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Gene-Editing for Resistance to Influenza A Virus in Swine

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Declaration

I declare that this thesis has been composed by myself and that the work has not been submitted for any other degree or professional qualification. All the work and data submitted is my own, including the work which has or will form part of jointly-authored publications. My contribution and those of the other authors to this work have been explicitly indicated below. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

The work presented in Chapter 1 is an adjusted version of a review published in *Porcine Health Management* as *Current and Prospective Control Strategies for Swine Influenza A Virus* by myself (Hamish Salvesen) and Bruce Whitelaw. This study was conceived by all of the authors. I, Hamish Salvesen, wrote the initial manuscript.

The work presented in Chapter 3 is an adjusted version of a manuscript in preparation. This study was conceived by all of the authors. I, Hamish Salvesen, carried out the coding, modelling and manuscript writing.

Hamish Alexander Salvesen, March 2022

Abstract

Influenza A Virus (IAV) presents a major threat to human health and animal welfare. As pigs are susceptible to infection from avian and mammalian origin IAVs, they can be an intermediate host for onwards transmission and act as a mixing vessel in which novel IAVs are generated. ANP32 family proteins have been identified in humans and chickens as host proteins critical to the efficiency of viral genome replication and host factors involved in IAVs adaptation. Host factors recruited by IAV present potential gene-editing targets for controlling IAV transmission and the editing of ANP32 genes in swine represents a potential method of IAV control.

Using CRISPR/Cas technology, ANP32A and ANP32B were disrupted in a porcine tracheal cell line (NPTr) to determine whether they are recruited in the same manner as in humans and chickens by IAV polymerase to support viral genome replication. Our results show that human, avian and swine adapted IAVs can recruit ANP32 family proteins in NPTr, and that ANP32A and ANP32B are functionally redundant for IAV and must both be functionally knocked out to reduce the capacity for IAV to propagate.

To consider industrial applicability, we have modelled the introgression of IAV resistance alleles into a commercial pig breeding herd by one-step zygote gene-editing. Our model results show that more efficient gene-editing methods will reach fixation quicker, even with greater rates of zygote death, and that the level of germline transmission for the gene-edited alleles will have the largest effect on the flow of alleles to commercial breeders. Together, these results have identified genes for further consideration regarding IAV resistance in swine and that gene-editing will need optimisation in porcine zygotes for implementation in the near-term.

Lay Summary

Influenza A Virus (IAV) is the microbe responsible for the most significant loss of human lives, the pandemic of 1918-20. Several novel pandemic IAVs have since emerged, and seasonal IAV is a major cause of disease that contributes significantly to annual global deaths. It is a virus that is not restricted to humans. From its natural reservoir in wild birds, through to farmed species such as chickens and pigs, and onto household pets such as dogs and cats, the burden of disease from influenza rests with many species.

Because each influenza virus is made of so few components, it is a molecular parasite that relies on the contribution of host factors to be able to replicate effectively. ANP32 family proteins have been previously identified to play a role in supporting effective IAV replication. This research set out to understand the role of ANP32A and ANP32B in pigs. If the ANP32A and ANP32B factors identified are essential to IAV replication, we could reduce disease in pigs and prevent transmission of avian IAVs to humans, via pigs as an intermediate vessel through gene-editing.

In this project, we have successfully gene-edited ANP32A and ANP32B in a model system and demonstrated that without these factors present in swine cells, swine, avian and human origin IAVs are less effective at replicating. We also created a simulation model to assess the integration of genes for IAV resistance into a commercial pig herd and found that the decision to use gene-editing would depend on several factors, including the farming system concerned, gene editing efficiency and the rate at which offspring pass on the edited genes to their offspring.

Acknowledgments

What a journey this has been. Reaching the destination of submitting a thesis has relied upon support from many friends, family and colleagues. I will be eternally grateful to those who have helped me along the way.

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To the Digardians, as a lab, you are in incredibly intelligent and talented group. To Dr Nisha Kriplani, Dr Spring Tan, Dr Hui-Min Lee, if only a small part of the experimental virology wizardry you showed me has been imparted with me, I'll be delighted.

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Moving to Edinburgh to follow my education and research dreams would not have happened without the support from my amazing wife Lizzy. You have put up with my erratic hours, terrible planning, and unreliable organisation. Beyond putting up with it, you have actively supported it. You made Edinburgh and Scotland our home, where we made great friends and we lived such a fun life. Thank you for being who you are, and for helping me to become who I am.

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List of Abbreviations

AAV	Adeno-associated virus
AI	Artificial Insemination
AKO	ANP32A knockout NPTr
ANP32A	Acidic Nuclear Phosphoprotein 32A
ANP32B	Acidic Nuclear Phosphoprotein 32A
ANP32E	Acidic Nuclear Phosphoprotein 32E
ASF	African swine fever
ASFV	African swine fever virus
BKO	ANP32B knockout NPTr
CMI	Cell-mediated immunity
Cas	CRISPR-associated protein
cDNA	Complementary DNA
CCK-8	Cell counting kit-8
CPE	Cytopathic effect
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRM1	Chromosomal Maintenance 1
cRNA	Complementary RNA
crRNA	CRISPR RNA
Ct	Cycle threshold
DKO	ANP32A and ANP32B knockout NPTr
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERV	Endogenous retrovirus
ESNIP	European Surveillance Network for Influenza in Pigs
FAO	Food and Agriculture Organisation of the United Nations
FACS	Fluorescent activated cell sorting

FBS	Fetal bovine serum
FluPol	Influenza Polymerase
GDP	Gross domestic product
gDNA	Genomic DNA
GeCKO	Genome wide CRISPR knockout
GFP	Green fluorescent protein
gRNA	guide RNA
HA	Haemagglutinin
HDAC6	Histone deacetylase 6
HDR	Homology directed repair
HI	Herd immunity
IAV	Influenza A virus
ICE	Inference of CRISPR edits
IFITM	IFN-induced Transmembrane Proteins
IFN-I	Interferon Type I
Indel	Insertion or deletion of nucleotides
iPSCs	Induced pluripotent stem cells
ISAV	Infectious salmon anaemia virus
IVT	*In vitro* transcription
LB	Lysogeny Broth
LCAR	Low complexity acidic region
LRR	Leucine rich repeat
LMICs	Low and middle-income countries
LOD	Limit of detection
M1	Influenza A virus protein Matrix 1
M2	Influenza A virus protein Matrix 2
MDCK	Madin Darby canine kidney cells
MMEJ	Microhomology-mediated end-joining
mRNA	messenger RNA
NA	Neuraminidase
NCBI	National Centre for Biotechnology Information
NEP	Influenza A virus protein Nuclear export protein
NHEJ	Non-homologous end-joining
NMD	Nonsense mediated decay

NP	Influenza A virus Nucleoprotein
NS-1	Influenza A virus protein Non-structural 1
NPTr	Newborn pig tracheal cells
NPV	Net present value
NXF1	Nuclear export factor 1
OIE	World Organisation for Animal Health
OMIM	Online Mendellian inheritance in Man
ORF	Open reading frame
pH1N1	pandemic H1N1
PA	Influenza A virus protein Polymerase acidic
PAM	Protospacer adjacent motif
PB1	Influenza A virus protein Polymerase basic 1
PB2	Influenza A virus protein Polymerase basic 2
PA	Influenza A virus protein Polymerase acidic
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PK15	Porcine kidney-15 cells
PGC	Primordial germ cells
PPE	Personal Protective Equipment
PRDC	Porcine respiratory disease complex
PRRS	Porcine Reproductive and Respiratory Syndrome
PRRSv	Porcine Reproductive and Respiratory Syndrome virus
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNAi	RNA interference
RNP	Ribonucleoprotein
RT	Reverse Transcriptase
RT-qPCR	Reverse Transcriptase quantitative PCR
RVD	Repeat variable diresidue
SCNT	Somatic cell nuclear transfer
sgRNA	single guide RNA
shRNAs	short hairpin RNAs
SNPs	A nucleotide polymorphisms

*sp*Cas9	*Streptococcus pyogenes* Cas9
SRSF10	Serine and Arginine Rich Splicing Factor 10
ssRNA	Single-stranded RNA
SUMO	Small ubiquitin-like modifier
swIAV	Swine Influenza A virus
TAE	Tris-acetate EDTA
TALENs	Transcription activator-like effector nucleases
TIDE	Tracking of indels by decomposition
TGEV	Transmissible Gastroenteritis Virus
tracrRNA	*trans*-activating CRISPR RNA
TRIG	Triple reassortment gene cassette
USA	United States of America
USD	United States dollar
USDA	United States Department of Agriculture
VAERD	Vaccine-associated enhanced respiratory disease
vRNA	viral RNA
vRNP	viral RNP
WHO	World Health Organisation
WT	Wildtype
YWHAZ	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta
ZFNs	Zinc finger nuclease

Contents

1 Introduction

Disclaimer – This introduction is a reformatted and altered version of the manuscript for *Current and Prospective Strategies for Control of Influenza A Virus*, published in *Porcine Health Management* (Appendix 1).

1.1 Feeding the World

As the *Homo sapiens* population nears 8 billion members, one of the biggest challenges we face as a species is to ensure that every individual brought into this earth has access to a balanced diet that can support normal development and an active life. In 2020, malnutrition contributed to nearly 45% of deaths in children under 5. Over 9 million deaths from 2020 were attributed to hunger and hunger-related disease, a total that is more than the cumulative total of cancer, AIDS, malaria and tuberculosis. The impacts of these statistics is significantly biased towards low- and middle-income countries (LMICs), and it is particularly in these regions where changes to the availability of a high quality nutritional source, such as livestock protein, would help in alleviating hunger-related suffering. There are negative environmental impacts from an over reliance on animal-based products for nutrition, but in some regions, livestock represent the optimal land-use and as such they have a role to play in alleviating poverty, malnutrition and hunger.

1.1.1 Animal Breeding

Domesticated livestock species are a cornerstone of global food security. Over 1 billion people rely on farmed animals, of which 70% are women, for their livelihoods worldwide, and in many regions there is a cultural significance to the presence of livestock that should not be disregarded when considering their role in the future of an equitable and nourished global society (Global Agenda for Sustainable Livestock, 2014).

Livestock domestication began with the capture of wild animals and their exclusion from their native gene pool. Beginning 12,000 years ago, before Charles Darwin's publication of *the theory of evolution by natural selection*, and

before any understanding that genes were passed on to progeny as a hereditary element, selection was performed by these original stockmen through breeding males and females that shared desirable traits and behaviours. Their genomes have now been moulded over millennia to lead to the domesticated species we have today (Figure 1-1).

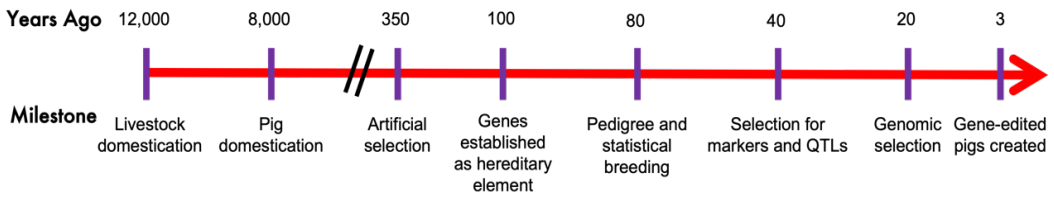


Figure 1-1: A timeline of seminal points in animal agriculture. Between domestication and selective breeding programs several millennia passed. With just over a century since the discovery of genes as the essential hereditary element, genetic knowledge and technologies has revolutionised livestock breeding.

Artificial selection became formatively established as a livestock breeding concept in Western societies with Robert Bakewell around 350 years ago. Bakewell bred elite sires with elite dams with the intention of enhancing specific market relevant traits such as carcass weight. Significant productivity and efficiency improvements were gained when selective breeding programs began taking detailed records of trait data and pedigrees were created. Because genetic gains made by breeding are heritable and cumulative, significant progress has since been made.

A second revolution in breeding came along with the development of genomic technologies. Taking a quantitative view of livestock genomes, traits are selected for through their association with genome regions harbouring single nucleotide polymorphisms (SNPs). This has significantly improved the accuracy of selection and led to a drastic boost in productivity (Georges et al., 2018). However, because the focus was initially only on productivity, unintended selection for genomically linked negative traits, such as reduced fertility was incident. Breeding programs are now more holistically focused to select for animals that are better balanced for health, fertility, environmental

impact and productivity. Through selection based upon genetic knowledge over presuming phenotypic inheritance we can also select for difficult to observe traits such as feed conversion ratios and environmental footprint. Currently, the most effective tool available for minimising the negative impacts of livestock is through increasing their productive lifespan and breeding years. Increases in animal productivity can alleviate the need to use more land to create more food, which will potentially allow a reversal of deforestation and land reconversion back to natural and not cultivated states.

Animal breeding can now incorporate qualitative genetic tools alongside the quantitative genomic selection tools. The genetic toolkit of the 21st century includes methods of making specific changes in the genome down to a single base pair of DNA (Tait-Burkard et al., 2018). Identification of genes associated with resistance to livestock diseases has opened the door to the use of genome-editing as a tool for improved productivity (Crispo et al., 2015; Zou et al., 2018), disease control (Burkard et al., 2017; Whitworth et al., 2019) and to improve animal welfare (Carlson et al., 2016). Integration of naturally occurring disease resistance alleles into a herd via selective breeding is possible, however the allele may be genomically linked with less desirable traits creating genetic drag, and the animal carrying the resistance allele may not be from a genetically elite breeding pool (Proudfoot et al., 2019). Using gene-editing technologies *de novo* resistance alleles or natural variants can be integrated into the genome of elite breeding animals, circumventing the issue of genetic drag by selective breeding from less desirable animals.

In the face of a growing human population and an increasingly unpredictable climate, agriculture needs innovative solutions to ensure that productivity is maintained in volatile weather patterns and that adequate diets are available in an globally equitable manner. Alongside genomic technologies and pedigree analysis, an ability to edit the genome to introduce alleles directly into desirable breeding animals could add to the eligibility for animals to continue in their role in providing food security, maintaining the livelihood of farmers, and allowing animal agriculture to remain as a feature across diverse cultures (McFarlane et al., 2019; Proudfoot et al., 2020).

1.1.2 Pigs

The domestic pig, *Sus scrofa domesticus,* is a staple species in the global agricultural landscape. Belonging to the non-ruminant, even-toed, hoofed family of *Suidae,* the ancestors of modern pigs are European wild boar that were domesticated at least 8000 years ago in the Near East (Larson et al., 2007). By 1500 BC they were being kept throughout Europe and Asia, before becoming globally distributed through the ages of colonialism and exploration as an animal that could tolerate long periods on ships and the diverse habitats where explorative ships landed. In Pacific Islands, Australia and the Americas, introduced pigs are destructive within fragile ecosystems where native organisms have not evolved to withstand the pressures that wild pigs can have. There are several species of indigenous pigs that have significant cultural value, and could play a significant role in research for traits such as heat and disease tolerance, however the 'pig' referred to here on is exclusively the domesticated pig.

Pork accounts for over a third of meat produced and over a quarter of protein consumed worldwide, and thus is a critical for global food security (OECD-FAO, 2021). Their omnivorous diet, large litter size and ability to perform well in varying climactic conditions has made them popular across the globe in both small and large farming systems. In small-holder farming scenarios, pigs can complement humans effectively as they do not necessarily directly compete with humans for their nutritional sustenance and they are efficient at converting low value energy into a nutritionally dense meat product. Because of this robustness, their practicality in a circular food system, and fast growth rates, pigs play an integral role in providing nutrition in vulnerable communities and LMICs. Pigs on small scale farms live an extensive, free-range life that often has minimal inputs for animal welfare or nutritional benefits. In contrast, pigs in intensive systems, farms with over 100 pigs (as described by (Gilbert et al., 2015), are living under tightly regulated conditions with monitored breeding cycles and controlled dietary inputs. Beyond personal nutrition and food security, swine production is important due to its contribution to global trade

markets, mainly through the larger intensive farming operations. There are now over a billion pigs in the production market worldwide, creating an industry worth over $200 billion USD (OECD/FAO, 2016). Hog rearing has dramatically expanded in the last half century, with the pig population growing 2.5 times (Figure 1-2) (Gilbert et al., 2015). The design of and infrastructure required for this scaling up and transition to industrial breeding systems has meant that the intensification has occurred in regional hotpots and has a heavy reliance on international trading (Figure 1-3A) (Gilbert et al., 2015).

When affluence increases, as measured by Gross Domestic Product (GDP) growth, there is a correlated increase in demand for animal food products (Espinosa et al., 2020). And this is a demand that is projected to increase. To meet the increased demand for meat, farmers have transitioned to high-density herds, which now comprise over 50% of the total global pig population (Gilbert et al., 2015). As GDP increases, the proportionate amount of intensive farming increases (Espinosa et al., 2020; Gilbert et al., 2015). If the trend for GDP growth continues and the corollary with intensification remains, intensive farming will become ubiquitous, with extensive or small-holder farming featuring in the livestock landscape as a hobby practice and with pockets of culturally significant rearing.

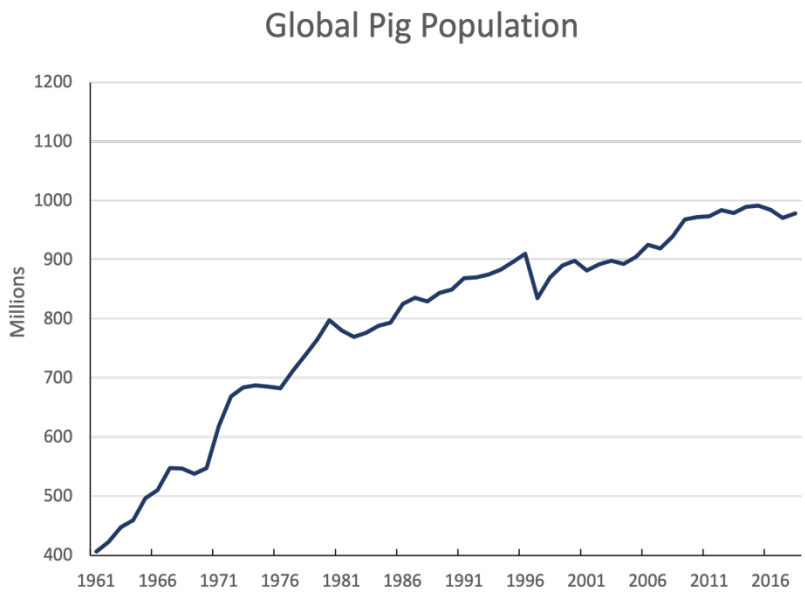


Figure 1-2: The global population of pigs from 1960-2018. Data from Food and Agriculture Organisation of the United Nations (FAO).

1.2 Disease in Swine Farming

Infectious disease is one of the main economic constraints in livestock production and concerns from an animal welfare perspective. Industrial farming requires larger herds and frequent movement of animals, which has obvious impacts in potentiating disease transmission due to more animals being in close proximity that can provide an abundance of potential hosts (Drew, 2011). This is particularly true for viral pathogens, and outlined by the fact that in regions with predominantly intensive farming systems, there is a clear prioritisation towards viral disease in research publication, whilst regions that are predominantly small-scale farms are more focused on bacterial, helminth and protozoal pathogens (VanderWaal and Deen, 2018). Viral diseases can rapidly become endemic in intensive farming systems. Porcine epidemic diarrhoea virus was first spread to the USA in 2013, and within 1 year over 50% of USA herds were affected (Scott et al., 2016). It cannot conclusively be stated that this increasing disease prevalence is solely a result of intensification however, as the gradual

transition to high-density herds has coincided with better diagnostic tools and increased surveillance, factors that make recognition of a pathogen being present far more likely.

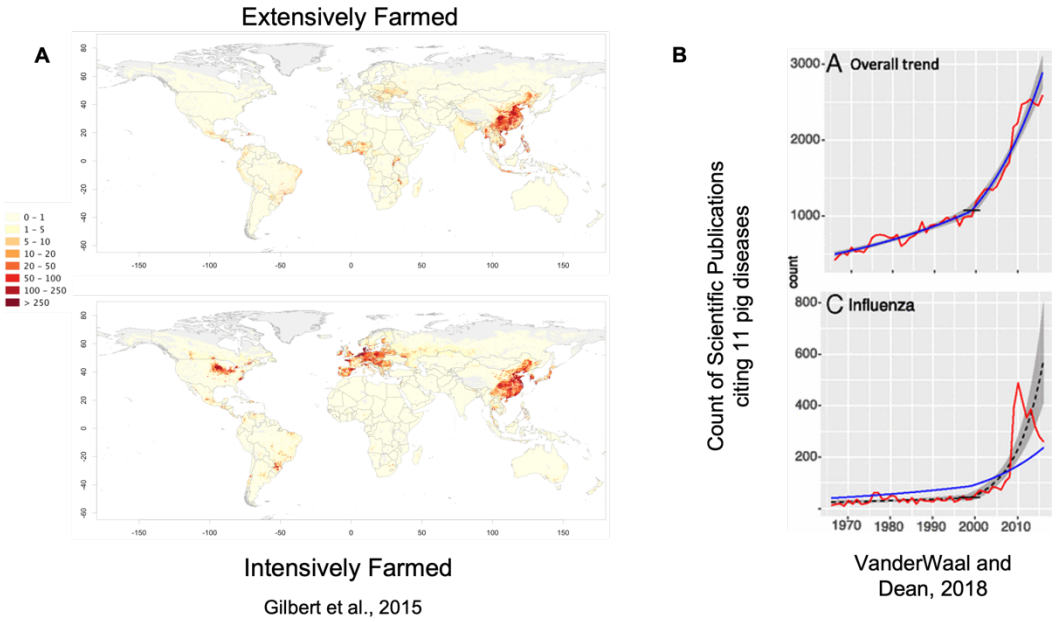


Figure 1-3: A) The global distribution of pigs shown farmed in intensive (>100 pigs) and extensive (<10 pigs) farming systems. Intensive systems are pigs farmed. Heat map coloured by head of pigs per square kilometre. Adapted from Gilbert *et al*., 2015. **B)** The publication count of swine diseases over time. The overall trend of 11 swine disease (top) and the trend of publications for swine IAV (swIAV) (bottom). The red line represents count per year, the blue line represents expected trend based on overall publication counts of the 11 investigated pathogens. The red peak in Influenza is in 2009/10 following the Swine Flu pandemic.

Large farms should not be considered the only present hazard for disease transmission. Outdoor rearing and the lack biosecurity measures presents an alternative risk, and transmission between small farms is considered a major contributing factor for the 2018 African Swine Fever (ASF) swine epidemic in China. This had devastating consequences, with up to 200 million pigs purportedly culled, ramifications were felt in all economic sectors through a reduction of 0.78% in Chinese GDP (You et al., 2021). Furthermore, small farms can more easily get infections or pass them on due to the sharing of boars with

unknown infection status, the admixture of multiple different livestock species and not preventing wildlife and livestock interacting.

Infectious diseases in swine are not homogenously prioritised globally, with some pathogens being isolated or adapted to specific regions. Concern for disease, as determined through publication records, is naturally heightened where a pathogen is endemic, and also where past outbreaks have left scarring (VanderWaal and Deen, 2018). The same study also notes that infectious diseases in swine that are also zoonotic have increased proportionately more than nonzoonotic pathogens over the last 59 years, probably as a result of a better understanding of public health implications as well as funding to prevent swine disease in order to placate public concerns of swine farming (Figure 1-3B).

When considering disease control on farms, it is important that a *One Health* approach is taken to ensure optimal benefits to animals, humans and the environment (Mackenzie and Jeggo, 2019). Disease in farm animals has a significant impact on their productivity and quality of life, but the impacts are not restricted to individual infected animals. If an infected animal is placed as the middle point within three concentric circles, the impacts of disease can be considered to fall within the three following groups from each circle; direct effects from a pig being ill and upon the animal itself, effects that the infected animal will have on farm operations and individual humans, and from effects to systems outside the farm, such as trading, the environment or human society (Figure 1-4). These effects can be described separately, but because the outcomes of each are intimately intertwined, when one factor is in imbalance there are reactions across all three.

