of cytoplasmic motor proteins bound by Rab11a. Furthermore, using mutant Rab11a in a GST pull-down assay, our study also provides the first evidence that IAV RdRP potentially binds to the same site on Rab11a as Rab11-FIPs. While our study successfully identified residues on Rab11a that are involved in the interaction with the IAV RdRP, we could not identify the important residues on the PB2 subunit of the RdRP. Instead, the results of our study demonstrate that Rab11a does not determine the host specificity of IAV. Namely, our study did not identify adaptive mutations in PB2 that affect the interaction between RdRP and Rab11a. However, our results using PB2-C proteins from different strains of IAV shed further light on a high flexibility of the PB2 peripheral domains. Using these purified PB2-C proteins in further structural analyses would, therefore, expand our understanding of IAV RdRP conformational flexibility. Moreover, further structural characterisation of IAV RdRP in complex with Rab11a will undoubtedly be required to identify the exact site of Rab11a binding on the viral RdRP.

While crystallography has been responsible for a lot of the full heterotrimeric RdRP structures available, it has proven rather difficult for obtaining a structure of either RdRP–Rab11a or PB2-C–Rab11a complex. The formation of salt crystals due to the presence of magnesium chloride, which is required for Rab11a activity, has prevented us from obtaining a protein crystal in the time course of this study. As recent advances in cryo-electron microscopy (EM) made it a suitable method by which to study RdRP structure, we also employed this method to obtain a structure of the RdRP–Rab11a complex. Unfortunately, the data we present here are not of the IAV RdRP but rather the influenza B virus RdRP. As the cryo-EM grids were most likely mixed up during the data collection, we tried to generate new samples; however, issues with sample preparation have so far prevented us from achieving this. Nevertheless, multiple options remain available to address the issues encountered with sample aggregation, which should make it possible to obtain high-resolution structures by this method in the near future. In terms of obtaining a structure of the RdRP–Rab11a complex, protein crosslinking has proven to be excellent for stabilising macromolecular complexes (Drulyte *et al.*, 2018). Furthermore, the data presented in this study demonstrating that Rab11a binds to the RdRP from different subtypes of the IAV, make further sample optimisation possible. Namely, RdRPs from different IAV subtypes in complex with Rab11a might behave differently on cryo-EM grids; hence, while the NT60 RdRP was prone to aggregation, this might not be the case with either 1918 or Fj RdRP in complex with Rab11a. As to obtaining a structure of the PB2-C–Rab11a complex, I have tested single-chain antibodies available in our laboratory, known as nanobodies, which bind to PB2-C and do not disturb the PB2-C–Rab11a interaction. Cloning these nanobodies into a megabody backbone (Uchański *et al.*, 2021) could enable

overcoming the major obstacle that limits the use of single-particle cryo-EM reconstruction, i.e., particle size, and allow determination of the binding site of Rab11a to the PB2-C by cryo-EM.

Overall, study of the interaction between the IAV RdRP and Rab11a presents an interesting avenue for future investigations towards identifying the exact site of Rab11a binding on the viral RdRP. When identified, this could uncover a novel target site for the development of an influenza antiviral drug.

9 References

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