Direct costs of infected animals are not only incurred from reduced productivity in terms of how long an animal takes to reach market weight (Bennett, 2003). Feed conversion rates can be reduced which means more feed is required to be bought by the farm. The litter size of infected animals is reduced, which reduces the efficiency of farm production through lower average number of piglets per sow (Gumbert et al., 2020). Animals with disease, whether clinically visible or not, will be experiencing a reduced quality of life, and in fighting the initial infection other pathogens can become more likely to become established.

Figure 1-4: The cost of disease in swine. The direct impacts of disease are a result of the individual pig being ill. But the effects are not limited there. There are herd costs and management costs for the farmers that have economic impacts. The impacts also go beyond the farm gate, where swIAV affects international trade markets, which in turn impact on local communities' income and national GDP growth. The impacts of swIAV are multi-sectoral.

Every disease that has the potential to become endemic within a herd represents a threat to animal welfare, human health or negatively affects farm economic performance, and should therefore be given attention. Each medical treatment that needs to be bought, veterinary expert paid, and employee hired for administration of medicines or vaccines reduces farm profitability. All the traditional biosecurity measures discussed in this introduction, quarantine,

isolation, culling and surveillance have associated costs, and increases in costs that make it difficult for a farm to be financially viable can reduce attention to welfare and create a product for human consumption.

Finally, the impacts that are outside the farmgate could be the most significant threat to the trade and presence of pigs as livestock. With over 75% of emerging diseases being zoonotic livestock pathogens, effective control of swine disease also provides major benefits to humans in a multi-sectoral capacity through healthcare, agriculture, tourism, investment, that warrants management for the benefit for the whole of society (Rassy and Smith, 2013; Smith et al., 2019).

Diseases that have zoonotic capacity in swine can cause serious illness in humans. Some, such as Influenza A Virus (IAV), also have the potential to become human pandemics. If serious disease outbreaks or pandemics have an origin in pigs, the import and export swine meat markets are affected (Morgan and Prakash, 2006). Reduced trade affects GDP, which at a national scale can have a large impact on state economies that are heavily reliant on pork production. At a more local scale, and relevant to large farms and extensive rearing in LMICs, if consumer abstention from pork or large scale culling occurs there will be a removal of pork related income from communities and there could be a flow on reduction in spending and employment. The threat of a global pandemic emerging from swine farming is difficult to anticipate for the pork industry, but with increasing concern for the way in which animals are farmed, it seems possible that many consumers would lose trust and decide against buying swine products. For livestock farming to continue with a social license to operate, and with consumer connectivity to products increasing, it will become progressively important that both endemic and zoonotic diseases are well controlled in livestock.

The animal-human interface is absolutely critical for controlling disease. This interface is not exclusively relevant to farming, but long exposure times and regular close contact with livestock is a significant point of contact. Farmed pigs can be the conduit for disease to transmit to humans via pigs from wildlife origin pathogens such as has occurred with Nipah Virus, Hepatitus E Virus and

Influenzas (Smith et al., 2011). Unless pigs are biologically resistant to infection, they will maintain the ability to become a vector for disease because wildlife will remain pathogen reservoirs (Pulliam et al., 2012). Research in Myanmar has identified a priming theory for zoonotic infection of Nipah Virus in pigs. When transmitting from bats to pigs, an infected herd can develop disease tolerance through partial reisitance. This partial resistance increases the likelihood that subsequent infections in the herd can become endemic, which in turn means that humans are faced with a more present concern for zoonotic transmission (Pulliam et al., 2012).

Detection of swine origin IAVs in humans in the USA over the last 10 years demonstrates that swine farming is a frontier of IAV zoonosis (Figure 1-5). The regions of the highest incidence of swine IAV (swIAV) detection in humans conspicuously overlaps with the locality of intensive farming identified previously in Figure 1-3A. Zoonotic outbreaks may be symptomatic of 21st century lives. We live condensed in urban areas, in a globally connected world, with increasing contact to wild animals through changing ecosystems and farmed animals through intensive farming – controlling disease at the animal-human interface will be critical in reducing the likelihood of future pandemics.

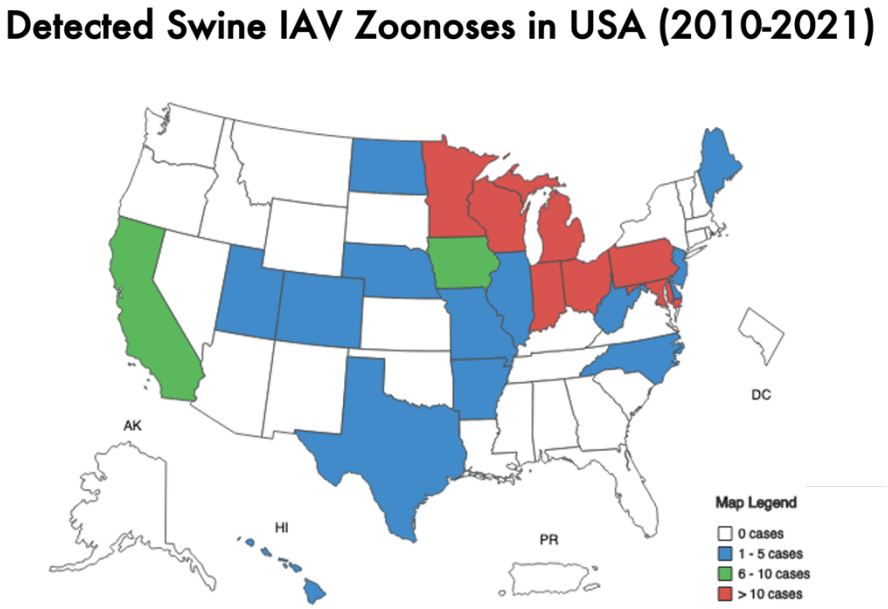


Figure 1-5: The distribution of swine-origin IAV cases detected in humans over the last decade in the USA. These are cases confirmed by molecular diagnosis through the Centre for Disease Control, USA. The states with the most regular identification of cases overlaps closely with the regions in Figure 1-3A that are intensively farmed.

1.3 Influenzas

One such emerging zoonoses is the family of Influenza viruses. Influenza viruses are significant pathogens of humans, livestock, and a multitude of wild species. They have a diverse and complex ecology stemming from their ability to cross species barriers. Comprising four genera within the *Orthomyxoviridae* family, Influenza A Virus (IAV), Influenza B Virus (IBV), Influenza C Virus (ICV) and Influenza D Virus (IDV) are enveloped virions with segmented negative-sense single-stranded RNA genomes (-ssRNA) (Figure 1-6A). Seasonal epidemics of IAV and IBV occur in humans whilst only IAV has been attributed to cause epidemics in swine (Janke, 2014). IBV (Ran et al., 2015), an ICV-related pathogen (Hause et al., 2014, 2013) and IDV (Foni et al., 2016) have been reported and associated with mild morbidity in domestic pigs (Brown et al., 1995; Hause et al., 2014). Herein, the focus will be on IAV due to its more significant historical impacts and greater potential for emergence as a swine or human pandemic.

It was recognised in the 1918-1919 Influenza outbreaks that swine were affected with similar influenza morbidities to humans, but it was not until 1931 that the influenza virus was isolated and identified as the aetiological agent of this illness in swine (Shope, 1931). IAV has a remarkable capacity to infect a wide range of clades. It is a quintessential emerging zoonotic pathogen, surfacing in annual epidemic cycles as antigenically novel subtypes (Buckland, 2015). The natural reservoir is in migratory Anseriformes (i.e waterfowl), where the greatest diversity of viruses is found. All known subtypes of IAV have been found to infect avian species. These viruses spill over into other non-waterfowl species and are dispersed globally through migratory routes. Subsequently, mammals become infected through their interactions with infected birds. As well as pigs, other domestic species that are infected with IAV naturally includes dogs, cats and horses (Figure 1-7) (Krammer et al., 2018). This means that IAV in mammals can be regularly antigenically refreshed from a wild reservoir and means that its evolution can be difficult to predict.

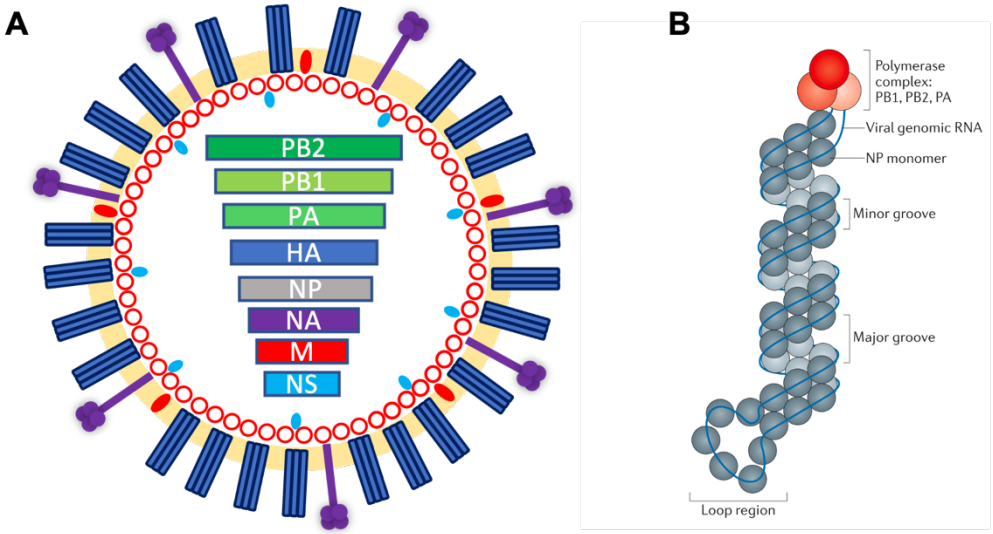


Figure 1-6: The Influenza A Virus and genome. HA = Haemagglutanin. NA = Neuraminidase. PB1 = Polymerase Basic 1. PB2 = Polymerase Basic 2. PA = Polymerase Acidic. NP = Nucleoprotein. M = Matrix. NS = Non-structural. **A)** A schematic of IAV. The 13.6 kb genome is comprised of 8 discrete segments that are contained within a bilipid membrane envelope. **B)** An IAV vRNA bound by nucleoprotein with homologous pairing of the 5' and 3' ends encased within the heterotrimeric Influenza A Polymerase (FluPol) complex. Adapted from Eisfeld et al., 2015

The IAV genome is ~13.6 kb of single stranded negative-sense RNA encoded upon 8 discrete segments. These anti-sense segments encode for at least 10 proteins (HA, NA, PB1, PB2, PA, NP, M1, M2, NS1, NEP) (Hutchinson et al., 2010; Palese and Schulman, 1976). Nomenclature for IAV derives from antigenic subtypes determined by the major surface glycoproteins haemagglutinin (HA) and neuraminidase (NA) (HxNx). The segmented genomic architecture and the functional role of proteins is important in understanding its evolutionary history and continued adaption, particularly concerning intraspecies and zoonotic transmission.

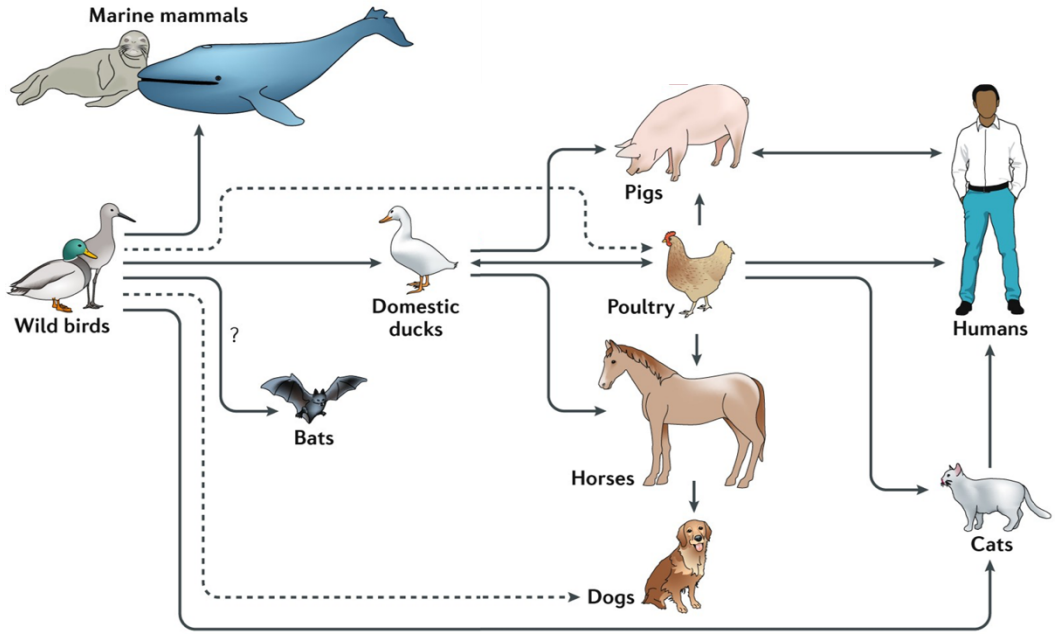


Figure 1-7: The panzootic transmission of IAV. The natural reservoir of waterfowl can be infected with all IAV subtypes. Swine can be infected with avian and human origin strains and could be a critical node for control of avian IAV transmission to humans. Adapted from Krammer *et al*, 2018.

1.3.1 Influenza Replication Cycle and Protein Roles

Considering a static IAV virion, the viral envelope is a lipid bilayer that is derived from the hosts cellular membrane and contains the IAV transmembrane proteins haemagglutinin (HA), neuraminidase (NA) and matrix 2 (M2). Encompassed within the envelope is the viral genome, proteins that manipulate

the host cell response and proteins that replicate or help facilitate replication of the viral genome. Matrix 1 (M1) is located beneath the membrane and maintains the virion structure through polymerisation and acts as anchoring points for the viral ribonucleoproteins (vRNPs) (Figure 1-6B) (Selzer et al., 2020). The vRNPs are mostly nucleoprotein (NP) that has viral -ssRNA (vRNA) bound in a twisted, anti-parallel double helix with small amounts of nuclear export protein (NEP) that acts as an adaptor between vRNPs and host nuclear export proteins to facilitate nuclear export of the vRNPs under specific conditions (Eisfeld et al., 2015). At one terminus of the vRNPs are the polymerase proteins (polymerase basic protein 2; PB2, polymerase basic protein 1; PB1, polymerase acidic protein; PA). This heterotrimeric influenza polymerase (FluPol) complex binds with the 5' and 3' end of vRNA that have base pair complementarity (Figure 1-6B). The non-structural 1 protein (NS1) is an inhibitor of the hosts immune response that contains an RNA binding domain and an effector domain (Rosário-Ferreira et al., 2020). Through its interactions with host RNA and proteins it hampers the immune response and affects multiple host pathways, removal of functional NS1 can be an effective strategy for attenuation in vaccine production (Richt et al., 2006; Solórzano et al., 2005).

The span of an influenza virion's existence begins when it buds from a host cell, and can be considered complete when it has infected a new cell and contributed to the generation of new virions budding from that subsequently infected cell (Figure 1-8). Host cell entry is primed by attachments of the HA homotrimer to specific sialic acid (SA) residues on host membrane bound glycoproteins. The carbohydrate linkage between the SA moieties and the glycoprotein is a key factor for HA binding affinity (Rogers and Paulson, 1983). Antigenic evolution of HA occurs to improve binding affinity for the prevalent SA residue and the physiological environment they are found in (Mair et al., 2014; Matrosovich et al., 2000). SA residues α(2,3) and α(2,6) are distributed differently in different species. Humans predominantly have α(2,6) SA in the respiratory tract, whilst chickens predominantly have α(2,3) SA in the gastrointestinal tract. Swine have both α(2,3) and α(2,6) present in their

respiratory tract (Suzuki et al., 2000). This variation in sialic acid is a key determinant in zoonotic transmission (Wilks et al., 2012).

Interaction of HA with the SA residue instigates viral entry into the host cell by receptor mediated endocytosis. The low pH environment of an endosome induces cleavage of HA into two peptides. HA2 is inserted into the endosome membrane, leading to fusion of the endosome and viral membrane. The acidic environment also causes a conformational change in the M2 protein tetramer, causing it to open as a proton-selective ion channel. A decrease in the internal pH of the virus catalyses dissociation of the bond between M1 and the vRNPs, allowing diffusion into the host cytoplasm.

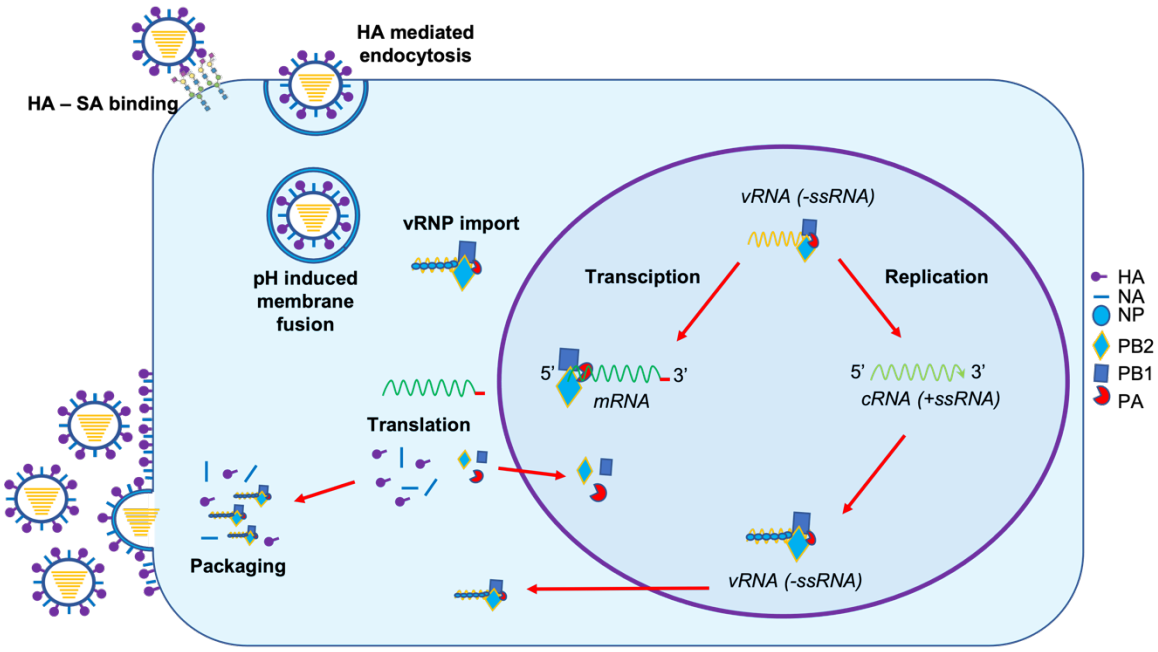


Figure 1-8: The replication cycle of a swine IAV. The beginning of the IAV interaction with the host cell is the HA-SA interaction. Conformational change of HA leads to virus uptake by endocytosis. Internal acidification of the virion results in release of the vRNPs. The vRNPs are then transported into the nucleus for transcription and replication. Viral mRNAs are exported for translation. Once translated, viral proteins are either imported into the nucleus or transported to the cell surface. vRNPs are exported from the nucleus in bundles and migrate to the cell surface to be packaged and emerge as an infectious virion. HA = Haemagglutanin. NA = Neuraminidase. PB1 = Polymerase Basic 1. PB2 = Polymerase Basic 2. PA = Polymerase Acidic. NP = Nucleoprotein. M = Matrix. NS = Non-structural.

The IAV genome is transcribed and replicated in the host nucleus and thus the vRNP complexes must be transported from the cytoplasm to nucleus. The vRNP gene segments from infecting particles travel as a bundle to the nucleus (Chou et al., 2013). Nuclear localisation signals (NLS) present in all proteins of the vRNP complex support interactions with proteins from the importin family that facilitate active transport through membrane pore complexes. The import and export of vRNPs between the cytoplasm and nucleus is an intricately regulated process that requires exposure and camouflaging of the NLS for unidirectional transport depending on the stage of infection (Boulo et al., 2007; Tarendeau et al., 2007).

Once the genomic contents are in the nucleus, it must be converted to positive sense RNA, called complementary RNA (cRNA), which can act as a template for conversion back to the negative sense vRNA. This ensures there is more vRNA to serve as templates for creating proteins to instigate a severe antiviral response and more rapid production of progeny virions. The viral RNA dependent RNA polymerase (RdRp) initiates replication of the vRNA through the presence of genetic complementarity present at the 5' and 3' ends that now functions as a primer. For transcription of mRNA from the vRNA templates, IAV steals 5' methylated caps through PB2 endonuclease activity from host mRNAs to prime viral transcription. This has dual benefits of initiating viral transcription and disrupting translation of host mRNAs that could otherwise contribute to the host antiviral response (Krug et al., 1979). Viral repression of host transcription and translation is part of a multifaceted response known as host shutoff (Khaperskyy et al., 2016; Levene and Gaglia, 2018). Ultimately, this leads to host cells being becoming viral protein factories.

For viral transcripts that require splicing, host splicing machinery is recruited. This has the dual purpose of subduing host splicing activity and negating a normal antiviral response and apoptotic pathway induction (Thompson et al., 2018). To the nascent viral mRNAs, a poly (A) tail is added through stuttering of RdRp. The mature mRNA is then ready for nuclear export, complete with 5' caps from the host and a poly (A) tail. They are exported and

translated by host ribosomes in the same fashion as canonical eukaryotic translation occurs.

Import of the viral RdRp proteins, NP, and NEP back into the nucleus for further transcription and genome replication precludes formation of vRNPs for nuclear export. To reverse the nuclear localisation properties of mature vRNPs, M1 binds to the N-terminal of NEP to mask its NLS, with NEP then having a binding affinity for CRM1 (also known as XPO1) that stimulates nuclear export via the CRM1 dependant pathway (Bui et al., 2000; Martin and Helenius, 1991). An interaction between NP and NXT1 also stimulates export via the CRM1 dependent pathway (Chutiwitoonchai and Aida, 2016). This process is well reviewed in (Eisfeld et al., 2015). Other proteins that are not imported into the nucleus or required in viral replication dynamics, such as HA, NA and M2 migrate to the plasma membrane. The remaining requirement to complete the viral replicative cycle is for infectious progeny virions to bud from the host cell.

For packaging of virions, stochastic and specific models of packaging 8 segments have been proposed. Packaging signals in viral segments and experimental analysis suggests that the latter model of specific packaging occurs, through an arranged pattern of 7 + 1, with a central vRNP surrounded by 7 segments as an octameric complex. Each vRNP segment is not exported individually, but as bundles that form during transport to the cell surface (Haralampiev et al., 2020; Lakdawala et al., 2014). Biological misdemeanours do occur in the packaging process that result in the creation of non-infectious virions that do not contain each of the 8 segments (Nakatsu et al., 2016). As a virion is budding at the surface of the plasma membrane, NA cleaves the SA from the glycoprotein to allow release of budding virions, and if packaged correctly, these virions can infect cells in the same organism or be transmitted onwards to a new host (Li et al., 2021).

Alongside the core proteome, proteins that are essential for persistently infectious virions or their absences severely attenuates infection, there are accessory proteins (Pinto et al., 2020). These are proteins, generated through alternative translation initiation sites and alternative splicing, which are nonessential, expressed in a relatively low abundance and identified

inconsistently between strains. Their inconsistent presence does not mean that they have inconsequential roles for pathogenicity however, and their presence can affect pathogenicity and infection outcome *in vivo*.

1.3.2 Swine IAV Evolution

Genetic variation of IAV arises via two mechanisms: (1) from reassortment of the eight genomic segments when multiple subtypes co-infect the same cell (antigenic shift), or (2) through accumulation of point mutations due to a lack of viral RNA proof-reading following genome replication (antigenic drift) (Yang et al., 2017). Amino acid changes are selected according to their physiological contexts which vary between different hosts, thus promoting rapid divergence and adaptation following zoonotic transmission.

Antigenic shift can occur in the instance of two distinct IAVs co-infecting a single host cell, whereby genomic segments from distinct original virions can become reassorted, leading to progeny virions having different genomic compositions to the ancestral infectious particles that will have antigenic novelty (Figure 1-9B) (Rose et al., 2013; Vijaykrishna et al., 2010). Termed antigenic shift, this phenomenon supports rapid generation of novel IAV subtypes, that in turn promotes the circumvention of the host immune response by evading recognition (Mostafa et al., 2018).

Because the respiratory tract of pigs has both α2-6 and α2-3 on the surface of epithelial cells, this creates the opportunity for both avian adapted and mammalian adapted IAVs to enter the same cellular environment (Gambaryan et al., 2005; Trebbien et al., 2011). The presence of multiple IAV subtypes concurrently infecting a single host cell means there is the potential for novel viral emergence by antigenic shift, this has led to pigs being coined as "mixing vessels" (Nelson and Worobey, 2018; Neumann et al., 2009). The original source of infection in swine may come from humans, chickens and/or wild Anseriformes, undergo swine adaptation and genomic reassortment to create novel antigenicity, and transmit back to humans with immune naivety making pigs an intermediary host with concerns of their role in transmitting

mammalian-adapted and IAV strains carrying more pathogenic avian flu segments.

Genome reassortment alone does not drive IAV emergence, with the error-prone viral RNA-dependent RNA polymerase (RdRp) contributing through the introduction of mutations during RNA replication (Rodriguez-Frandsen et al., 2015). In a constant evolutionary arms race between IAV and their hosts, the adaptive pressure exerted by host immune systems upon the virus is the response that stimulates IAV phenotypic diversity and ultimately drives their evolution. Lacking a proof-reading function, diversity in the viral genome gradually accumulates, providing a source of variation for the forces of natural selection to be imposed on, in a process known as antigenic drift (Figure 1-9A) (Morris et al., 2020). The viruses that can transmit between hosts and replicate within a host most efficiently then become pervasive by superior propagation.

The 1918 Spanish 'Flu is suggested to have emerged in humans from birds potentially via intermediary pigs, and the 2009 pandemic "Swine 'Flu" arose after antigenic reassortment of endemic avian, human and porcine influenza strains re-emerged from swine into a human host (Girard et al., 2010; Smith et al., 2009). Understanding the genetic evolution of IAV is critical to disrupting the IAV ecosystem and determining the approach of controlling both intraspecies and zoonotic transmission.

The nomenclature bestowed on HA and NA proteins are examples of host restrictive proteins, with antigenic variation of these viral surface proteins being essential for cell entry and viral budding respectively and are associated with the species specificity of IAV subtypes (Capitanio and Wozniak, 2012; Watson et al., 2015). IAV genomic adaptations are generally associated with specific hosts as they confer a selective advantage in a particular physiological context, with specific amino acid changes in HA of avian derived IAVs known to be associated with an increased potential for successful mammalian transmission (Suzuki et al., 2000; Vijaykrishna et al., 2010).

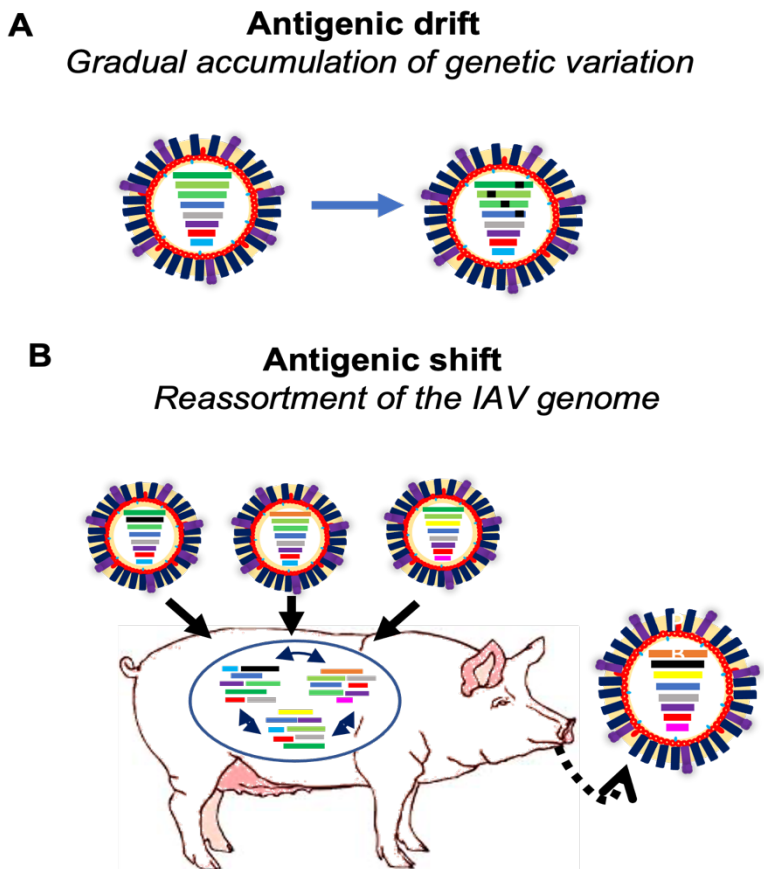


Figure 1-9: swIAV evolves two mechanisms known as antigenic shift and antigenic drift. **A)** Antigenic Shift. Genomic reassortment can occur with multiple distinct IAV strains infecting a single host cell concurrently. The 8 segments can interchange from what was in each original virus to create a novel swIAV. **B)** Antigenic drift. Without a proof-reading function, the IAV polymerase creates variation through unfaithful replication of template RNA. This leads to the gradual accumulation of genetic changes that eventually results in novel strains emerging.

1.3.3 Swine Influenza Epidemiology

Livestock species, primarily pigs and chickens, play a central role in the ecology of IAV. Birds which undertake migratory travel disseminate IAVs from their wild reservoirs and opportunities for cross species transmission arises in scenarios where wild infected birds mix with free-range chickens or other birds en route (van der Kolk, 2019). The close phylogenetic relationship of chickens and waterfowl means that there are few biological hurdles for avian-avian

zoonotic events. However, for avian adapted IAV subtypes there are greater barriers for transmission onto mammals due to the physiological differences between hosts.

Spill overs of avian origin IAV into porcine hosts occurs more frequently than into humans, but transmission between pigs is limited without mammalian adaptations (Baudon et al., 2017; Bhatt et al., 2013). Key steps to overcome include host cell entry, transport of viral RNA into the nucleus and replication of the viral genome, each of which requires adaptation to the host environment (Gabriel et al., 2008; Long et al., 2016; Moncorge et al., 2013; Resa-Infante et al., 2008; Watanabe et al., 2014). Because of IAVs error-prone RNA replication, beneficial genetic changes can be rapidly acquired in the new host if the full viral cycle is completed. The infectious ability of IAV in specific hosts is associated with the acquisition of signature amino acid substitutions following zoonotic transmission (Long et al., 2019b).

For 80 years there was a single known strain of swIAV in North America, the 1918 H1N1 strain (cH1N1). Our inability to control swIAV in a globalised world has provided the basis for further evolution and divergence into distinct clades within each subtype. H3N2 emerged in 1998 as a result of triple genome reassortment with HA, NA and PB1 from human seasonal influenza, PB2 and PA from and avian IAV and NP, M1, M2, NS1 and NEP from cH1N1. The acquisition of this triple reassortment gene cassette (TRIG) has driven further emergence of multiple strains (H1N1, H1N2 and H3N2) that are now globally endemic in pigs (Baudon et al., 2017; Torremorell et al., 2012). H3N1 has emerged most recently in Europe, Asia and the United States but has not yet been associated with large outbreaks (Lekcharoensuk et al., 2006; Moreno et al., 2009; Shin et al., 2006).

An epidemiological meta-analysis determined the global pig and herd-level seroprevalence as 49.9% and 72.8%, respectively (Baudon et al., 2017). With an average lifespan of under 2 years and a population over a billion, 500 million pigs that have been infected represents a significant IAV reservoir and turnover of susceptible hosts. Epidemics of swIAV usually circulate in a seasonal manner, with a large peak in Autumn and a smaller peak in Spring (Janke, 2013). In the USA, 48% of pigs are seropositive for at least one IAV subtype, however

only 30 – 40% of pigs are symptomatically identified as being ill (Detmer et al., 2012). This suggests subclinical infection is widespread and the impacts of IAV are likely to be under reported. Implementation of control measures are hindered by low diagnostic rates, as transmitting animals will remain in the same herd as uninfected pigs. Sustained infection is made possible by subclinical transmission to naive pigs or antigenic IAV variants evading the acquired immune response (Detmer et al., 2012).

The most recent IAV pandemic alert released by the World Health Organisation was for the eponymous 2009 H1N1 pandemic "Swine 'Flu" (pH1N1) that caused an estimated 500,000 human deaths. This outbreak brought the role of pigs in IAV ecology to the fore. Despite their role not being clearly understood, it was quickly appreciated that the human-swine interface played a major role not only in regard to the original emergence, but through continued bidirectional swine-human transmissions (Chastagner et al., 2019b). Distinct antigenic derivations emerged in North and South America, Asia and Europe as a result of divergent evolution following establishment in local swine populations (Torremorell et al., 2012; Vijaykrishna et al., 2010).

Regional divergence and reassortment events of pH1N1 exemplifies how genetic drift and selective sweeps affects the evolution of IAV (Smith et al., 2009). Recent examples of further swine-human transmission include an H3N2 strain containing the pH1N1 matrix protein (M1) that has largely been detected in attendees of agricultural fairs in the USA, and seropositivity in Chinese swine workers against a novel strain reassorted from avian-like H1N1 and pH1N1 identified (Nelson et al., 2016; Sun et al., 2020). Given that these examples are from regions with existing swIAV surveillance infrastructure, this suggests frequent cross-species transmission and where bidirectional human-swine IAV transmission has not been identified it should be considered whether it is genuinely a case of it not occurring or whether it has just not yet been detected. This level of subclinical and unrecognised infections presents a cause for concern as there cannot be controls in place for an issue that remains unrecognised.

1.3.4 Swine IAV Pathogenesis and Transmisison

Influenza resides in the respiratory tract of infected pigs, inducing characteristic symptoms of fever, loss of appetite, lethargy and nasopharyngeal issues such as nasal discharge, coughing and sneezing. Most often, but not exclusively, it manifests in young pigs as a seasonal respiratory disease. Whilst mortality rates are generally low, morbidity rates can reach up to 100% (Gramer et al., 2007; Watson et al., 2015). These symptoms cause weight loss, which significantly affects the productivity of growing pigs and the reproductive performance of breeding sows (Gumbert et al., 2020; Janke, 2013). Most animals will fully recover after 14 days of infection, with herd fatality rates of between 1% and 4% in uncomplicated cases (Janke, 2013). The impacts of endemic swIAV are a contrast to highly pathogenic avian influenza, which can have mortality rates nearing 100% in poultry (Hubbard et al., 2017).

Pathogenesis of swIAV is generally observed between 5 and 7 days following the initial infection, whilst transmission can occur within two days of infection (Janke, 2014). Virions are transmitted by the nasopharyngeal route via airborne droplets or direct physical contact. Zoonotic transmission of IAV occurs in the same manner as intra-species transmission. Swine Influenza A virions can persist in the environment for over 170 days at 4°C and for 30 days at 23°C, contributing to the increase in infection seen during winter months (Poulson et al., 2016).

Infection with swIAV is often compounded the presence of other respiratory infectious agents, such as PRRSV, coronavirus and *Mycoplasma hyopneumoniae*. Known as Porcine Respiratory Disease Complex (PRDC), it increases the burden of disease, complexity of management and risk of mortality associated with infection (Janke, 2013). The reduced productivity from swIAV infection is compounded by PRDC to an amount greater than the additive effect from individual infections (Deblanc et al., 2013; Fablet et al., 2012).

The impact to farmers of pigs afflicted with IAV is sex and age dependent. For boars, the quality of sperm is affected, reducing their fertility. Pregnant females are prone to abort litters, sows on heat are less receptive to fertilisation due to a disrupted oestrus cycle and lactating sows can struggle to

nurture their piglets through to weaning. Piglets are most commonly infected as they have a naïve immune system, and their growth and development is perturbed by the burden of disease. Specific targeting of control to piglets could represent an effective strategy for preventing swIAV establishing an endemic reservoir on farms (White et al., 2017).

1.3.5 Swine IAV Economic Impacts

The presence of viral infections are a significant influence on the profitability of a farm (Fablet et al., 2018). The primary economic impacts in finishing pigs are a result of retarded weight gain from a loss of appetite. This causes an increased time to market for finishing pigs and longer time to maturation for breeding sows. In the USA, the costs of a pig infected with swIAV has been estimated to be $3.23 USD. With PRDC co-infections included, the cost of this complex infection is greater than the additive cost of individual infections (Haden et al., 2002). The major economic concerns for hog farmers from swIAV stem from the reduced productivity that entails a longer time to slaughter and the lower number of average piglets per sows in IAV endemic herds (Donovan, 2005). A German study identified that 80% of farms with clinical presentation of swIAV had reduced reproductive performance prior to implementation of a vaccination program and found higher abortion and preweaning mortality rates that result in the average of piglets weaned per sow annually being reduced by more than one, a factor that will seriously impact the economic performance of affected farms (Gumbert et al., 2020).

The economic impacts of swIAV are not restricted to those felt by farmers. If spill over into humans occurs, as was observed in 2009, impacts are far reaching and cross-societal (Smith et al., 2019). As well as direct effects to humans such as medical care and indirect effects such as time off work, a swine influenza pandemic also presents a major threat to global trade. Public misconceptions about the safety of eating pork and concerns of sustained swine to human transmission caused losses to the US pork industry estimated to be over $1 billion USD (Pappaioanou and Gramer, 2010). The Mexican economy is estimated to have suffered financial impacts of >$3.2 USD billion as a result of

culling pigs to manage disease transmission, a ban on the importation of pork products by countries including China and Russia and consumer abstention, despite IAV transmission from processed meat products being unfounded (Attavanich et al., 2011). Further to these losses there was a $2.8 USD billion loss to the Mexican economy from slowing in the tourism trade (Rassy and Smith, 2013). These examples demonstrate the fragility of consumer pork demand in the instance of a swIAV zoonotic transmission and provides a warning of the potential damage to the pork industry from indirect costs. Especially given that the reversal of the Chinese import ban conspicuously coincided with a severe Porcine Reproductive and Respiratory (PRRS) Virus outbreak in China, with the import of North American pork products being necessitated to quell local demand.

With pig farming becoming more industrialised to meet consumer demands for pork, the higher density of pigs is likely to increase swIAV prevalence if left uncontrolled (Baudon et al., 2017). In Brazil, Almeida *et al*., 2017 detected almost 25% swIAV prevalence in intensive farming systems, and no swIAV in their extensive farms investigated. The extensive farms were selected by having a total lack of biosecurity measures (Almeida et al., 2017). Close and regular interactions between swIAV endemic pigs and humans creates an environment that could lead to the emergence of novel strains through bi-directional transmission and subsequent reassortment events (Chastagner et al., 2019a; Fragaszy et al., 2016; Mine et al., 2019). Furthermore, the limited but present international trade of pigs and global movement of people exacerbates the potential for co-infection with multiple distinct IAV strains (Lycett et al., 2012; Trovão and Nelson, 2020).

In this introduction we consider current and prospective control strategies that aim to reduce the prevalence of swIAV across all pig farming systems in the face of the increasing threat posed to animal welfare and productivity from swIAV (Figure 1-10). Each farming system will have differing practicalities and cost/benefits prospects for each method, and therefore it is only suggested that farms implement the maximum that is possible in an effective way within their system to control swIAV transmission.

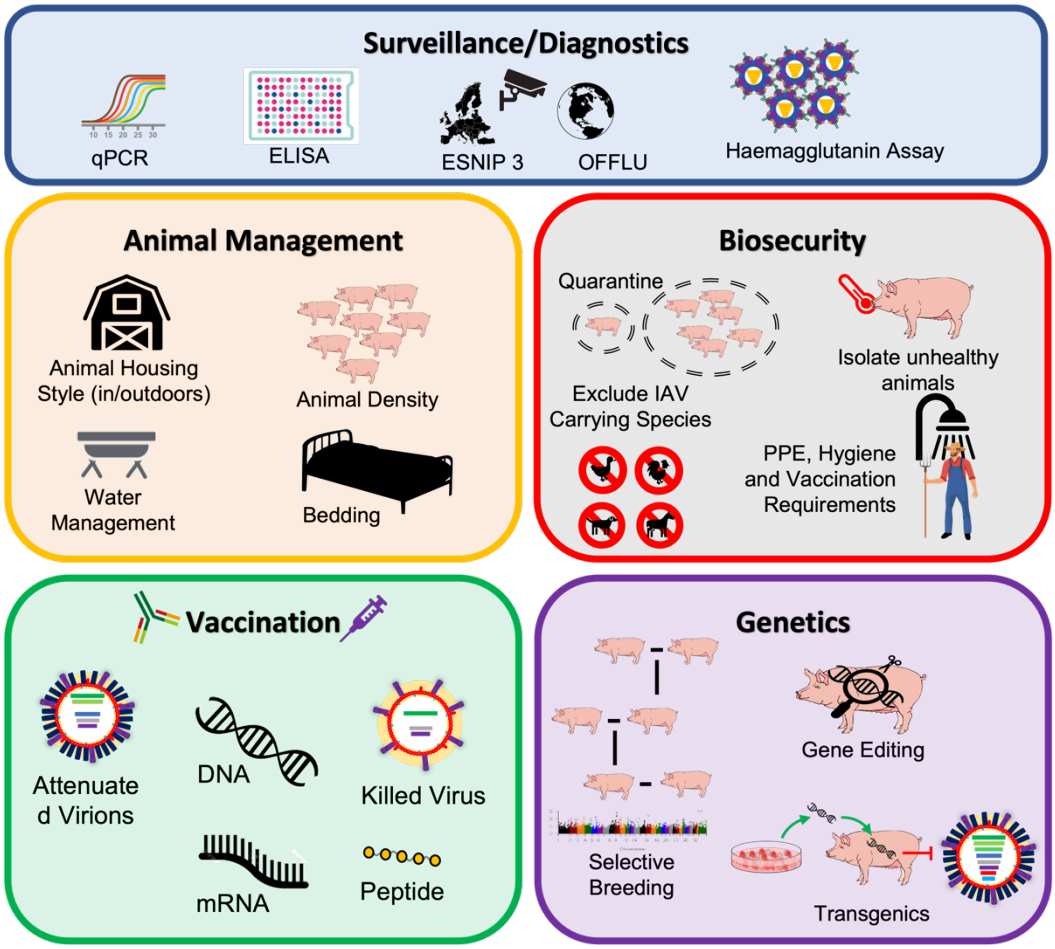


Figure 1-10: Current and prospective control strategies for Influenza A Virus in swine.

1.3.6 Control of Swine IAV in Pigs

Because of the lag between symptom presentation and transmission, once infection is established on farm it can be difficult to remove. Biosecurity measures such as quarantine of new arrivals, segregation of weaners and good hygiene practice can reduce the spread. IAV in the environment can be inactivated by temperature, low pH and chemical disinfectants (Poulson et al., 2016). Drugs for medicinal intervention are available for human use, however because IAV is highly mutable, resistance to drugs on the human market is increasing and there is limited appetite for their use in agricultural species (Neumann et al., 2009; Watanabe et al., 2014). These steps are effective as

responsive containment measures, however they do not effectively control the transmission.

Pre-emptive control measures such as vaccination are better suited to transmission prevention, but because of the low mortality rates resulting from IAV infection vaccination is not financially incentivised enough for farmers to justify its implementation at scale. This is particularly true of vaccine programs in developing regions where storage, access and financial restrictions limit their usage. Swine vaccinations are annually developed for the endemic H1 and H3 strains in a predictive manner with the intention of anticipating the major strains circulating (Buckland, 2015). Due to the species-jumping ability of IAV, control and surveillance of IAV must take into account human-pig and other environmental interactions which carry the risk of IAV transmission (Gray and Kayali, 2009). With their status as a 'mixing vessel', pigs could operate as an effective node of IAV control for both pig and human health. With difficulties associated with the aforementioned control strategies, the use of genetic technologies and genomic knowledge to complement current measures could be an important component in helping to reduce the potential of an IAV pandemic outbreak originating from hogs.

1.4 Current Control Measures

To control the spread of swIAV, the number of onwards infections must be reduced to be below 1 per infected animal. The number of onwards infections per animal in an entirely susceptible population is known as an R_0 value. If it is below 1 the pathogen will subside in a population. Control strategies can be reactive, which aim to clear a virus infection after clinical presentation or detection, or proactive, which aims to prevent a pathogen becoming established. With swIAV having an R_0 value of 10.66 it will rapidly spread in if no control programs are implemented (Romagosa et al., 2011). Effective control is vindicated through improved future economic performance on swIAV free farms because of better productivity efficiency and reduced veterinary costs.

From a human health perspective, the reduction in swIAV prevalence will lower the potential for the emergence of a pandemic, which have historically had huge societal and economic impacts. Controlling IAV in swine should be approached with 'One Health' considerations due to its significant role in humans, livestock, and the environment (wild species). As a quintessential zoonotic pathogen, successful control in one species will have knock-on effects throughout the swIAV ecological web. All control measures discussed here refer to their application in farmed pigs, as although wild pigs are infected (Baker et al., 2011; Hall et al., 2008; Kaden et al., 2008) they cannot be managed in the same manner and the low prevalence and minimal interactions with humans or pig farms means they present a low risk. Reduced swIAV prevalence may also benefit by less erroneous use of antibiotics due, which have incidentally increased along with adoption of industrial farming practices (Lekagul et al., 2019).

1.4.1 Animal Management

The most basic swIAV control strategy is through evidence-based animal husbandry methods that will concomitantly benefit animal welfare standards. Animal management control measures are most effective when applied pre-emptively to prevent swIAV establishment rather than retrospectively to clear an endemic outbreak (Chamba Pardo et al., 2018; Mastin et al., 2011). With high levels of subclinical presentation and variable results from medical interventions, once an outbreak has begun it can be difficult to intervene.

In favourable conditions, such as in cold water or with cold temperatures on a hard surface, IAV can remain infectious outside a host for beyond a year (Dublineau et al., 2011; Poulson et al., 2016). The increased persistence in water is particularly relevant as Mastin et al., 2011 observed reduced odds ratios for swIAV in pigs where there were less than 18 finishers for each water access. The main route of transmission postulated is via droplets (Brookes et al., 2010; Lange et al., 2009; Tellier, 2006) during physical contact with another pig or from contaminated surfaces, so it is expected that increasing the incidences of nasal contact and the sharing of mucus will assist the perpetuation of swIAV. As

follows, the size and density of pig herds is a risk factor for increased prevalence (Ewald et al., 1994; Poljak et al., 2008; Suriya et al., 2008). Furthermore, overcrowding creates stressful conditions that can lead to a depression of the immune response that increases the susceptibility and severity of swIAV across a herd (Fablet et al., 2013). The type of farm and housing system also plays a role in swIAV epidemiology. Measures such as using straw for bedding as opposed to having a slat floor system have been associated with a lower seroprevalence (Ewald et al., 1994; Mastin et al., 2011), whilst indoor housing (Mastin et al., 2011) and open partitions between pens in barns (Simon-Grifé et al., 2011) have been associated with an increase. With indoor housing, slat floor systems and high-density herds increasingly common, these factors may contribute to the increasing swIAV prevalence observed on pig farms.

Effective animal management includes collecting data on husbandry and animal movements for use in supporting effective trace and isolate protocols as required. If an outbreak occurs, movement restrictions can then be applied quickly, but only if appropriate information is available to make an informed decision. Regarding movements, fallow periods for pens are recommended but are not realistic to apply in economically optimised systems. More feasible is sanitation of pens between groups with disinfectants that inactivate viruses to ensure transmission does not happen between groups on arrival in a new pen. Disinfecting pens further benefits by killing other pathogens of PRDC (Maes et al., 2000). In a worst-case scenario, the culling of entire herds and having a fallow period to clean farms is a viable method of animal management to eradicate disease, however the repercussions of eradicating pigs could not be more detrimental financially or emotionally to farmers. These drastic measures were taken in response to pH1N1 in only Norway and Egypt, primarily as preventative measures for swine-human transmission, however it was not considered widely effective as human-human transmission was significantly the main source of infection (Keenliside, 2012). In 2009 the World Health Organisation (WHO) announced that trade restrictions on pigs or pork products were unnecessary, but reflecting this decision with our current knowledge that bidirectional transfer can occur, the continued movement of pigs which

disseminated the virus between pigs (Chastagner et al., 2019b; Nelson et al., 2015) may also have posed a limited but present threat to humans who had close contact with swine farms.

1.4.2 Biosecurity

As well as on-site management of animals, swIAV control must account for how multi-site pig systems require regular movement of pigs between stratified breeding farms through to finishing farms. Intuitively, movement of an infected animal into a naïve herd is a primary source of infection, and so once pathogen free status is attained the aim is to prevent reimportation. To reduce the likelihood of swIAV establishing in a naïve population from new arrivals, quarantining before mixing with the original herds is recommended since the presentation of clinical symptoms occurs subsequently to the peak of time of viral shedding (Krueger and Gray, 2012). Given that many animals are asymptomatic carriers that present subclinical infections (Heinen et al., 2002; Janke, 2013; Yoo et al., 2018), a lack of visible symptoms should not be considered definitive confirmation of no infection and excuse of quarantine before clearance for herd integration.

All-in-all-out systems would reduce the risk of new arrivals becoming infected; however, the logistical complexity and dynamism of pig breeding does not lend itself to this being practical. If the breeding farms pathogen status is known, receiving farms can appropriately have either confidence or mitigation strategies in place. Given the time and cost of using the available diagnostic tests, and the potential for subsequent infection between testing and movement, quarantine is the most effective blanket measurement to take for new arrivals.

Observations that higher replacement rates are associated with higher seroprevalences of swIAV outlines the potential risk of farming with high replacement rates that are inherently necessary in multi-site farming systems (Simon-Grifé et al., 2011; Suriya et al., 2008). However, the impact of replacement is not well defined, as data from swine belt states in the USA observed no reduction of endemic swIAV prevalence associated with the closure

of breed to weaning herds, suggesting further work is needed to understand whether inter-herd transmission or within herd transmission is the main driver infectious cycles (Chamba Pardo et al., 2018).

The inherent movement requirements of multi-site production systems mean the entire system is more efficient with higher localised farm densities to reduce transport costs and permit hubs of abattoirs and feed production. Although beneficial for ease of coordination and economic efficiency, proximity to other hog farms and density of pig farms has been positively associated with increased swIAV seroprevalence (Poljak et al., 2008; Suriya et al., 2008). An unseen risk of farm density is present in barn exhaust air that has purportedly been detected to still contain swIAV over 1 mile away (Torremorell et al., 2012). With multi-site pig production systems expected to become increasingly popular, these conditions that are more permissive to swIAV transmission will lead to a higher prevalence (Baudon et al., 2017). Beyond regional boundaries, international transport of live pigs encourages the global dissemination of swIAV (Nelson et al., 2015), with North American and European swIAV lineages now circulating in China and Africa (Fan et al., 2012; Nelson et al., 2011; Vijaykrishna et al., 2011).

Biosecurity control strategies should not be limited in their focus to pigs. Workers on swine farms are regularly handling pigs and there is therefore potential for transmission of IAV between pigs and humans (Chastagner et al., 2019b, 2019a; Gray and Kayali, 2009). If an employee or pig is infected with an endemic strain to their species and then a zoonotically transmitted strain, genomic reassortment can occur. Restricting entry onto pig farms of only essential people (*e.g* vets, employees, suppliers), ensuring all employees and their families are vaccinated, and implementing good hygiene across all farm practices will help reduce the risk of bidirectional transmission and the emergence of a novel strain by zoonoses (Ramirez et al., 2006). Good hygiene practices include wearing Personal Protective Equipment (PPE) and strictly enforcing ill employees not to attend farms. A lack of biosecurity measures can be seen to have an effect as agricultural fairs in the USA are an identified hotspot for swine-human transmission (Baudon et al., 2017; Nelson et al., 2016;

Watson et al., 2015). The close contact required for showing pigs, minimal attempts at mitigation compared to the farm setting and the mixing of pigs from distinct origins creates the ideal opportunity for IAV strain admixture and bidirectional transfer.

Biosecurity protocols should also consider the threat of IAV infections from beyond humans and pigs. There have been instances of what were thought to be swIAV free herds in well ventilated and secure barns detecting avian influenza strains (Brown et al., 2007; Karasin et al., 2004, 2000). It was noted following detection of avian IAV strains in these herds that they use surface water from nearby ponds to clean out the pens between replacing stock. As the surface water was used by ducks and other waterfowl species that are known to excrete IAV in their faecal matter, it is plausible the water acted as the vector for IAV transmission (Dublineau et al., 2011; Poulson et al., 2016). The biosecurity hazard of pooling water near-by, especially when used as a resource for cleaning pens exemplifies that anything entering a pig farm could be considered a risk for swIAV introduction.

For small scale holders the practical biosecurity measures differ from indoor systems. Although indoor housing systems have been observed to assist in harbouring endemic swIAV (Baudon et al., 2017), on outdoor farms there is a scenario of not being able to exclude other IAV susceptible animals such as birds, cats and mustelids which creates a cauldron for IAV mixing from multiple hosts. An example of modifying nearby habitats to reduce wildlife vector interactions with livestock is from Myanmar where there are restrictions on tree planting surrounding pigsties to remove flying fox habitats from proximity to reduce the potential for bats transmitting Nipah Virus to pigs (Pulliam et al., 2012). Regardless of the farming system, knowing where breeders come from, quarantining new arrivals, receiving information of the previous owners swIAV management protocols and maintaining good hygiene practice and excluding wildlife intrusions where possible will contribute to reducing the opportunity for swIAV transmissions and it becoming endemic.

1.4.3 Surveillance

Because of the low mortality, high morbidity and generally self-limiting nature of swIAV infections, it is not considered as a notifiable disease by the World Organisation for Animal Health (OIE) or the United States Department of Agriculture (USDA) (Archive, n.d.; "PANDEMIC H1N1 2009 Questions and Answers: OIE - World Organisation for Animal Health," n.d.). However, surveillance data optionally collated does help to understand the epidemiology and evolution of swIAV and can be applied to inform policy decisions. Because of its diversity and intrinsic mutability, knowledge of the prevalent genetic and antigenic landscape enhances the effectiveness of control measures in place. The more we understand about swIAV, the more targeted control measures can be. Up to date information on swIAV's distribution and prevalence also plays an important role in ensuring that current vaccines remain relevant to the prevailing endemic strains (Detmer et al., 2012).

Although knowledge acquired from surveillance does not directly control swIAV, the timely sharing of virology and epidemiology data to identify infection hotspots and transmission events within and between herds/species, allows us to gain insight into where novel strains are likely to emerge and respond to prevent further transmission and establishment in a population (Detmer et al., 2012; Watson et al., 2015). Prior to 2009, swIAV was present but under recognised beyond Europe and the USA, as seen by 34% of samples from a Chilean study of respiratory infected pigs being confirmed to be infected retrospectively (Mena et al., 2021). If better surveillance had been in place for H1N1 detection, it may have been less likely to emerge into humans as pH1N1.

Because of this the USDA established a national swIAV surveillance program after 2009 (Anderson et al., 2013) and Europe followed suit with the European Surveillance Network for Influenza in Pigs (ESNIP) (Simon et al., 2014). Further supporting surveillance is the establishment of OFFLU (www.offlu.net), a collaborative effort of experts on animal influenza aiming to promote animal influenza research and data sharing that is supported by the OIE and Food and Agriculture Organisation of the United Nations (FAO) (Rajão and Pérez, 2018). Given that the dynamics of swIAV infections have been reported to be both

cyclical as in humans (Chamba Pardo et al., 2018, 2017), and persistent throughout the year (Kyriakis et al., 2013), we should be aware that swIAV epidemiology is not necessarily directly translatable from human research.

Despite the overt swine health and swIAV control benefits derived from mass surveillance, efforts are sometimes impeded by concerns that public knowledge of the distribution or identification as a swIAV hotspot would reduce consumer appetite for pork products and damage the pig industry's reputation. Concerns are also raised that even with the knowledge there is no cheap and genuinely reliable treatment and individual farms do not directly feel the benefit. If the surveillance data is held privately because of these concerns, it is only useful for understanding swIAV in a particular herd. Collaborative efforts will contribute much more to the efforts of the pig farming community to control swIAV.

Surveillance data can be from simple diagnostics such as clinicopathology and post-mortem assessment of a carcass, however, these methods are limited by not providing definitive detection of IAV (Detmer et al., 2012). For more reliable results, molecular diagnostic tools can assess viral antigens, nucleic acids or host antibodies that bind swIAV. Quantification of swIAV RNA by Reverse Transcriptase quantitative PCR (RT-qPCR) from a nasal swab measures the current viral load. Using conserved primers the RT-qPCR assay can accurately and relatively rapidly detect a broad range of swIAV subtypes (Detmer et al., 2012). Downstream sequence analysis of amplified viral RNA can also provide insights into the evolution of swIAVs detected and can easily identify genomic reassortment if the full genome is sequenced (Diaz et al., 2017; Lycett et al., 2012). The falling costs and increasing ease of nucleic acid sequencing makes it increasingly attractive as a detection method as it has broader benefits of providing insights into the evolution and epidemiology of swIAVs as well as the capacity to identify genomic reassortment events (Chauhan and Gordon, 2022). Current infections can also be detected with non-nucleic acid assays such as an enzyme-linked immunosorbent assay (ELISA) or during post-mortem by immunostaining fixed tissues for swIAV antigens (Hurt et al., 2007).

ELISA can also be utilised to retrospectively assess whether pigs have recently been infected with swIAV (Detmer et al., 2012; Hurt et al., 2007). Antibodies circulating in blood plasma can be detected from 1-2 weeks post infection and peak after 4-7 weeks (Van Reeth et al., 2006, 2004). Other retrospective swIAV tests such as the haemagglutination assay and serum neutralisation assays are not available commercially and therefore less relevant for widespread surveillance but have been demonstrated to be effective at distinguishing endemic swIAV strains (Van Reeth et al., 2006).

A major limitation in testing for serum antibodies to swIAV compared to RT-qPCR is that vaccination stimulates an antibody response and a vaccinated animal cannot be discriminated from a naturally infected animal (Detmer et al., 2012). Current surveillance measures are also limited because commercially available diagnostic tests are presently only available for specific H1N1 and H3N2 subtypes (Detmer et al., 2012).

1.4.4 Medical Strategies

The application of medical strategies to reduce the prevalence of swIAV is a complementary tool to the traditional strategies and should not be considered a substitute. The epidemiological data from surveillance must be considered to assist the targeted application of medical strategies. Prophylactic treatment for swIAV is performed by individual farms to remove endemic swIAV and thereby improve the productivity of their pigs, however if applied effectively, its broader benefits will contribute to a reduction in the likelihood of transmission between farms, as well as the potential for reassortment and zoonotic transmission (Corzo et al., 2012; Richt et al., 2006; Romagosa et al., 2011).

1.4.5 Vaccination

The principal prophylactic strategy for controlling swIAV is vaccination. As demand for pork products increases, pig production has intensified, and with this the uptake of vaccination to reduce swIAV has as well. After the 2009 Swine ‘Flu outbreak vaccine uptake improved as pig farmers moved to negate the threat of another zoonotic event occurring that had outlined the fragility of

consumer confidence in pork consumption and the economic impacts of a swine origin IAV outbreak (Pappaioanou and Gramer, 2010; Verikios et al., 2011). Viral shedding and disease is significantly reduced in vaccinated animals, but transmission from vaccinated animals that are directly infected to naïve pigs will still occur, meaning blanket vaccinations and good record keeping is required for it to be a fully effective strategy (Everett et al., 2021).

The ability of swIAV to evolve by antigenic shift and antigenic drift complicates the creation and vaccine design process, making it essential that effective surveillance is in place to ensure optimal vaccine design. Vaccination has been observed to reduce the reproduction ratio (the number of secondary infections caused by an infected animal) in naïve pigs from 10.66 to below 1, making it an effective strategy for swIAV control (Romagosa et al., 2011). Vaccination for swIAV in swine principally works by inducing the production of virus-specific antibodies via a humoral adaptive immune response which has two mechanisms of function; through antibodies circulating in the host serum that neutralise/opsonise infectious swIAVs and through priming the host immune system to clonally produce antibodies following the detection of the epitope (Rahn et al., 2015). All licensed vaccines that are currently commercially available are inactivated whole viruses that target the major porcine endemic strains H1N1, H1N2 and H3N2 in various combinations prepared with oil-adjuvants (Sandbulte et al., 2015). Because Europe and North America swIAV differ significantly in genetic and antigenic composition, even of the same HA and NA subtype/nomenclature, they must be designed for the prevalent regional strains (Sandbulte et al., 2015).

The major challenge to effective Influenza vaccination is swIAV's complex ecology and incessantly mutable genetic and antigenic composition (Carrat and Flahault, 2007). The more closely matched the vaccine and infectious strain are the better the immune response will be. Antibodies will only bind to closely matched target epitopes, thus if a mismatch between the vaccine strains and the infecting swIAV strain is present, serum antibodies will fail in neutralising the infectious virions (Everett et al., 2019). HA is by far the most abundant viral protein, comprising about 80% of IAV membrane proteins

(Samji, 2009) and is highly accessible to antibody binding as a protruding protein. Because of this, the modus operandi associated with whole inactivated swIAV vaccine mounted responses is to produce antibodies that target the exposed head region of the HA protein (Kitikoon et al., 2006; Lee et al., 2007; Loving et al., 2012). Antibody binding with HA interferes with its ability to bind to the host receptor SA, preventing endocytic uptake and therefore viral infection and transmission. However, as the exposed head region of HA is heterogenous (25% genetic distinction within subtypes) and has a high mutation rate, positive selection of swIAV that do not have an HA match with an antibody promotes evolution of swIAV virions that circumvent the vaccine induced immune response (Morris et al., 2020). This evolution is a major cause of low vaccine efficacy rates and reason why vaccines must continually be updated and modified to match circulating strains. If antibodies target a more conserved region, such as the stem region of HA they will have activity against a broader range of swIAV and assist in counteracting the circumvention of host immune systems by swIAV evolution (Steel et al., 2010). Antibodies have been identified in humans that cross react with the HA stalk region of pH1N1 and also divergent H1N1 and H5N1 influenza strains, suggesting that an effective vaccine could mimic this response in humans at least (Wrammert et al., 2011).

Nucleoprotein (NP) and M2 have been considered for vaccine efficacy, however the smaller amount of protein and their localisation make them difficult to effectively target using whole inactivated virus technology (Gao et al., 2013; Vander Veen et al., 2013). The ultimate goal for Influenza control is for a universal vaccine with reactivity against all strains, however even for humans this is not on the immediate horizon (Sautto et al., 2018).

Using different strains of swIAVs for subsequent vaccination has been observed to protect against both strains better than concurrent bivalent administration (Van Reeth et al., 2017), however the complexity of distributing specific vaccines strains to different farms/regions for use at specific times makes heterologous vaccination programs administratively difficult and so has led to the less effective but simpler bivalent vaccines becoming ubiquitous. Although swIAV antibodies wane annually and if present will not always match

subsequent strains due to HA evolution, there are suggestions that swIAV specific B lymphocytes might be maintained over a pig's life (Sandbulte et al., 2015). In humans, people vaccinated with the 1976 swine-origin H1N1 strain showed a slightly enhanced neutralisation response to the 2009 H1N1 pandemic, albeit in a small cohort (Van Reeth et al., 2017).

The other major complication for vaccine efficacy is the presence of maternal derived swIAV antibodies (MDAs) in piglets. Typically, 2 doses of vaccine are given intramuscularly to gestating sows prior to farrowing. Although no swIAV specific antibodies transfer through the placenta, maternal antibodies in colostrum confers immunity in neonates and with passive transfer from sows' milk immunity is usually maintained until around 14 - 16 weeks old (Loeffen et al., 2003; Markowska-Daniel et al., 2011). As neonates do not have a well enough developed immune system to respond well to vaccination this is the most effective way at preventing new-born piglets of becoming a large reservoir for swIAV. As the piglets' MDA titre declines, susceptible piglets become a reservoir for swIAV, transmitting to other pigs in the herd as their own antibodies wane or the virus evolves (White et al., 2017). Piglets in comprehensive vaccination programs, are vaccinated at weaning, however the biological variation in reducing antibody titres means that under this system a proportion of piglets will either not have a well enough developed immune system to respond to the vaccine or have no MDAs remaining well before vaccination, meaning a naïve reservoir is likely to remain.

The presence of MDAs in piglets presents a conundrum to the farmers. Kitikoon et al., 2006 observed that piglets in their study which had MDAs also had increased infection rates and prolonged presence of clinical symptoms as a result of suppressed serum antibodies and T-cell response compared to piglets without MDAs (Kitikoon et al., 2006). An impaired humoral immune response has also been identified after vaccination of piglets against Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) in the presence of MDAs (Fablet et al., 2016).

Furthermore, vaccine-associated enhanced respiratory disease (VAERD) has been experimentally observed whereby more severe disease is caused if the

infecting virus does not match the inactived swIAV strains contained in the vaccine due to an obfuscated immune response (Gauger, 2012). As the mechanism for VAERD is not well-understood and it has only been observed under experimental conditions, the consideration it should be given when considering on-farm swIAV vaccination strategy is contested and dependent on the vaccine type (Holzer et al., 2019; Rajao et al., 2014). There is a desire to move away from whole inactivated vaccines due to their need for annual updating, inconsistent efficacy in the field and potential VAERD.

An alternative vaccine strategy that is increasingly popular in US commercial settings are autogenous vaccinations, inactivated swIAV isolated from strains endemic to the herd (Corzo et al., 2012; Sandbulte et al., 2015). The major drawback to autogenous vaccines is that they are created from presently circulating strains, and pigs are therefore likely to be immunologically naïve to any new swIAV introductions. However, as commercial vaccines often have low efficacy rates, up to date veterinary knowledge of local swIAV epidemiology can mean autogenous vaccines offer an improved solution to the endemic strains over the broad based commercial vaccines (Kitikoon et al., 2013). In a longitudinal study from the USA, there was no significant difference in the reduction of swIAV in herds using either commercial or autogenous vaccinations in sows, however as the autogenous vaccine strains used were not genetically identified, mismatches between the vaccine strain and circulating strains may have reduced the effectiveness of the autogenous vaccination programs (Chamba Pardo et al., 2020). This underlines why it is important to have a robust swIAV surveillance program prior to vaccination to target the right strains and also post vaccination to evaluate if it has been successful and whether the vaccination program needs to be improved.

Vaccination programs in pigs are specific to porcine endemic strains, and therefore will not prevent avian or (most) human IAV strains from infecting pigs. Immunising swine farm employees is important to prevent human to swine transmissions, rather than preventing the zoonotic transmission to humans (Chastagner et al., 2019b; Gray and Kayali, 2009; Myers et al., 2007). Reducing the potential human-pig transmission diminishes the risk of a swine endemic

strain genomically reassorting with the human strain and transmitting back to humans. This was observed in 1998 events when swine viruses acquired human genes, ultimately leading to the emergence of novel swine viruses (Anderson et al., 2013; Watson et al., 2015). Vaccination of pigs against the classical H1N1 swIAV strain did not prevent the emergence of pH1N1, and subsequent transmission between humans and pigs has been identified multiple times (Anderson et al., 2013; Chastagner et al., 2019b; Nelson et al., 2016, 2015). The threat of a swine-origin IAV pandemic emerging from reassorted strains is epitomised with the repeated appearance of Swine 'Flu outbreaks ("Search ProMED Posts – ProMED-mail," 2020). With the occurrence of antigenic divergence commercial vaccines need to be updated to remain effective against the novel strain. Given that the commercial vaccines are most effective as trivalent concoctions, the addition of another strain would threaten their functionality (Sandbulte et al., 2015).

Considering that effective vaccination programs require surveillance to be implemented fully effectively, have a cost of labour with two intramuscularly administered doses 3 weeks apart and are likely to entail veterinary consultation, the development of vaccines with broader heterospecific activity and efficacy are essential to improving farmer uptake. Vaccination of piglets at weaning is further complicated by the movement of pigs between breeding and finishing farms, whereby swIAV resistance in piglets does not directly benefit breeding farms as they leave soon after weaning. This results in a dichotomy in the incentives for vaccination between breeders and growers in that the benefit is mostly received by the grower of weaned piglets, but the breeder would ideally vaccinate piglets to reduce the likelihood of swIAV being transported between farms. If the responsibility of vaccination falls on the growing farms, some piglets are likely to already be infected on arrival and will act as a fresh source of swIAV. Because of this there will always be susceptible pigs in the production chain.

For vaccines to be adopted by farmers they need to be cost effective or incentivised in an alternative means to ensure the broader benefits of controlling IAV ecology are realised. It is notable that in countries that are

endemically infected with highly pathogenic avian influenza there are blanket vaccination programs against specific avian IAV strains across complex production systems that could be helpful when considering a framework of how to implement blanket swIAV programs (Domenech et al., 2009; Spackman and Pantin-Jackwood, 2014).

1.5 Pipeline Control Measures

The control measures described above work best when applied with coordinated discretion. Likewise, novel strategies for IAV control will operate most effectively when integrated alongside traditional control strategies that are prescribed and implemented in an accurate manner. If infection is prevented, transmission is prevented, hence IAV control is most effective when applied prophylactically. Reactive measures can only be administered when symptomatic signs are seen in an animal or at the herd level, and by that stage of the infective cycle viral shedding has commenced, spreading to naïve hosts who will continue the infective cycle. For a virus that has high rates of subclinical infections the optimal result is to entirely prevent infection, thereby removing a reservoir for the emergence of a human pandemic.

1.5.1 Novel Immunostimulant Strategies

As an alternative to vaccination, novel swIAV control strategies being developed are focussed on improving the host immune response through enhanced adjuvants to improve delivery efficacy or alternative vaccine mechanisms that will prime or induce the host immune response (Rahn et al., 2015; Rajão and Pérez, 2018). Innovative immune stimulation strategies include novel swIAV vaccine strategies aiming to induce cell-mediated immunity (CMI; T lymphocytes) alongside the antibody mediated response as well as innate immunostimulation therapies. Much of the research into swIAV vaccines piggy backs research into human strategies, in particular research for a universal vaccine that would target all IAV strains. For a full review on novel IAV control strategies in humans see Wei et al. (2020) (Wei et al., 2020).

Live vaccines are effective, however because they carry a risk of genome reassortment with coinciding infectious swIAV they are not appropriate for commercial use. Richt et al., 2006 demonstrated their effectiveness with a modified live-influenza vaccine based on an H3N2 containing a Non-Structural 1 (NS1) gene expressing a truncated protein. The virus is greatly attenuated in pigs as a result of not being able to suppress the innate immune response without a functional NS1 (Solórzano et al., 2005). Inoculation of pigs with the virus led to complete protection against H3N2 strains, however only partial protection against H1N1 strains, further demonstrating the difficulties faced by swIAV heterogeneity.

The commercially available and autogenous vaccines used in the pig industry are inactivated swIAVs and do not typically stimulate extensive CMI, which is recognised as being more effective against heterologous IAV infections (Balz et al., 2020). DNA vaccines circumvent these hurdles as they stimulate both CMI and antibody mediated responses and can be polyvalent in nature by expressing genes of multiple swIAV strains (Borggren et al., 2016; Gorres et al., 2011; Olsen, 2000; Ulmer et al., 1998). Furthermore, they are comparatively easy to manufacture and DNA can be readily substituted to evolve with swIAV heterogeneity (Borggren et al., 2016). Experimental results from Karlsson et al. (2018) (Karlsson et al., 2018) provides continued promise of realistic DNA doses and intradermal delivery strategies transitioning DNA vaccines into a commercial setting. Administering mRNA that encodes specific antigens of interest has also been demonstrated in pigs to elicit a humoral and cellular immune response (Petsch et al., 2012). Another method of directing nucleic acids to epithelial cells of the lung is through viral vectors such as recombinant adenoviruses expressing swIAV antigens (Wesley et al., 2004). It is not known whether these vaccine administration methods will prevent the issue of VAERD but from the smaller samples in research settings it is conspicuously unidentified.

Nucleic acids may not be restricted to being vaccines for therapeutic application. Co-administration of interleukin-6 DNA was observed to enhance anti-IAV activity in mice (Olsen, 2000). It has been observed that SUMOylation

(Small Ubiquitin-like Modifier) changes at the genomic level occur following IAV infection (Domingues et al., 2015; Pal et al., 2011). Schmidt et al. (2020), identified that endogenous retroviral (ERV) expression is enhanced by specific SUMOylation changes, and ERVs that are usually epigenetically silenced are de-repressed with IAV infection following epigenetic reprogramming (Schmidt et al., 2019). Expression of viral dsDNA, albeit endogenous, induces a heightened immune response and is therefore postulated to assist in clearing viral infections. Direct administration of dsDNA to induce the same effect could therefore be a plausible IAV therapy.

Concentrated and timely delivery of therapeutics to lung epithelial cells using improved adjuvants will improve their efficacy. Currently, oil based adjuvants are used with commercial vaccines to improve their immunogenicity (Rajão and Pérez, 2018). Intranasal administration of immunogenic antigens with a porcine lung surfactant and Poly I:C with inactivated virions have separately been observed as effective in a research setting limited to H1N1 inactivated virions (Thomas et al., 2015; Vinson et al., 2019). Intranasal delivery is further supported as a simple and effective administration technique using nanoparticles as a delivery vehicle for M2 antigens (Hiremath et al., 2016). Here, pigs developed immunity to H1N1 through CMI without any detectable antibody response observed. This suggests that combination therapies specifically targeting CMI alongside humoral immunity could be effective.

The adoption and combination of new technologies alongside novel administration methods that reduce the skilled labour and costs required for vaccination will go a long way to improving the uptake from producers and assist in improving control of swIAV.

1.5.2 Genetic Technologies

Genetic technologies offer the prospect of broad acting, permanent and heritable resistance to swIAV. Where vaccines are too expensive and there is poor distribution infrastructure the possibility of resistance by selective breeding could be particularly appealing. However, there are no genetic markers currently identified in pigs that could be selected for via traditional

breeding programs. Genetic polymorphisms that confer phenotypic resistance to swIAV must therefore be introduced to breeding animals to provide the basis for disseminating resistance by breeding. To reduce the likelihood of mutation escape from genetic resistance in the porcine host, using multiple genetic methods for resistance is recommended to increase the barriers to escape. Here, the discussion of genetic technologies is restricted to relevant viral resistance examples in pigs and how they relate to prospective swIAV resistance.

1.5.3 Transgenics

Transgenic pigs have been created using various genome engineering technologies to develop *in vitro* and *in vivo* porcine models for swIAV resistance. Here, a transgenic animal is defined as one containing DNA not native to that species. Type I Interferon (IFN-I) is an important mediator of the innate immune response to viral infections, and Mx1 (mice and pigs, MxA in humans) is an integral downstream effector protein of the IFN-I antiviral response (Le Bon and Tough, 2002). Allelic variants of Mx1 confer variable susceptibility to IAV in pigs (Nakajima et al., 2007; Palm et al., 2007). Fibroblasts isolated from transgenic pigs generated by Somatic Cell Nuclear Transfer (SCNT) containing multiple copies of porcine Mx1 cDNA were observed to have a 10-fold reduction in IAV titres compared to wildtype controls (Yan et al., 2014). IFN-induced Transmembrane Proteins (IFITM) are virus restriction factors stimulated by the innate immune response that inhibit cellular entry of several viral pathogens (Brass et al., 2009). Constitutive overexpression of porcine IFITMs in a pig tracheal cell line was observed to reduce IAV infection by Lanz et al. (2015), whilst reduced expression of endogenous IFITMs led to an increase in viral titre. These findings were corroborated Benfield et al. (2015), who reciprocated these findings specifically for IFITM3 (Lanz et al., 2015).

In the fight against PRRSV, Histone deacetylase 6 (HDAC6) has been identified as having anti-viral properties (Husain and Cheung, 2014; D. Wang et al., 2015). Lu et al. (2017) (Lu et al., 2017) created transgenic pigs overexpressing porcine HDAC6 and found PRRSV gene expression was reduced

and virus production impeded. These examples of transgenic animals are important in improving our understanding of how we can fight viral infections and the knowledge on these proteins may have applications related to therapeutic drugs. Because constitutively inducing an anti-viral innate immune response is likely to have unintended biological effects on the systemic health of the organism these transgenic strategies are unlikely to ever be realised commercially for ethical reasons.

1.5.4 RNA interference (RNAi)

A transgenic strategy more plausible in gaining regulatory approval would have specific antiviral activity as opposed to inducing a systemic immune response. Expression of RNA interference (RNAi; short interfering RNA, micro RNAs, short hairpin RNAs (shRNAs)) products can be introduced to knockdown transcription or translation of key genes for viral infections (Bradford et al., 2017; Karlas et al., 2010; König et al., 2010). *In vitro* research has observed reductions in gene expression and subsequent impaired replication capacity for swine endemic coronaviruses (Li et al., 2019), Classical Swine Fever (Li et al., 2011), PRRSV (Oh et al., 2018), African Swine Fever (ASF) (Keita et al., 2010), Foot and Mouth Disease (Gismondi et al., 2014; Jiao et al., 2013) and IAV of swine and avian origins with RNAi (Stoppani et al., 2015). *In vitro* success has been translated *in vivo*, with PPRSV (Li et al., 2014), FMDV (Hu et al., 2015) and CSF (Xie et al., 2018) transgenic pigs expressing shRNAs showing resistance to the relevant viral infection. Importantly, Li et al. (2014) (Li et al., 2014) only observed an increase in the IFN-I response when foreign viral RNA is detected and not constitutively against the RNAi molecules. RNAi mediated immunity against IAV has been developed in chickens (Lyall et al., 2011), but has not been demonstrated yet in pigs. However, the success in chickens and the established use of lung specific promoters for RNAi and its effectiveness against other viral pathogens in pigs suggest that it could be a potent inhibitor of swIAV replication with minimal unintended biological consequences. However, the highly mutable nature of swIAV and its genetic heterogeneity would mean transgenic RNAi swIAV resistant pigs would need to target multiple genes to reduce the

likelihood of escape by mutation and remain effective, which would increase the risks associated with expressing non-native RNA products by the multitude of transgenic transcripts.

Despite being discussed for use in livestock dating back to at least 2003 (Clark and Whitelaw, 2003), it is clear that the promising results of RNAi in research face significant hurdles in transferring the technology to a commercial scenario for public consumption. Delivering the RNAi technology using nanoparticles (Hong and Nam, 2014; Shi et al., 2011) or viral vectors such as Adeno-Associated Viruses (AAVs) (Shen et al., 2015; Xu et al., 2012) as a therapeutic as opposed to constitutive expression as a defence against swIAV infection could be an alternative delivery mechanism with less regulatory pushback to consider. Transient delivery of RNAi moecules through these methods would permit rapid therapeutic adaptation to the genetic identity of swIAV strains diagnosed in particular regions and would reduce the potential for unintended consequences such as resistance emergence and off-target effects. However, despite the benefits of improved productivity at the farm level and reducing the burden of swIAV in pigs, using RNAi in a therapeutic manner is reactive and would therefore create a reservoir of persistent subclinical infections. For a more comprehensive review of the applications and risks of RNAi in animal agriculture see Bradford et al., 2017.

1.5.5 Gene-Editing

A more viable strategy for creating permanent and heritable resistance than creating transgenic organisms may be in modifying endogenous host genetics to prevent viral exploitation of host proteins. In the microbial evolutionary arms race of bacteria against viruses, the discovery of a molecular mechanism in bacteria that acts against viral infections in a targeted and specific manner has been redefined to be a critical molecular research tool. This was originally identified as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) with the CRISPR-associated protein 9 (CRISPR/Cas9). Comprising of a guide RNA that targets nucleic acid sequence through homologous base pairing,

and a Cas enzyme that has endonuclease activity, this site-specific break in DNA sequence allows specific genes to be edited (Figure 1-11).

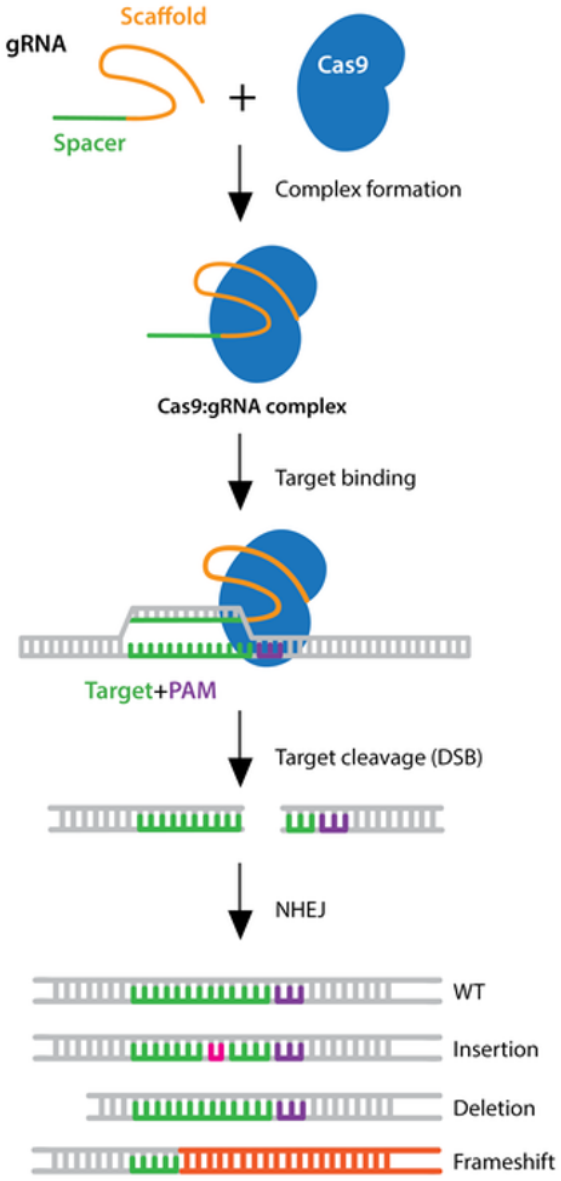


Figure 1-11: Gene-editing with CRISPR/Cas9.The gRNA component targets specific sequences of DNA through homologous base pairing. The gRNA has a contains a tracrRNA that interacts with Cas9 and recruits endonuclease activity within the heteroduplexed gRNA:DNA. The editing of genes for this pathway displayed occur due to the error-prone host DNA repair pathways. Insertion of non-native nucleotides at the Cas9 induced double-strand break disrupt the gene ORF, leading to a putatively knocked out allele. Alternative host repair pathways can be exploited to introduce specific changes at the target site. Image adapted from Addgene.com/guides/crispr

Viruses require a host organism for replication and transmission. This reliance on a host, usually specific in type and/or organism, has led to the evolution of host proteins being exploited during the virus replicative cycle. By altering the DNA sequence of host genes that code for proteins which are recruited by a virus, such as SA that promotes swIAV endocytosis, we can perturb the viral replication cycle, confer resistance to the host and prevent onwards transmission. The crudest form of gene-editing for viral resistance is to delete an entire gene or cause an insertion or deletion (indel) in the coding sequence that introduces a premature stop codon. Introducing an indel into the coding sequence of host cell receptors that lead to phenotypically null pigs has been demonstrated to work effectively for viral resistance to PRRS (Prather et al., 2017; Whitworth et al., 2016, 2014) and specific coronavirus strains (Whitworth et al., 2019).

A more nuanced approach to prevent PRRSV interaction with the host cell receptor, CD163, was taken by Burkard et al. (2017) (Burkard et al., 2017). Here, they deleted an exon of the CD163 gene that codes for the protein domain that PRRSV specifically interacts with. Further research of the biological impact of lacking this CD163 domain has thus far identified no unintended biological consequences (Burkard et al., 2018). The data has not been published for the CD163 null pigs displaying PRRSV resistance and thus unfortunately a complete comparison cannot yet be made between the biological impacts of losing a single exon or the entire protein of CD163. For IAV specific resistance, the host cell receptor (SA) is less appropriate as a target for gene-editing due to its crucial role for normal function (Varki, 2008). It is important to consider the systemic effect of edited genes in an organism when selecting a target and to avoid thinking that protein function is always restricted to its described nomenclature from what has been identified in a laboratory environment.

A family of nuclear proteins, acidic nuclear phosphatase 32's (ANP32s) has been identified in chicken and mammalian *in vitro* studies to have strong pro-viral effects by enhancing the efficiency of IAV genome replication (Long et al., 2016). Human ANP32 null cell lines inhibit the infection of swIAVs, and when porcine ANP32s expression is recapitulated with cDNA constructs the ability of

the virus to replicate is returned. These findings, corroborated by avian and human *in vitro* results (Long et al., 2019a; Staller et al., 2019; Zhang et al., 2019), suggest that the conserved gene family of ANP32s supports viral RNA polymerase function and enhance the efficiency of genome replication (Baker et al., 2018; Baker and Mehle, 2019). Conservation of IAVs exploitation means that successful resistance could be against multiple IAV subtypes to variable extents dependent upon the infected species and IAV polymerase genetics (Peacock et al., 2020b). Specific amino acids in ANP32 proteins that affect the function of viral polymerase activity have been identified to confer a similar reduction in activity as the full loss of function mutations and offer potential targets for substitution (Peacock et al., 2020b; Staller et al., 2020; Zhang et al., 2019).

Research and development of the CRISPR/Cas systems has led to a revolution of gene-editing technologies. As a tool for generating research models, Cas9 transgenic pigs and chickens have been developed (Rieblinger et al., 2021; Wang et al., 2017). Alternative Cas protein-based strategies include using Cas13 which endonuclease activity specifically associated with RNA. A transgenic animal expressing Cas13 and guide RNAs that target the swIAV genome could therefore conceptually be more resistant to infection. Inactivated Cas proteins that have transcription activating or repression domains fused could be therapeutically prescribed to affect the expression of immune response genes (Shen et al., 2015). Base-editing and prime-editing are more recent developments that have enhanced the specificity and make small changes to the host genome have not been well assessed in pigs but hold promise for reducing the potential for unintended biological impacts (Anzalone et al., 2019; Rees and Liu, 2018).

A major hurdle to the implementation of gene-editing in livestock for disease resistance is gaining regulatory approval and broad public acceptance (Ishii, 2017). From a public health perspective for IAV it is difficult that the application of genome-editing in livestock may only be favoured retrospectively following an outbreak of disease that may have been controllable in the primary instance through genome-editing. Pigs that have no foreign DNA are much more likely to be permitted for animal welfare benefits and economic demands from

producers, with regulations in Argentina, Japan and Canada already having legislated to regulate the animal and not the process by which the DNA was altered (Holman, 2019; Whitelaw, 2019). Therefore, if the gene-edit has been validated to be benign other than the intended effect they are likely to be allowed for production. These positive steps towards regulatory acceptance alongside the development and optimisation of gene-editing strategies that could be applied at scale in a commercial setting (McFarlane et al., 2019) provide optimism for a case by case approach to the acceptance of gene-edited livestock.

Gene-editing could also be applied outside directly editing the infected host genome for vaccine production through changing the genome of chickens that lay eggs used as bioreactors for the propagation of whole IAVs. Ectopic expression of swine factors (such as SA or ANP32s) that support the replication of swine adapted strains in an avian environment could boost the efficiency of replication in eggs, reducing the cost of vaccine production and thereby potentially making uptake of vaccination more accessible. A risk to gene-editing of pigs comes from the corporate nature of pork production, meaning producers that are not integrated with breeding companies offering IAV resistance alleles in their population could lead to IAV risk farms being isolated, causing small holder producers to be perceived as less safe. The economic factors regarding the introduction of new innovative technologies will hopefully not impede improved safety for humans and welfare for pigs from being available.

1.6 Thesis Rationale

Influenza A Virus pandemics are a present threat to swine welfare and have the potential to emerge from swine into humans as a pandemic strain. The observations of how pig farming and international economics were affected by this outbreak from what turned out to be a moderately virulent strain, and the shockwaves in terms of death, illness and disruption to our lives that we experience today due to the SARS-CoV-2 pandemic should set the precedent for understanding the threat pandemics present. Relating to endemic strains that annually circulate in pigs causing flu', for farming to retain a social license to

operate, it must ensure first-rate animal health practice, including stringent IAV control practices, to achieve the highest possible standards of animal welfare.

With industrial farming practices increasingly adopted and multi-site systems necessitating more pig movement, there will be more opportunities for viral reassortment that enhances the potential for novel IAV strains to emerge. Implementing effective control measures to reduce the intraspecies and zoonotic spread of swIAV will improve the economic performance of pig production, improve farmer and pig health and negate the potential for pigs to act as a mixing vessel for emergent strains of IAV.

Each approach in swIAV control will be variably relevant according to different regions and the dominant farming system, with the available local infrastructure affecting the implementation of each strategy. The application of genetic technologies, such as gene-editing, in livestock offers potential in providing pre-emptive disease control in a known risk source. Research into how gene-editing can be used effectively, with considered biological consequences could provide not only a swIAV resistant pig, but contributes insights into host-pathogen interactions that could be extrapolated for insights into disease control through disruption of viral of host pathways.

1.7 Thesis Aims

The aims of this project are to investigate the potential for gene-editing to be implemented in pigs to improve resistance or resilience to IAV. In a research environment, the first steps are to gene-edit a small cohort of organisms. Given that most pigs are reared in large commercial systems, the parameters of efficiency may be restrictive to implementing gene-editing at a commercial scale. In the first results chapter, we set out to model the introgression of monogenic and digenic sAIV resistance into a multi-herd commercial breeding system. This considered how different gene-editing techniques would affect the scale of editing required and how different gene-editing efficiencies affects the flow of resistance alleles to commercial farmers. The data generated here was to contribute to understanding how gene-editing could be effective in a real-life scenario. The data here helped to translate

knowledge of how to do gene-editing academically, to how it could be done commercially.

With an understanding of how gene-editing could be effectively implemented as a tool for swIAV control, the second aim of this project and focus of Chapter 4 was to select gene targets for editing and to create an *in vitro* model for swIAV resistance in pigs. We selected to use a pig tracheal cell line as the native site of a respiratory infection in pigs and validated a model system that would be suitable for investigating the role of ANP32A and ANP32B, our identified target genes, during IAV infection in swine.

Having validated our model as a functional knockout of the target genes, in Chapter 5 it was investigated whether the loss of ANP32A and ANP32B affected the replication dynamics of IAV in swine and whether they were functionally redundant as host cofactors for IAV exploitation. The development of a two gene resistance model retained relevance for the initial modelling work that assessed monogenic and digenic integration into swine herds.

2 Materials and Methods

2.1 Cell Culture

2.1.1 Eukaryotic cells

Mammalian cells were cultured in Nunc™ cell culture treated flasks with filter caps in a humidified atmosphere containing 5% CO_2 at 37°C in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose (4.5 g/L), GlutaMAX™ and sodium pyruvate (0.11 g/L) that was supplemented with 10% Foetal Bovine Serum (FBS) (v/v) (Life Technologies) and Penicillin/Streptomycin (PenStrep) (10,000 U/mL, Life Technologies). This medium is defined hereafter as 'Full Medium'. All cell culture work was performed in a Class II biosafety cabinet. Newborn Pig Tracheal (NPTr) cells (Ferrari et al., 2003) were received from Dr. Christine Tait-Burkard. Madin-Darby canine kidney (MDCK) cells were received from Dr. Nisha Kriplani.

2.1.2 Cell passaging

To passage cells, medium was aspirated from the culture flask and cells were washed with molecular biology grade phosphate buffered saline (PBS) (Sigma-Aldrich). In Nunc™ T75 Cell Culture flasks, 5 ml of TrypLE™ Express Enzyme (Gibco) was overlayed on cells before incubation for 8 minutes at 37°C. The volume of TrypLE™ Express Enzyme used was scaled accordingly to flask size. To detach all cells, the flask was firmly tapped, with further incubation performed as required. The TrypLE™ was deactivated with Full Medium at a volume ratio of four times Full Medium to TrypLE™. The suspended cells were transferred to a 15 mL Falcon tube cells and centrifuged at 500 x *g* for 5 minutes in a swing-bucket centrifuge. All centrifugations at volumes > 1.5 mL were performed in swing-bucket centrifuges, whilst volumes < 1.5 mL were centrifuged at a fixed angle. The supernatant was discarded before the cell pellet was resuspended in Full Medium. To count cells, 10 μL of the cell-containing medium was added to a haemocytometer and cell concentration was calculated as follows:

$$Number\ of\ cells/ml\ =\ n\ X\ 10^4$$

Cells were then reseeded at the concentrations required for subsequent experiments.

2.1.3 Freezing cells

Once cells had reached 80% confluence in a T75 culture flask, the protocol described in Section 2.1.2 was followed to detach and pellet cells. The cell pellet was resuspended in 8 mL DMEM with 2 mL FBS (20% v/v), without PenStrep. 900 μL of the resuspended cell solution was aliquoted to a 1.5 mL cryovial and 100 μL DMSO (10% v/v) was added and mixed well before transfer to an isopropanol filled cell freezing container. These cells were kept for 1 week at -80°C before transfer to -150°C for long term storage.

Resuscitation of frozen cells was performed by rapid thawing of the cryovial in a 37°C water bath before transfer into a 15 mL Falcon tube containing 10 mL of prewarmed Full Medium. To remove the DMSO, cells were centrifuged at 500 x *g* for 5 minutes, the cell pellet was resuspended in fresh Full Medium and seeded in a T25 cell culture flask.

2.1.4 Cell Counting Kit (CCK)-8 Assay

To compare the proliferative capacity of gene-edited NPTr cell lines to Wildtype (WT) NPTr, Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich) assays were performed. A CCK-8 assay is a colorimetric method for measuring the number of cells proliferating. WST-8 gets reduced by dehydrogenase activity in cellular electron transport chains to WST-8 formazan. The amount of coloured WST-8 formazan produced is proportional to cellular metabolism and can be measured spectrophotometrically.

In triplicates for each cell line at each time point, 9 x10^3 cells were seeded in a Nunc™ 96-well plate. The outside wells were not used to avoid interference of the absorbance readings. At the specified time points, 10 μL of the CCK-8 solution was added directly to the well with care being taken to not introduce bubbles. After incubation for 1 hour at 37°C, the Optical Density 450 (OD_{450}) was measured at 0.1 second intervals using a microplate reader. Results

were analysed by a two-way ANOVA with a post-hoc Tukey's test to determine differences in proliferation between knockout cell lines and the WT control.

2.1.5 Plasmid transfection

Transfection of plasmid DNA was performed with Lipofectamine® 3000 according to the manufacturer's protocol (Invitrogen). In a Nunc™ 6-well plate, 3 x10^5 NPTr were seeded in each well with 2 mL of Full Medium. For each sample, 125 μL Opti-MEM was mixed with 1.5 μg plasmid DNA and 3 μL of the P3000 Enhancer reagent in a microcentrifuge tube, and a second solution of 125 μL Opti-MEM was mixed with 7.5 μL Lipofectamine® 3000 in another microcentrifuge tube. For dual transfections, plasmids were in equimolar proportions, with the 1.5 μg as the final concentration. The DNA solution was added dropwise to the Lipofectamine® 3000 solution and pipetted up and down 5 times before incubation for 15 minutes at room temperature.

For each transfection, the combined 250 μL of DNA/Lipofectamine® 3000 solution was added dropwise to freshly seeded cells and gently distributed by movement in a figure of 8 motion, before placement in the incubator with cell culture conditions. Medium containing lipofection reagents was replaced with fresh Full Medium after 24 hours, and after 48 hours, either the DNA was extracted, or the cells were sorted by Fluorescent Activated Cell Sorting (FACS) according to Green Fluorescent Protein (GFP) positivity. For transfections in 12-well plate, the same protocol was followed but with the reagent volumes/ concentrations halved.

2.2 Bacterial Molecular Biology

2.2.1 Gel Electrophoresis

Agarose gels were made by dissolving UltraPure™ agarose (Invitrogen) in 1X Trisacetate-EDTA (TAE) buffer (Roslin Stores). For example, to make a 1.5% agarose gel, 1.5 g of agarose was completely dissolved in 100 mL of 1X TAE by heating in a microwave on low setting. Once the agarose gel mixture had cooled slightly, 2 μL of 10,000X SYBR Safe DNA stain (Thermo Fisher Scientific) was added, and the gel was cast in a transparent gel tray. Agarose and TAE

measurements were adjusted accordingly for different gel percentages and gel tray sizes. DNA samples were loaded alongside a well containing 5 μL of GeneRuler Mix or GeneRuler 1 kb DNA Ladders (ThermoFisher), and the electrophoresis gel box was set to at 90-100 Volts to discriminate DNA product sizes and approximating sample concentration. Bands were visualised and imaged on a UV Transilluminator Gel Doc XR (Bio-Rad Laboratories).

2.2.2 Bacterial Transformation

Plasmids were transformed into chemically competent *Escherichia coli* (TOP10), generated by Dr. Vrushali Patil. Competent cells were thawed on ice and 2 μL of the exonuclease treated plasmid reaction was added to the 25 μL aliquots of the competent cells before incubation on ice for 30 minutes. A 30 second heat shock step was then followed by incubation at 42°C in a PCR thermocycler, and a 2-minute incubation on ice. If the plasmid encoded for beta-lactamase (ampicillin resistance), the bacteria were spread on Lysogeny Broth (LB) agar plates containing 100 μg/mL carbenicillin (ampicillin analog; diluted in 50% EtOH:50% ddH_2O to a concentration of 10 mg/mL) using a sterile plate spreader and incubated overnight (12 – 16 hours) at 37 °C.

To screen the colonies for gRNA integration, half of selected colonies were processed for a PCR reaction, as described in Table 2-1, using an oligonucleotide of the gRNA as the forward primer and a region within the CMV promoter as the reverse primer (oSL35), under the PCR conditions described in Table 2-2. PCR products were run on a 2% agarose gel. The alternative half of the colony was transferred to 5 mL LB containing ampicillin (100 μg/mL) and shaken at 37°C for 14-16 hours at 220 rpm. Plasmids were extracted by miniprep (Qiagen), as described in section 2.2.3. The quality and quantity were measured by Nanodrop (Section 2.4.3) and submitted for Sanger sequencing (Section 2.4.5).

Reagent	Volume	Supplier
DreamTaq Mastermix (2X)	12.5 µL	ThermoFisher
oSL35 (Table 2-6) @ 10 µM	1.5 µL	IDT
Forward Primer (gRNA-F)	1.5 µL	IDT
Fi-Red	5 µL	Roslin
ddH2O with colony	10 µL	QIAGEN
Total	25 µL	

Table 2-1: Colony PCR reagents

	DreamTaq		Phusion		
Step	**Temperature**	**Time**	**Temperature**	**Time**	**Cycles**
1	94 °C	5 mins	98 °C	2 mins	1
2	94 °C	30 secs	98 °C	30 secs	30
3	55 °C	30 secs	See primer tables	30 secs	30
4	68 °C	30 secs	72 °C	30 secs	30
5	68 °C	5 mins	72 °C	5 mins	1

Table 2-2: PCR thermocycler conditions for DreamTaq and Phusion

2.2.3 Small scale preparation of plasmid DNA

Low volume bacterial culture (<5 mL) plasmid preparations were performed using the alkaline lysis based QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's protocol and using a fixed angle table-top centrifuge at 16,000 x *g* unless otherwise stated.

From the overnight culture, 1 mL was transferred to a 1.5 mL microcentrifuge tube and centrifuged for 3 minutes at 6800 x *g*. The supernatant was discarded, and this step was repeated with another 1 mL of the overnight culture. The supernatant was discarded again, and the bacterial pellet was thoroughly resuspended in 250 µL Buffer P1. For bacterial lysis, 250 µL of Buffer P2 was added, and the solution was mixed by careful inversion until homogenous. The lysis reaction was incubated at room temperature for 5 minutes before neutralisation with 350 µL of Buffer N3. The neutralised solution was mixed by gentle inversion and centrifuged for 10 minutes. The resulting

supernatant was transferred to a QIAprep spin column and centrifuged for 1 minute. The flow-through was discarded and 750 μL of Buffer PB was added. Samples were centrifuged for 1 minute and the flow-through discarded. 750 μL of Buffer PE was added for washing of the spin column and again centrifuged for 1 minute with the flow-through discarded. The spin column was then centrifuged without any solution to draw through any residual wash buffers. The spin column was transferred to a fresh 1.5 mL microcentrifuge tube for collection of plasmid DNA and 50 μL of Buffer EB was added directly to the column membrane and incubated at room temperature for 1 minute. A final 1 minute centrifuge step eluted the plasmid DNA into the microcentrifuge tube. DNA quality and concentration was measured by NanoDrop spectrophotometer (Section 2.4.3) to ensure it was suitable for downstream applications.

2.2.4 Large scale preparation of plasmid DNA

For larger scale plasmid preparations, a higher volume (50-100 mL) of overnight bacterial culture was inoculated and midipreps were performed using either the PureLink® HiPure Plasmid DNA Purification kit (Invitrogen; Mp1) or the PureLink® HiPure Plasmid Filter DNA Purification kit (Invitrogen; Mp2). Apart from where stated, the protocols are identical and are both based on alkaline lysis. Both were performed according to the manufacturer's instructions. RNase A was added to the Resuspension Buffer (R3) prior to starting.

Overnight cultures were dispensed into 50 mL falcon tubes and centrifuged at 4,000 x *g* for 10 minutes. While cultures were centrifugating, 10 mL (Mp1)/15 mL (Mp2) of Equilibration Buffer was added to the HiPure Midi Column and allowed to drain by gravity flow. The supernatant from the bacterial culture was removed and the pellet was resuspended in 4 mL (Mp1)/ 10 mL (Mp2) of Resuspension Buffer until the solution was homogenous. To lyse bacteria, 4 mL (Mp1)/ 10 mL (Mp2) of Lysis Buffer was added, and the solution was mixed by gentle inversion and incubated at room temperature for 5 minutes. To separate out cellular proteins and genomic DNA, 4 mL (Mp1)/ 10 mL (Mp2) of Precipitation Buffer was added and mixed by careful inversion.

For Mp1, the solution was centrifuged at 4,500 x *g* for 30 minutes at room temperature. The supernatant was then transferred to the equilibrated Midiprep column and allowed to flow through by gravity. For Mp2, the precipitated lysate was added into the filtration column and allowed to filter through by gravity flow. The inner filtration cartridge with Mp2 could then be discarded. In both versions, columns were then washed by adding 10 mL of the Wash Buffer and allowing it to drain by gravity flow twice. To elute the DNA, 5 mL of Elution Buffer was added directly to the silica membrane. The eluted plasmid DNA was then precipitated by addition of 3.5 mL isopropanol before collection by centrifugation at 4000 x *g* for 1 hour at 4°C. Without disrupting the DNA pellet, the supernatant was removed and the pellet was gently washed in 70% ethanol before centrifugation at 4000 x *g* for 10 minutes at 4°C. The supernatant was carefully discarded, and the pellet air-dried before resuspension in 200 μL TE Buffer and storage at -20°C. Plasmid DNA quality and quantity was assessed by NanoDrop spectrophotometer (Section 2.4.3).

2.2.5 Restriction Digests

Restriction digests were performed to confirm size and orientations of plasmids that were propagated in bacterial culture. 1μg of plasmid DNA was incubated with 1 Unit of the selected NEB High Fidelity restriction enzyme with the buffer designated in the manufacturers protocol and at the appropriate temperature for at least 1 hour. Plasmid were discriminated by gel electrophoresis on a 1% TAE agarose gel.

2.3 CRISPR Development

2.3.1 gRNA Design

For gRNA design, genes were originally annotated using sequences downloaded from the NCBI gene database through Geneious software (Geneious Prime). The gRNA sequences were then selected through the CRISPOR online tool (http://crispor.tefor.net; Haeussler et al., 2016). To summarise CRISPOR, the target region for gene-editing was entered in the query box and the reference genome, *Sus scrofa - Pig - Sscrofa 11.1* and the PAM

sequence of 20bp-NGG-SpCas9 was selected. Consideration was given to gRNA specificity (Hsu et al., 2013) and the predicted efficiency with expression from U6 promoters (Doench et al., 2016) If there were off-target predictions within one or two base pairs elsewhere in the reference genome, these gRNAs were not considered for selection. The chosen SpCas9 gRNAs were ordered as ssDNA oligonucleotides from Integrated DNA Technologies with 5' overhangs appended for *BbsI* cloning into pSL70 (Table 2-3).

Guide RNA Name	***Target Sequence***	***Target Strand***	***Predicted Efficiency***	***Predicted Specificity***
gRNA A1	AACGGATTCATTTAGAGCTG	(+)	58	76
gRNA A2	CATTTGACCGACAATTGTCC	(-)	47	95
gRNA B1	AGTGACAATAGAATCTATGG	(+)	70	76
gRNA B2	CAATAGAATCTATGGAGGTC	(+)	45	82

Table 2-3: Guide RNAs targeting swine ANP32A and ANP32B for cloning into pSL70 and testing in NPTr.

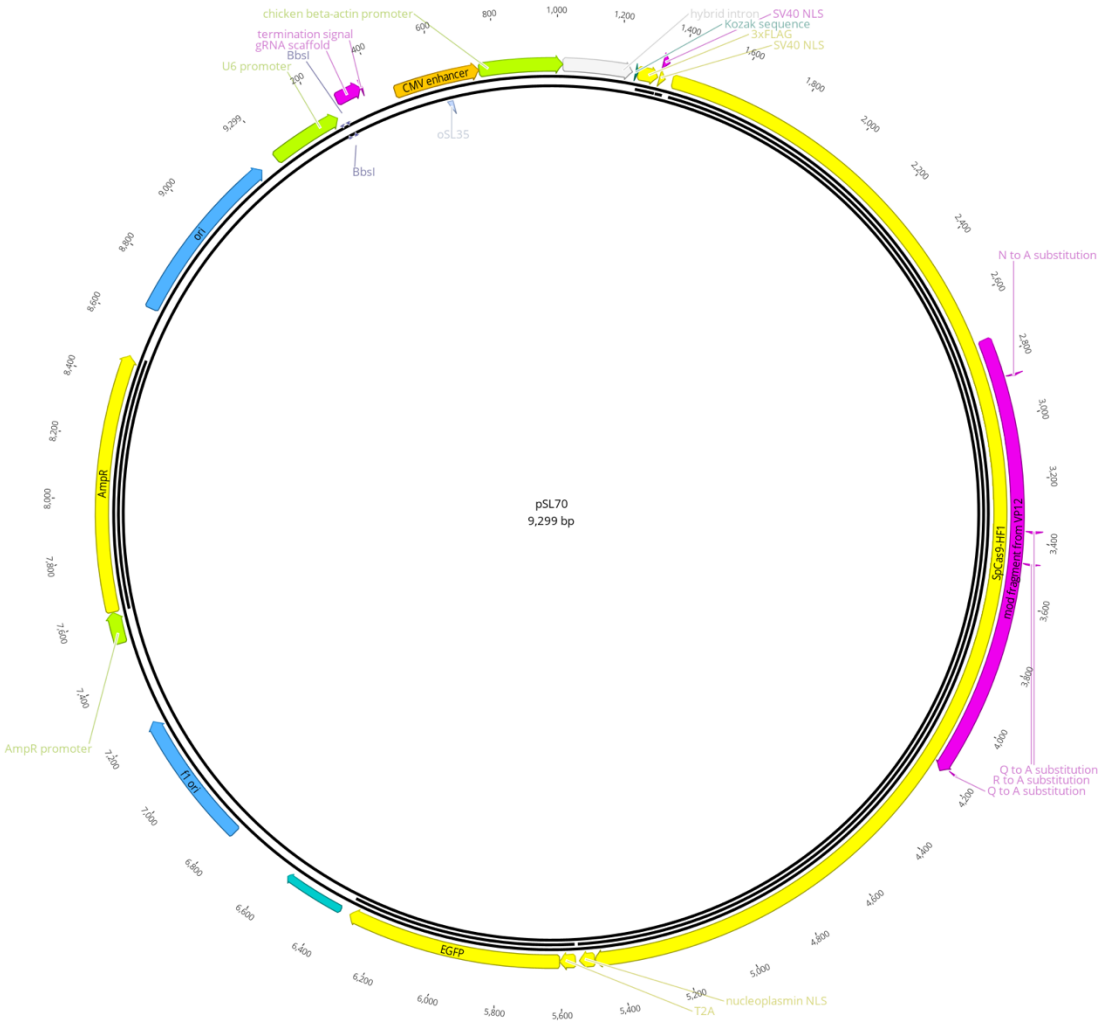


Figure 2-1: Annotated map of pSL70. pSL70 has a gRNA scaffold with U6 promoter and cloning site, and cDNA expression vector *sp*Cas9, eGFP and *ampicillin* resistance. From Dr. Simon Lillico.

2.3.2 gRNA Cloning

Integration of gRNA's into the Cas9-GFP plasmid was performed as outlined in Ran et al., 2013. pSL70 is a modified vector that originated as pSpCas9(BB)-2A-GFP (PX458; Addgene plasmid #48138; *Streptococcus pyogenes* Cas9 with 2A-EGFP, an ampicillin selection cassette and gRNA cloning backbone; a gift from Prof. Feng Zhang), with 4 amino acid substitutions to enhance Cas9 nuclease specificity (VP12) (Kleinstiver et al., 2016). This was modified by Dr. Simon Lillico (Figure 2-1).

For gRNA integration, 1 μL of the forward and reverse oligonucleotides (100 μM stock) were ligated together in a reaction with 1 μL of NEB buffer 2 and 7 μL of ddH_2O at 98°C for 1 minute, before a 95 °C incubation for 5 minutes with a 5 °C/minute reduction to 25°C. Annealed oligonucleotides were diluted 1:200 in ddH_2O for cloning into pSL70. Cloning reactions containing the reagents of Table 2-4 were incubated for 6 cycles of 37 °C for 5 minutes and then 21 °C for 5 minutes to digest the vector and ligate the sticky ends from the annealed oligonucleotides.

Reagent	Volume	Supplier
pSL70	1 μL @ 100 ng/μL	Dr. Simon Lillico
Annealed oligos	2 μL	IDT
T4 Ligase Buffer	2 μL	NEB
BbsI - HF	1 μL	NEB
T7 Ligase	0.5 μL	NEB
ddH_2O	13.5 μL	Qiagen
Total	20 μL	

Table 2-4: Reagents for cloning gRNAs into pSL70

Residual linearised DNA was digested with Plasmid-Safe™ exonuclease (Epicentre, USA) by incubation at 37 °C for 30 minutes and then 70 °C for 30 minutes, with reagents as described in Table 2-5. These plasmids were subsequently transformed into bacteria for propagation (Section 2.2.2).

Reagent	Volume	Supplier
Ligation Reaction	11 μL	
10X Plasmid-Safe™ Buffer	1.5 μL	Epicentre
10 mM ATP	1.5 μL	Epicentre
Plasmid-Safe™ Exonuclease	1 μL	Epicentre
Total	15 μL	

Table 2-5: Reagents for the digestion of linearised DNA

2.3.3 Fluorescent Activated Cell Sorting (FACS)

NPTr transfected with pSL70 were selected for by the presence of eGFP. The presence of eGFP indicates plasmid expression and translation, and

therefore enriches for cells for cells that are expressing the Cas9/gRNA casette. FACS was performed with a BD FSCS Aria III machine around 48 hours after NPTr were transfected. NPTr were detached from the 6-well plate as described in Section 2.1.2 and pelleted by centrifugation in a 1.5 mL microcentrifuge tube for 5 minutes at 500 x *g*. The cell pellet was resuspended in 300 μL of serum-free DMEM (SFM) and filtered into a Falcon 5 mL round bottom test tube with a cell strainer snap cap (Corning™) and kept on ice until cell sorting commenced. FACS was operated by a member of the Roslin Bioimaging facility with forward and side scatter gating's set according to a non-transfected negative control sample and positive control sample transfected with the pSL70 vector containing no gRNA casette.

For collection of pooled GFP-positive cells, samples were sorted into screw-top microcentrifuge tube tubes containing 200 μL PBS. These samples were immediately ready for genomic DNA (gDNA) extraction following the protocol from Section 2.4.1. For isolation of single cells that were to be expanded as clonal populations, each cell was sorted into a well of a 96-well tissue culture plates which contained 90 μL of Full Medium supplemented with 10 μL of conditioned medium (NPTr medium culturing cells for one day previous, filtered through 0.22 μM filter).

2.3.4 T7 Endonuclease Assay

For approximate analysis of gRNA efficiency, gDNA was harvested from pooled FACS cells. The presence of indels was inferred by T7 endonuclease activity through restriction digestion of mismatched DNA. PCR products that were designed to include the target gRNA region were amplified and quantified by agarose gel estimation. 100 ng of the PCR products was incubated with 1 μL NEB buffer 2 and mixed to 9.5 μL with ddH_2O before denaturing at 95 °C for 5 minutes and gradual cooling to 25°C (-0.1 °C/sec) to allow rehybridisation of PCR products. 0.5 μL of T7 endonuclease (NEB) was added to the 9.5 μL solutions, with 0.5 μL ddH_2O added to a separate negative control. Following a 20-minute incubation at 37°C to catalyse T7 endonuclease activity in a PCR thermocycler, 8

µL was ran on a 1.5% agarose gel to visualise whether mismatches in DNA base pairing had been introduced by the presence of indels.

2.3.5 Inference of CRISPR Edits

After PCR products of the gRNA target regions were Sanger sequenced (Section 2.4.5), traces were analysed using Inference of CRISPR Editing (ICE) software (Synthego) (Conant et al., 2022). Through decomposition of the Sanger sequence traces, ICE can discriminate the type of indels present by proportions and determine whether the indel will disrupt the ORF. This method requires that the edited sequences are compared to a WT reference sequence.

For batch analysis of Sanger sequences when determining the CRISPR editing efficiency in clonally isolated cells, the Geneious 'Analyze CRISPR Edits' tool was used. This automatically determines the cut site based on the majority of the variant locations and clusters the edited alleles for downstream analysis. Sequences with R^2 values below 0.8 were discarded. Cross referencing of both data sources was performed to support reliability.

2.4 General Molecular Biology

2.4.1 Genomic DNA extraction

Genomic DNA was extracted from NPTr, aside from when in 96-well plates, using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol for cultured cells. Briefly, cells were collected in a 1.5 mL microcentrifuge tube and pelleted by centrifugation at 500 x *g*. The cell pellet was resuspended in 200 µL PBS that contained proteinase K for removing DNase's and other protein contaminants. To lyse the cells, 200 µL of Buffer AL was added and the solution was vortexed. After 10 minutes incubating, DNA was precipitated in 200 µL of 100% ethanol and vortexed. The solution was transferred to a DNeasy spin column and centrifuged for 1 minute at 6000 x *g* and the flow-through was discarded. To wash the spin column, 500 µL of Buffer AW1 was added. After centrifugation for 1 minute at 6000 x *g*, the flow-through was discarded and 500 µL of Buffer AW2 was added before centrifugation for 3 minutes at 20,000 x *g*. The spin column was transferred to a fresh 1.5 mL

microcentrifuge tube and 200 µL Buffer AE was added directly to the membrane and incubated for 1 minute at room temperature. The DNA was eluted by centrifugation for 1 minute at 6000 x *g*.

For extraction of gDNA from 96-well plates QuickExtract™ (QE) DNA Extraction Solution (Epicentre) was used. The medium was aspirated from each well containing cells and 50 µL of QE Solution was added. To assist with lysis, cells were scraped with the pipette tip and the QE solution was pipetted up and down. The lysed cells solution was then transferred into a 96-well PCR plate. Samples were briefly vortexed and centrifuged at 200 x *g* for 30 seconds before heating at 65°C for 6 minutes. Another brief vortex and centrifugation step preceded heating at 98°C for two minutes. The extracted DNA was stored at -20°C and was added directly to PCR reactions for use.

2.4.2 Polymerase Chain Reaction (PCR)

PCR was performed with Phusion® High-Fidelity PCR Mastermix with HF Buffer (New England Biolabs (NEB) using the primers described in Table 2-6 and components outlined in Table 2-7. Conditions for the thermocycler are outlined in Table 2 with the annealing temperatures for each primer pair determined by gradient PCR. For different total volume PCR reactions, reagent volumes were adjusted accordingly. Fi-Red solution (Ficoll-400 10% (v/v) and a trace of cresol red dye in ddH_2O, was used as a marker for visualising DNA bands.

Name	Function	Strand	Sequence 5'→ 3'	Annealing °c	Amplicon length
ANP32A exon 2	PCR	Sense Antisense	GAATGTGGCAGTTGGGTCTT GCAGGGAGAGAAAAAGCAAA	62.6	508
ANP32B exon 3	PCR	Sense Antisense	TTTGTGTTTGTGTGCCGTGT GGGAGAGTAATGCCACAGGA	56	513
ANP32A	RT-PCR	Sense Antisense	CGACAAGGAGGCCTCTGACT CATCCTCCTCTTCACCTTCC	58	127
ANP32B	RT-PCR	Sense Antisense	GCAGAAAAACTTCCAAATCTCA CCAGGCTTTTCAGACACTCC	59	106
YWHAZ	RT-PCR	Sense Antisense	TGATGATAAGAAAGGGATTGTGG GTTCAGCAATGGCTTCATCA	60	203
oSL35	oSL70 seq.	Antisense	GTCAATAGGGGGCGTACTTG	55	

Table 2-6: Primers for PCR amplification of genomic DNA and RT-qPCR amplification of cDNA.

Component	Volume (30 µL)
Phusion	15 µL
Fi-Red	6 µL
Primer Forward	1.5 µL @ 10 µM
Primer Reverse	1.5 µL @ 10 µM
gDNA	100 ng
Nuclease-free H_2O	To 30 µL

Table 2-7: Reagents for individual PCR reaction with genomic DNA

2.4.3 Nucleic acid quantification and quality assessment

The concentration of DNA and an estimation of contaminants present was measured using a NanoDrop 1000 spectrophotometer with ND-1000 software (Thermo Fisher Scientific). The NanoDrop was initialised with 1 µL of ddH_2O before a blank measurement was taken using 1 µL of the suspension solution. 1 µL of the nucleic acid solution was then added to the sensor and the spectral measurement was taken. Nanodrop-1000 software calculates the concentration of DNA through absorbance of light at the wavelength of 260nm.

The amount of light absorbed allows inference of DNA concentration in the drop. The purity of the nucleic acid was inferred from the 260/280 and 260/230 ratios. The 260/280 ratio indicates the presence of protein, phenol or other contaminants that have an absorbance close to 280 nm, and a ratio ~1.8 - 2 was considered pure. The 260/230 ratio is a secondary measure indicative of nucleic acid purity and organic compounds that can be detected at 230 nm absorbance. A 230/260 ratio of ~2 – 2.2 was considered acceptable.

2.4.4 Gel extraction and PCR Product clean-up

Digested plasmid fragments or PCR products that were electrophoresed for discrimination and isolation were cut out from the agarose gel with a clean scalpel and purified using GFX PCR DNA and Gel Band Purification kit (GE Healthcare Life Sciences) according to the manufacturer's protocol. Excised gel slices were incubated with 500 µL of Capture Buffer 3 at 60°C until the agarose was completely dissolved. The agarose solution was then loaded directly onto the GFX column. For PCR products that were to be Sanger sequenced, up to 50 µl of a PCR reaction was mixed thoroughly with 500 µL of Capture Buffer 3 before loading directly onto the GFX column. The GFX columns were then centrifuged at 16,000 x *g* for 30 seconds, and the flow-through was discarded. The columns were washed twice with 500 of µL of Wash Buffer and centrifuged at 16,000 x *g*, with the flow-through discarded after each step. To ensure all residual ethanol was removed the columns were centrifuged for 3 minutes at 16,000 x *g*. The spin columns were transferred into fresh 1.5 mL microcentrifuge tubes and 20 µl of either Elution Buffer 4 for cloning processes, or Elution Buffer Type 6 for PCR samples being prepared for Sanger sequencing was added directly to the column and incubated at room temperature for 1 minute. A final centrifugation for 1 minute at 16,000 x *g* eluted the DNA from the spin column.

2.4.5 Sanger Sequencing of DNA

Sanger sequencing was performed by Edinburgh Genomics or Source Bioscience (Table 2-8). PCR products were cleaned up using the GFX PCR Purification kit protocol (Section 2.4.4) prior to submission for sequencing.

Analysis and quality control of sequence results was performed using Geneious Prime.

	Edinburgh Genomics	**Source Bioscience**
PCR product	50 ng	50 ng
Plasmid	100 ng	100 ng
Primer	6.4 µM	3.2 µM
H_2O	To 5 µL	To 5 µL

Table 2-8: Reagents for individual Sanger sequencing reactions

2.4.6 RNA extraction

Total RNA was extracted using QIAGEN RNeasy Mini Kit. The protocol was followed according to the manufacturer's instructions for adherent animal cells, including the on-column DNase digestion. Briefly, < 1 x 10^6 NPTr were harvested and pelleted by centrifugation at 500 x *g* for 5 minutes. All medium was carefully aspirated and the cell pellet was homogenised and lysed in 350 µL Buffer RLT. 350 µL of 70% EtOH was mixed well with the cell lysate before the 700 µL was transferred into a RNeasy spin column and centrifuged for 15 s at 8000 x *g*. To wash the RNeasy membrane, 350 µL Buffer RW1 was added and centrifuged at 8000 x *g* for 15 s, with the supernatant being discarded. An on-column DNA digestion was then performed, with 10 µL DNase I (Qiagen) diluted in 70 µL Buffer RDD added to the column for a 15 minute incubation at room temperature. A repeat of the wash step with 350 µL of Buffer RW1 and centrifugation was performed to remove digested DNA. A further column wash was performed with 500 µL Buffer RPE and centrifugation at 8000 x *g* for two minutes. The supernatant was discarded, and this centrifugation step was repeated to ensure no residual ethanol remained. Finally, to elute the RNA from the silica membrane, the spin column was transferred to a fresh 1.5 mL microcentrifuge collection tube and 50 µL of RNase-free H_2O was added directly to the membrane before centrifugation for 1 minute at 8000 x *g*. RNA quality and concentration was assessed by Nanodrop and gel electrophoresis before use in downstream applications.

2.4.7 cDNA generation

RNA was reverse transcribed into complementary DNA (cDNA) using Invitrogen SuperScript™ III Reverse Transcriptase (RT) with random primers, according to the manufacturer's protocols. As follows, total RNA (500 ng), 0.5 µL random primers, 1 µL dNTPs (10mM) and ddH_2O were mixed by pipetting and incubated for 5 minutes at 65°C and then 2 minutes on ice. To this solution, 4 µL First-Strand 5x Buffer, 1 µL 0.1M DTT, 1 µL RNaseOUT and 1 µL SuperScript™ III RT was added and mixed by pipetting. After incubation for 5 minutes at room temperature and then 1 hour at 50°C to catalyse RT activity, before RT enzymatic activity was inactivated by incubation at 70°C for 15 minutes.

2.4.8 Quantitative Real-Time PCR (qPCR)

Quantification of gene expression was performed by analysing cDNA that has been reverse transcribed from total RNA extracted from NPTr cell cultures, using SYBR Green to detect the generation of double-stranded DNA (dsDNA). Primers used for qPCR are described in Table 2-6.

The cDNA stocks were diluted 100-fold in ddH_2O, and 8 µL of each sample was added to wells of a 96-well PCR plate in triplicate. A PCR master mix containing 10 µL SYBR Green (Agilent Brilliant III Ultra-Fast SYBR® Green, Agilent), 0.8 µL forward primer (10 µM), 0.8 reverse primer (10 µM) and 0.4 µL ROX reference dye (diluted 1:500 in ddH_2O) per reaction was made and 12 µL was added to each well and mixed by pipetting. The 96-well plate was briefly vortexed and centrifuged at 200 x *g* for 1 minute.

Using an Agilent MxPro cycler and the MxPro software, qPCR was set up to run with a dissociation curve and to use ROX as the reference dye for all reagent containing wells. The qPCR thermal profile was set up with an initial dissociation step at 95°C for 2 minutes, followed by a 40 cycle, 2-step PCR as follows: 15 second, 95°C DNA dissociation step, then a 30 second, 57°C primer annealing step. The time taken to heat from 57°C to 95°C was sufficient for polymerase extension of the small PCR products (>300 bp). The dissociation curves were measured by a final 1-minute 95°C dissociation step, followed by 30

seconds of annealing at 57°C, before detection of all PCR product sizes through measurement of SYBR Green as the reaction was incubated to 95°C. Results were analysed using MxPro software with the normalised value (dRn) for fluorescence and the fluorescence threshold set in the linear phase of fluorescent increase.

Gene expression was analysed by relative abundance of gene of interest transcripts after normalisation to the porcine reference gene (YWHAZ). The comparative Cycle threshold (Ct) method (ΔΔCt) was used to calculate the fold change in gene expression for each gene compared to wild type samples. Fold change in gene expression = $2^{-\Delta(\Delta Ct)}$, where ΔCt=(target) – Ct (Reference) and Δ(ΔCt) = ΔCt, (treatment) – ΔCt (control).

2.4.9 Protein Extraction

Total protein was extracted from cell lines and tissues using Cell Lytic MT (Sigma-Aldrich) with 100x HALT phosphate and protease inhibitor (Thermo Fisher Scientific) included. The tissue samples were gifted as samples not required as part of a study by Dr Gerry McLachlan that had ethical approval. Using sterile scissors, spleen and tracheal tissues were reduced in size before homogenisation with a tissue homogeniser. For homogenised tissues, 5 mg of tissue was isolated. For cells, protein was extracted from a NPTr and Human Embryonic Kidney cells at 70% confluence in a Nunc™ T75 Cell Culture flask. Tissue or cells were incubated with 100 µL Cell Lytic MT containing 1 µL HALT was incubated at 4 °C with gentle rocking for 15 minutes. In a pre-cooled 4°C centrifuge, the total lysate was centrifuged at 16,000 x *g*. The protein containing supernatant was removed and stored at -20°C until further use.

2.4.10 Bradford Assay

Protein concentration from cell lysates was quantified using the Quick Start™ Bradford Protein Assay Kit (Bio-Rad). Protein extracts were prepared as described in Section 2.4.9 and diluted at a ratio of 1:10. In a flat-bottomed 96-well plate, 200 µL of Bradford Dye Reagent was added to enough wells to account for measuring samples and standards in triplicate. Using BioRad Quick

Start™ prediluted BSA standards, 4 µL was loaded into appropriate wells. From the proteins lysates to quantify, 4 µL was loaded. The plate was agitated gently to mix and incubated at room temperature for 5 minutes.

The absorbance was read on a plate reader at 595 nm wavelength. The mean absorbency for the standardised samples was plotted to generate a standard curve. The concentration of the cell lysate samples was calculated using a polynomial equation to fit against the standard curve generated. The concentration for the stock lysates could calculated by multiplying the diluted lysate concentration by 10.

2.4.11 Western Blotting

Each sample for Western blotting contained 35 µg of total protein, 5 µL NuPAGE™ LDS sample buffer (4x), 1 µL 1M dithiothreitol (DTT) and dH_2O to 20 µL. Samples were heated at 95°C for 5 minutes. Immediately prior to running the samples, 0.5 µL of 1M DTT (50mM final) was added.

Using a ThermoFisher XCell SureLock gel chamber system and a NuPAGE™ 4-12% Bis-Tris gel, the 20 µL protein samples were loaded into each well, an end well was loaded with 10 µL BioRad Precision Plus™ Protein WesternC™ protein ladder. The entire chamber was filled with 1x NuPAGE™ MOPS SDS running buffer and ran at 200V for 1 hour. The NuPAGE™ gel was carefully removed from its casing and placed on a section of cut to size Whatman filter paper, before placement in a transfer cassette on top of blotting pads as shown in Figure 2-2. All components of the transfer cassette were pre-soaked in 1x NuPAGE™ transfer buffer, and the Amersham™ Nitrocellulose membrane (0.45 µM pores, Cytiva) was gently placed onto the SDS-PAGE gel. All other components were added, and the transfer cassette was placed in the blot module. Transfer buffer was added directly within the transfer cassette to submerge it, whilst dH_2O added to the external chamber to maintain a cool temperature. The transfer was carried out at 200 mA for 1 hour.

For ANP32A (ab189110), blocking of the nitrocellulose membrane was performed in 5% skim milk powder (SMP) (w/v) in 0.1% Tween® 20 (Sigma-Aldrich) in PBS (PBST). For ANP32B (ab4224), nitrocellulose membrane blocking

was performed in 2.5% BSA (w/v) in PBST. After protein transfer was confirmed by visualisation of the ladder on the nitrocellulose membrane, the membranes were blocked in the appropriate blocking agent for 1 hour at room temperature. In a Nunc™ square bioassay dish, Parafilm was laid in the base and the membrane placed on top. Primary antibodies were diluted in either the fresh aliquots of the SMP or BSA blocking agents at a concentration of 1:500 and incubated overnight at 4°C. Parafilm is aquaphobic and helped prevent the

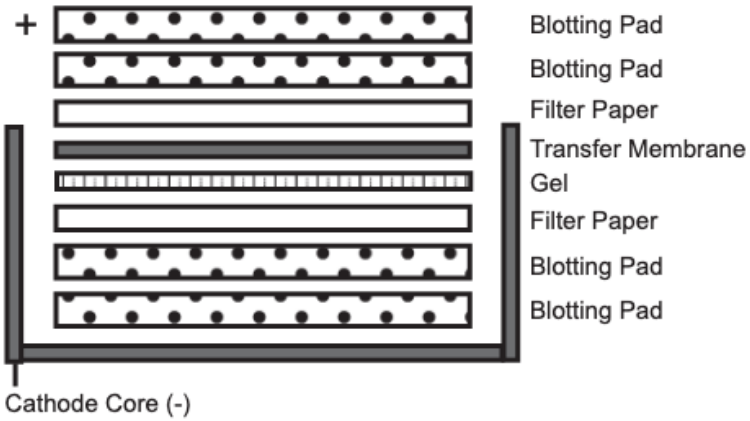


Figure 2-2: Set up of the Western blotting transfer module.

antibody dilution from dispersing in the dish.

The subsequent morning, the nitrocellulose membranes were washed 3 times with PBST to remove the primary antibody. For detection, horseradish peroxidase (HRP) conjugated secondary antibodies were used. Anti-rabbit for ANP32A (DAKO 1:1000) and anti-goat for ANP32B (DAKO 1:500), were added in a fresh aliquot of the blocking buffers and incubated in the bioassay tray for 1 hour at room temperature. Precision Protein StrepTactin-HRP Conjugate was included in the secondary antibody incubation at 1:5000 for chemiluminescent detection of the ladder. The membrane was washed 3 times in PBST for 5 minutes to remove residual secondary antibody. Solutions for Amersham ECL Western Blotting Detection Reagent (Cytiva) were mixed and added to the membrane. The ECL detection was incubated for 2 minutes. The nitrocellulose membrane was then carefully dried, and imaging was performed using a GeneGnome XRQ chemiluminescent imaging system.

2.4.12 Immunohistochemistry

The tissue samples were gifted as samples not required as part of a study by Dr Gerry McLachlan's that had ethical approval. Paraffin embedded tissues were cut onto microscopy slides (Nunc™ microscope slides, Thermo Fisher Scientific) at 8 μm thick and dehydrated. To remove paraffin and rehydrate tissues, slides were sequentially plunged into Xylene, 95% EtOH, 95% EtOH, 70% EtOH, 70% EtOH, ddH_2O and ddH_2O on automated settings using the Leica Autostainer ST5010.

Antigen retrieval was performed by heat retrieval in Citrate Buffer (10mM Sodium Citrate, 0.5% Tween-20, pH 6) in a pressurised cylinder heated for 20 minutes in a conventional microwave on high. After cooling, cells were permeabilised with 0.1% Triton™-X (Sigma-Aldrich) in PBS for 10 minutes then washed with PBST. Tissues were blocked with the appropriate blocking buffer (2% SMP in PBST for ab189110, 2% BSA in PBST for ab4224) for 1 hour at room temperature to reduce non-specific binding. Following blocking, 200 μL of antibody, diluted to the correct concentration (1:400 ab189110, 1:200 ab4224) in fresh aliquots of the same type of blocking buffer as previously used, was applied directly to the tissue and incubated overnight at 4°C in a covered box to prevent light exposure. Excess primary antibody was aspirated and the slides were washed 3 times in PBS. For secondary antibody staining, 200 μL of secondary antibody (AlexaFluor 488, ab150077 and ab150129 for ANP32A or ANP32B staining, respectively) diluted at 1:10,000 in the blocking buffer was added directly to the tissue. Excess secondary antibody was removed by washing 3 times in PBS before DNA was counter-stained with DAPI for 5 minutes (1:15,000 (Sigma-Aldrich)). A drop of fluorescent visualisation mounting solution (VECTASHIELD; Vector Laboratories) was overlayed on tissues before a cover slip was applied and the edges were sealed with nail varnish to secure the cover slip and reduce drying out. Visualisation was performed using a Leica DMLB with settings calibrated according to negative controls with no primary antibody. Images were analysed using FIJI software.

2.4.13 Immunocytochemistry

NPTr cells were cultured in Nunc™ 24 well tissue culture plates on cover slips (VWR) preincubated with 10% (v/v) collagen (Rat's tail, Sigma-Aldrich) in PBS. To make the original collagen solution, 100 mg of collagen was dissolved in 100 mL of 0.1M acetic acid, resulting in a 0.01% collagen w/v solution.

Cells were fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. Permeabilisation of nuclei with 1% Triton X-100: PBS for 10 minutes, followed by 3 washes with PBST and blocking with 5% (w/v) SMP: PBS for 1 hour at room temperature. To detect ANP32A cells were incubated with ab189110 (Abcam) diluted at 1:500 in 5% (w/v) SMP: PBS overnight at 4°C. Wells were washed twice with PBST before incubation with anti-rabbit AlexaFluor488 diluted at 1:10,000 (ab150077, Abcam). Staining of ANP32B was performed in the same manner except with ab4224 (Abcam), diluted at 1:400 in 2% FBS (w/v): PBST, which was used in place of SMP at all steps, and anti-goat AlexaFluor488 (1:10,000, Abcam, ab150131) used as the secondary antibody. In co-staining experiments the protocols for ANP32B antibody detection were followed. NP was detected using AA5H (ab20343) diluted at 1:500, following the same protocols, except with 1% FBS used in blocking steps and anti-mouse AlexaFluor488 (1:10,000, Abcam, ab150113) as the secondary antibody. For nuclei visualisation, cells were counterstained with DAPI (1:15,000, Thermo Fisher Scientific). A drop of fluorescent visualisation mounting solution (VECTASHIELD; Vector Laboratories) was placed on a fresh glass microscopy slide and each tissue culture cover slip was gently placed faced down in the solution. The edges were sealed with nail varnish to secure the cover slip and reduce drying out. Images were captured with a Leica DMLB fluorescence microscope using Micro-Manager software at 40x or 20x for DAPI and Phalloidin respectively. Images were processed using FIJI software.

2.3.14 Microscopy (DMLB)

All microscopy was performed on a Leica DMLB fluorescence microscope with images captured using micro-manager software. Post-image analysis was performed using FIJI (Image J).

2.5 Influenza Techniques

2.5.1 Influenza A Virus Stocks

IAV were initially created as p0 stocks with reverse genetics systems as previously described (Long et al., 2013). Virus replication assays using avian origin viruses (UDL and 50-92) were performed with recombinant viruses containing the HA, NA and M gene segments of PR8 (Long et al., 2013; Peacock et al., 2020b). For propagating PR8 and UDL viruses, MDCK were infected with p1 stocks (Figure 12). The Sw87 and 50-92 strains were gifted as p0 stocks and subsequently propagated by inoculation of the allantoic fluid of embryonated chicken eggs (Section 2.5.2). Infectious titres were determined by plaque assay on MDCKs (Figure 12). Aliquots of virus were stored at -80°C and defrosted on ice prior to use.

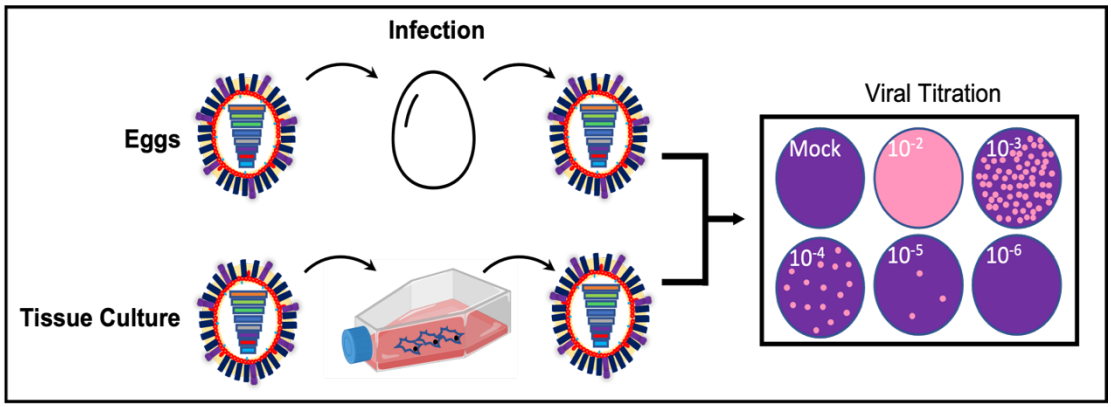


Figure 2-3: Propagation of IAV was performed either in embryonated chicken eggs or in NPTr tissue culture. After propagation the viruses were titred on MDCK.

PR8 (A/Puerto Rico/8/1934 (H1N1)) – MDCK culture

Sw87 (A/swine/England/163266/1987 (H3N2)) – received from Dr. Oliver Lin and propogated by culture in chicken eggs.

UDL (A/chicken/Pakistan/UDL-01/2008 (H9N2)) received from Dr. Abraham Lee

50-92 (turkey/England/50-92/1991 (H5N1)) received from Dr Hui-Min Lee and propagated in chicken eggs.

2.5.2 IAV Egg Propagation

Some IAV strains can be propagated in embryonated hen's eggs. To check egg viability, a flashlight was held close to eggs to confirm blood vessel integrity. For each virus, and for mock infections, 5 eggs were inoculated. Using an egg hole punch, a hole was made in the top of the egg and 100 μL of IAV diluted to 10^4 PFU/mL in pre-warmed SFM was inoculated into the allantoic fluid through a 25G needle. The pierced hole was sealed with tape and eggs were incubated at 37.5°C. After 72 hours, eggs were rechecked for viability and placed at 4°C overnight to kill the embryo.

The following day, the top of each eggshell was removed to a level just above the air sac. The allantoic fluid was aspirated into 15 mL Falcon tubes that were then centrifuged at 4000 x *g* for 10 minutes at 4°C before aliquoting the supernatant into 1 mL samples for storage at -80°C. To quickly screen for viral propagation a hemagglutination assay was performed to a sample from each egg that was not moved to long term storage (Killian, 2014). Samples that had haemagglutination were subsequently titred for infectious virions by plaque assay in MDCKs.

2.5.3 Influenza Infections

For IAV propagation in cells, MDCK were seeded in Nunc™ T25 flasks to be 80% confluent at point of infection (seeded at multiplicity of infection (MOI) = 0.01) or with 1 x 10^5 NPTr for experiments measuring IAV growth kinetics. For IAV growth experiments the infections were performed 6 hours post seeding. The MOI of viral infections was either 0.01 or 0.1.

Prior to infections cells were washed twice with SFM to remove residual serum. Infectious virus was diluted to the appropriate MOI in 1 mL SFM containing 0.14% (v/v) 0.22 μM filtered Bovine Serum Albumin (BSA, Life Technologies) and 1 μg/mL tosyl phenylalanyl chloromethyl ketone – treated trypsin (TPCK, Thermo Fisher Scientific) and overlayed on the appropriate cells for 1 hour at 37°C with gentle rocking every 15 minutes to distribute virions. 5 mL of the SFM/TPCK/BSA medium was added after 1 hour and cells were maintained in an incubator with a humidified atmosphere containing 5% CO_2 at

37°C. For propagation, the supernatant was aspirated for titration when all cells showed cytopathic signs. For viral growth experiments with time points, 150 μL of medium from flask was removed at 12-hour intervals and transferred to -80°C. All time points taken in triplicate from three separate flasks.

2.5.4 Plaque Assays

Plaque assays are a method of titrating virus from supernatant of infected cell cultures and provide a measurement of a viruses the ability to propagate (Figure 13). On day 1, MDCK cells were seeded in Nunc™ 6-well plates with full DMEM 12-24 hours before addition of IAV. On day 2, ten-fold serial dilutions of IAV samples were made in Nunc™ 96-Well Polypropylene DeepWell™ dilution plates with 500 μL DMEM in each well. 55 μL of virus stock was added to the first well and mixed by pipetting up and down. This well had a concentration of 10^{-1}. Using a fresh tip, 55 μL of the first well was transferred to the second well and mixed. This process was repeated down the dilution series to 10^{-6} or 10^{-7} as required.

The medium was aspirated from the 6-well plates before each well was washed twice with 2 mL of PBS (Roslin Stores). 400 μL of the serially diluted virus was added to the wells, starting with the most dilute first and moving up in concentrations. The infected cell cultures were incubated at 37°C for 1 hour to allow virus adsorption, with gentle rocking at 15-minute intervals to evenly distribute virions around each well. Overlay was then prepared to ensure that transmission of virions would only occur between neighbouring cells, and thus plaques would form. The overlay Avicel (Sigma-Aldrich) was used as follows per 6-well plate; 7 mL Avicel, 7 mL SFM, 14 μL TPCK (1 mg/mL) and 280 μL 7% BSA. Each well gently had add 2 mL overlay added along the wall without removing the 400 μL of virus containing medium. Plates were returned to the 37°C incubator. After 48 hours, 1 mL of 10% Neutral Buffered Formalin (NBF) was gently added to the Avicel overlay solution and incubated at room temperature for at least 30 minutes to fix cells. The NBF and Avicel solution was aspirated, and each well was gently washed twice with 2 mL of PBS. To the empty wells, 2 mL of 0.1% toluidine blue dissolved H_2O (w/v) was added and incubated for 20

minutes. Plates were poured off and washed in bowls of tap water to remove the remaining toluidine blue solution. The plates were inverted and left until dry, when counting of the plaques was possible Wells with 10-100 plaques were recorded and absolute viral titre was determined by calculating $2.5\ x\ number\ of\ plaques\ =\ plaque\ forming\ units\ (PFU)/mL$ to account for only 400 μL being initially added.

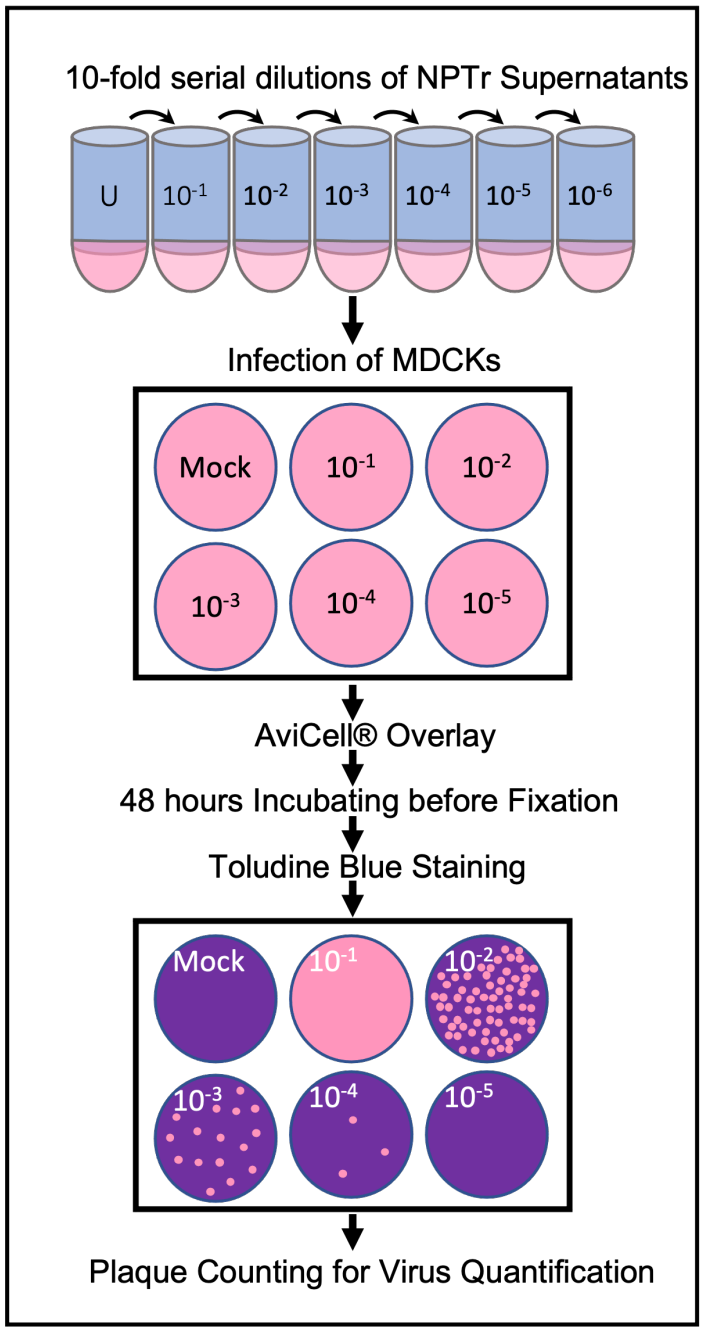


Figure 2-4: Schematic of the workflow for plaque assays for titring infectious virions.

2.5.5 Minigenome Assays

Minigenome assasy were performed in Nunc™ 12-well plates with the NPTr cell lines to measure the level of IAV polymerase activity. Minigenome reporter assays are also widely known as polymerase assays and minireplicon assays. Through transfection of plasmid vectors with mammalian promoter (pCAGGs) that express the minimal components required for Influenza polymerase (FluPol) to function in a cellular environment, as well as a reporter for vRNA production a proxy measurement of FluPol activity can be infered. All pCAGGs plasmids used were received from Dr. Thomas Peacock, Imperial College London, and the original swine reporter construct is described in Moncorge et al., 2013. The only vector created in this project was the pCAGGs_ANP32A-12bp which was cloned into the empty pCAGGs vector by T4 DNA ligation. T4 ligation was performed according to the manufacturer's protocols (Promega), using 100 ng gBlock insert (IDT), 50 ng pCAGGs vector, 0.5 μL T4 Ligase 1 μL T4 Buffer in a 10 μL reaction that was incubated at room temperature overnight before bacterial transformation as described in Section 2.2.2. Plasmids received were confirmed by diagnostic restriction digest as described in Section 2.2.4.

All transfections were performed in 12-well plates with 1.8 x 10^5 NPTr seeded immediately prior to the preparing the transfection reagents. Following transfection (Section 2.1.5), plasmids were mixed at the following ratios per well; PB1 (80 ng): PB2 (80 ng): PA (40 ng): NP (160 ng): pPolI-Luciferase (80 ng): p*Renilla* (80 ng): (pANP32*x* (100 ng) if required) (Figure 14A). The pPolI-Luciferase vector encodes for reverse complemented mRNA of firefly luciferase that is flanked by IAV promoter sequences. This transcript is then transcribed by a swine RNA promoter to create a -ssRNA (IAV-like) (Figure 14B). The firefly luciferase mRNA is then only generated in the presence of efficient FluPol activity. The expression of *Renilla* is from the constitutive pCAGGs promoter, and the ratio of firefly luciferase to *Renilla* luciferase provides a proxy for discerning the level of FluPol activity. The fold change between samples without PB2, an essential protein for FluPol activity, and those with all FluPol

components was assessed to ensure that there was more than a 50-fold increase in firefly luciferase expression to check that the data generated would be informative of changes in polymerase activity and not an artefact of low expression being highly variable (Figure 15).

24 hours after transfection, bioluminescence signal of firefly and *Renilla luciferase* were both measured using the dual-luciferase system (Promega). Briefly, cells were lysed by incubation with 200 μL of passive lysis buffer for 30 minutes on a slowly rocking platform at room temperature. After scraping carefully with a pipette tip to fully lyse cells, 20 μL of each sample was transferred to a white flat-bottomed 96-well microplate (Corning™ cell-culture-treated) in triplicate. LarII and STOPnGLO reagents were made according to the manufacturer's instructions and diluted 1:1 in Luciferase Dilution Buffer (Roslin; 5 mL 1M Tris (pH 7.5), 200 μL 0.5M EDTA (pH 8), 45 mL H_2O with final pH of 7.75).

Using a BioTek PowerWave HT microplate spectrophotometer, bioluminescence was measured with gain values set to 135, with measurements from each well taken 10 milliseconds (ms) apart. Data was collected through Gen 5 software (BioTek). First, using a multichannel pipette 20 μL LarII reagent (firefly luciferase measurement) was added to each sample. The plate was removed from the microplate reader and 20 μL of the STOPnGLO reagent (*Renilla* measurement) was added to each well and bioluminescence readings were taken on the same settings.

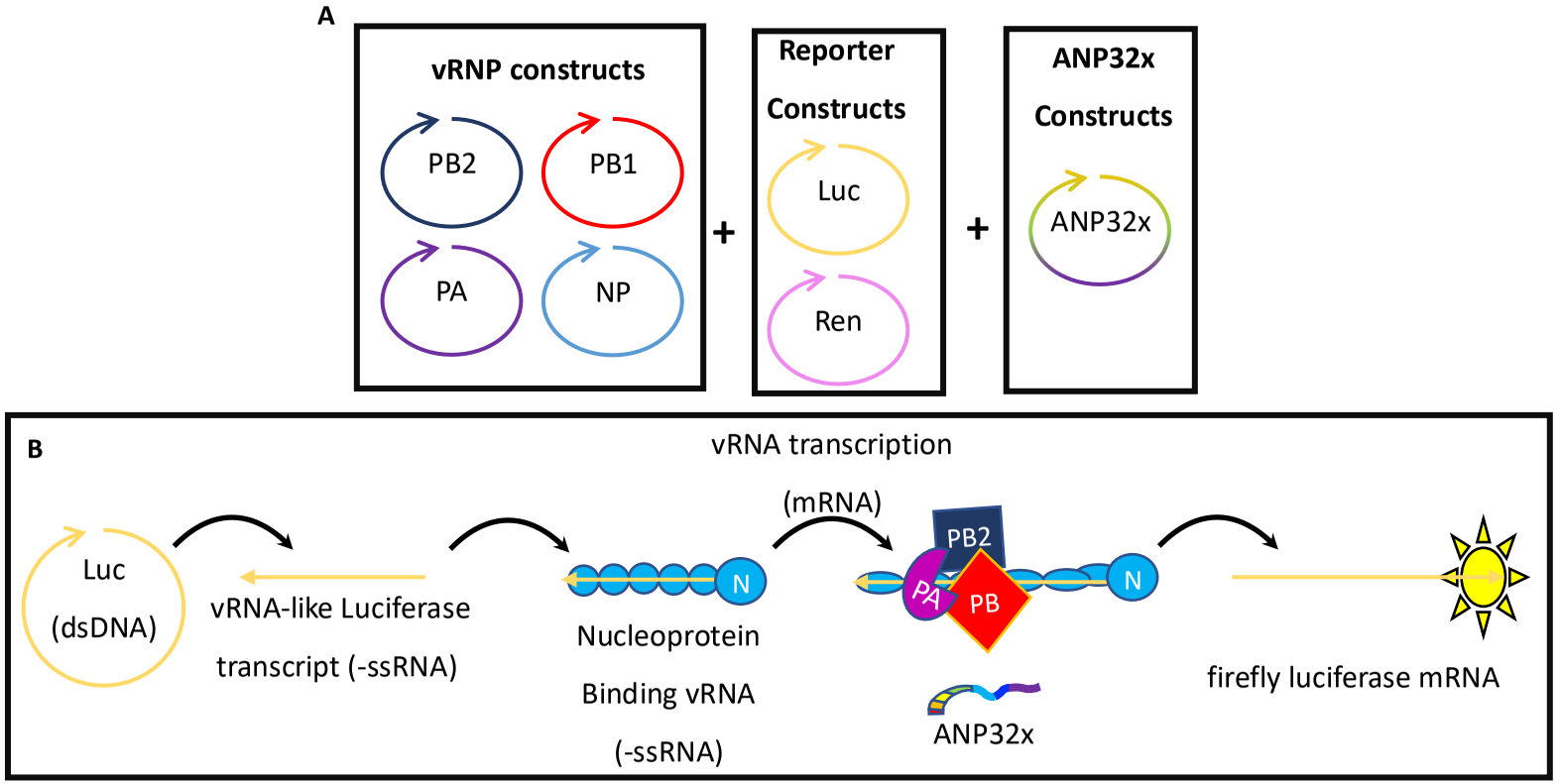


Figure 2-5: The premise of a minigenome assay that functions as a proxy for measuring IAV polymerase activity**. A)** The plasmid vectors transfected into each well in a minigenome assay. The ANP32x construct was either ANP32A cDNA, ANP32B cDNA or no sequence in pCAGGs vector. **B)** The premise of a minigenome assay is to determine IAV polymerase activity through a firefly luciferase gene that is a decoy for functioning FluPol. The more efficient the FluPol is in the cellular context, the more firefly luciferase mRNA will be generated. The firefly luciferase activity is normalised to activity of a *Renilla* reporter construct.

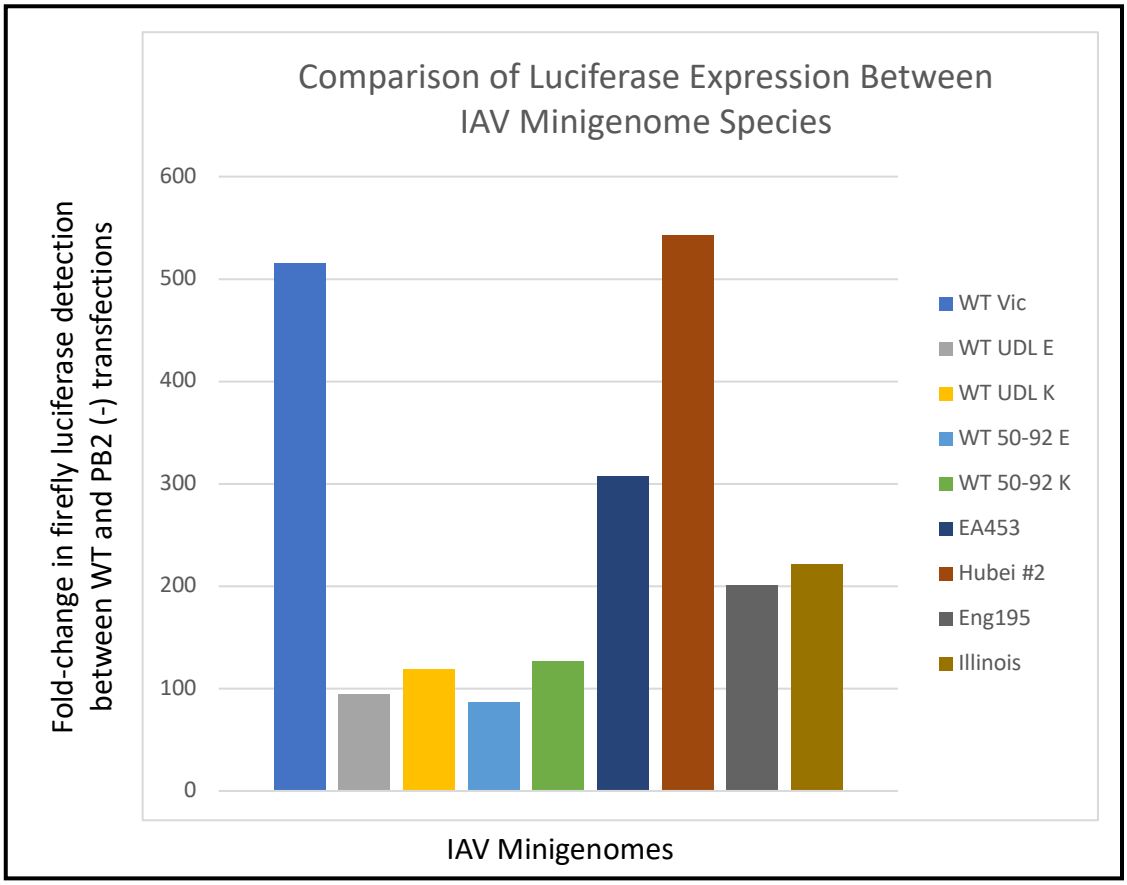


Figure 2-6: The fold-change in the detection of firefly luciferase with PB2 present and without PB2 present in NPTr to ascertain the level of firefly luciferase expression through 'leaky' plasmid gene promoters and to ensure that the entire FluPol complex was required to initiate FluPol activity.

2.6 Simulation Tools

All simulations were performed using RStudio (R core team, Austria). The methods applied in the simulations are described in detail within Chapter 3.

2.7 Software

Tool	*Producing Organisation*	*Utility*
Geneious Prime	Geneious	Sequence analysis and bioinformatics
CRISPOR	Tefor Infrastructure	gRNA design
TIDE	Bas Van Steensel lab	gRNA screening
ICE	Synthego	gRNA screening
PrimerBLAST	NCBI	Primer design
Ensembl	EMBL-EBI	Genome browsing
Image J (FIJI)	NIH, Contributors	Image analysis
I-Tasser	Zhang Lab, Michigan	Protein structure prediction
RStudio	R Core Team	Breeding simulations
Prism	GraphPad	Statistical and plot generation
MicroManager	Vale Lab, NIH	Microscopy image capturing
Robetta	Baker Lab, Washington	Protein structure prediction
Office	Microsoft	Text and presentation
MiniTab	Minitab LLC	Statistical Analysis
BLAST	NCBI	Sequence homology
Gen5	BioTek, Agilent	Miroplate reading

Figure 2-6: The software that contributed to during this thesis project.

3 Modelling the Introgression of IAV Resistance Alleles into a Commercial Swine Herd

The results contained within this chapter have been submitted for publication to *Genes*. The content has been modified slightly and includes a small amount of additional text for thesis context and has been reformatted to fit within this thesis.

3.1 Abstract

With a globally increasing prevalence, it is evident that current control measures for swine Influenza A Virus are not fully effective. Developing viral resistance with genetic technologies could complement current measures controlling endemic swine Influenza A Virus, helping to improve animal welfare standards and the economic efficiency of pig production whilst also reducing the potential for the emergence of a human pandemic by zoonotic transmission. We have created a simulation model to assess the genetic and economic implications of various gene-editing methods with the potential to be applied in a commercial, multi-tiered swine breeding system.

Our results demonstrate that extending the length of the gene-editing program was negatively associated with genetic progress in commercial pigs and that the time required to reach fixation of resistance alleles was reduced if the efficiency of gene-editing is greater. Previously unassessed by other gene-editing models were digenic gene-editing approaches, the inclusion of gene-editing associated genetic mosaicism in progeny, and the effects of selection accuracy. In a digenic resistance model there was an increased number of zygotes gene-edited and the time to reach fixation when compared to a monogenic model. In all scenarios, the likelihood that gene-edited alleles would be in the germline, determined by the level of genetic mosaicism, had a greater effect on the time required to reach resistance allele fixation and genetic progress of the herd than gene-editing efficiency and zygote survival. The economic analysis highlights that selection accuracy will not affect the duration

of gene-editing and the investment required compared to the effects of gene-editing associated mosaicism and the swine Influenza A Virus control strategy on farms.

These modelling results provide novel insights into the economic and genetic implications of targeting two genes in a commercial pig gene-editing program and the effects of selection accuracy and efficiency of successfully gene-editing the germline progenitor cells. Improving the likelihood that germline transmission in the gene-editing process will offer the largest improvements in outcomes associated with gene-editing programs in a multi-tiered pig herd. The economic analysis suggests that the presence of a vaccination program will be a major determinant of whether the breeding programs will be financially incentivised to incorporate gene-editing for swine Influenza A Virus resistance.

3.2 Background

Influenza A Virus

Influenza A virus (IAV) is a significant pathogen of humans and several keystone agricultural species, such as chickens and pigs. Its global distribution and ability to cross zoonotic barriers contribute to its potential as a source for emergent pandemics (Mostafa et al., 2018). This pandemic potential is exemplified by the swine originating 1918 Spanish 'Flu pandemic that is estimated to have claimed 50 – 100 million lives (Barclay and Openshaw, 2018). Having effective control measures to reduce IAV prevalence and transmission in swine herds will assist in mitigating the emergence of another pandemic strain (Thacker and Janke, 2008). Furthermore, although annual epidemics of swine IAV (swIAV) have low mortality rates, high morbidity rates are associated with lower animal welfare standards and reduced productivity that ultimately affects economic performance of the pig industry (Gumbert et al., 2020; Janke, 2013). With a global herd-level seroprevalence of 72.8%, swIAV is an endemic problem faced by most hog farmers (Baudon et al., 2017). The industrial expansion of pig farming has been associated with an increased swIAV prevalence (Baudon et al.,

2017), and a continuation of this trend will therefore likely contribute to an increasing prevalence.

With increasing swIAV prevalence, the likelihood of two distinct strains infecting a single host grows. In the event that multiple strains of IAV co-infect a host, the eight segmented RNA genome of IAV can be reassorted (Nelson et al., 2011; Vijaykrishna et al., 2010). Genomic reassortment generates a novel virus subtype, one that may have improved potential for intraspecies or zoonotic transmission into naïve hosts (Neumann et al., 2009; Watson et al., 2015). The difficulty of controlling swIAV stems from its heterogeneity and ability to rapidly evolve. Removing pigs as a reservoir for IAV infection will have the dual benefit of reducing the burden of disease in pigs and reducing the potential for pandemic emergence into humans following genomic reassortment in swine.

3.2.1 Influenza A Virus Control

Because swIAV has a low mortality rate, there is a large amount of variability in the application of control measures (Detmer et al., 2012). Herd management and basic biosecurity are the most widely applied measures, with quarantine of new arrivals and cleansing of pens between stock movements amongst the simplest methods. Where industrialised piggeries have been adopted, there is a wider uptake of proactive control in the form of vaccination programs (Sandbulte et al., 2015). Success of vaccination programs is variable due to the intrinsic evolutionary capability of swIAV. Additionally, because only endemic swIAV strains are targeted, vaccination does not prevent human-swine transmission (Vijaykrishna et al., 2011). With a limited arsenal of swIAV control techniques available, it is important we critically appraise the tools at our disposal. Genetic-based technologies such as gene-editing offer a novel and proactive control strategy that would complement current measures (Salvesen and Whitelaw, 2021).

3.2.2 Gene-editing for swIAV Control

As an intracellular parasite, IAV relies on host proteins to support their limited complement of proteins and therefore to complete their replicative

cycle (Han et al., 2018; Watanabe et al., 2014). Its reliance on host factors means that disruption of virus-host protein interactions by alteration of specific amino acids could impede viral replication, thereby reducing infection and/or transmission. Targeted and specific changes to the DNA sequence can be made using gene-editing technologies such as CRIPSR/Cas9 (Ran et al., 2013b). Examples of CRISPR/Cas9 being utilised for viral resistance includes pigs resistant to Porcine Reproductive and Respiratory Syndrome virus (PRRSV) (Burkard et al., 2017; Prather et al., 2017)and Transmissible Gastroenteritis virus (TGEV) (Whitworth et al., 2019), as well as chickens resistant to avian leukosis virus (Kheimar et al., 2021; Koslová et al., 2018). Identified genotypes which confer resistance to viral pathogens in pigs are haploinsufficient, and therefore successful editing of both alleles is necessary for full resistance (Burkard et al., 2017; Whitworth et al., 2019). *In vitro* data from human and avian cell models suggests that, by application of the same principles to IAV-relevant genes, there is promise for the creation of swIAV resistant pigs (Long et al., 2016; Moncorge et al., 2013).

Modelling the economic repercussions, considering negative factors such as the opportunity cost associated with reducing genetic improvement trends by biasing selection for disease resistance alleles and the direct costs of a gene-editing program, against positive factors such as the benefits of improved individual hog productivity from being swIAV resistant and a reduction in veterinary costs in commercial pig production is an important step in understanding the value proposition of gene-editing in commercial pigs. We have modelled the introgression of swIAV resistance alleles in a multi-tiered pig population, whereby editing a single gene confers full resistance (monogenic), as observed with PRRSV, and where digenic gene-editing on either the same or discrete chromosomes is required for full viral resistance.

From the available literature we have not identified a model for integrating alleles by gene-editing into a multi-tiered pig breeding pyramid, and for other species a digenic model has not been published (Bastiaansen et al., 2018; Mueller et al., 2019). In the pyramid breeding structure employed in commercial pig breeding, gene-editing could occur only in the top breeding tier,

with alleles flowing down by selection to the Finisher herd at the base (Figure 3-1), making it a particularly efficient breeding system for allele dissemination.

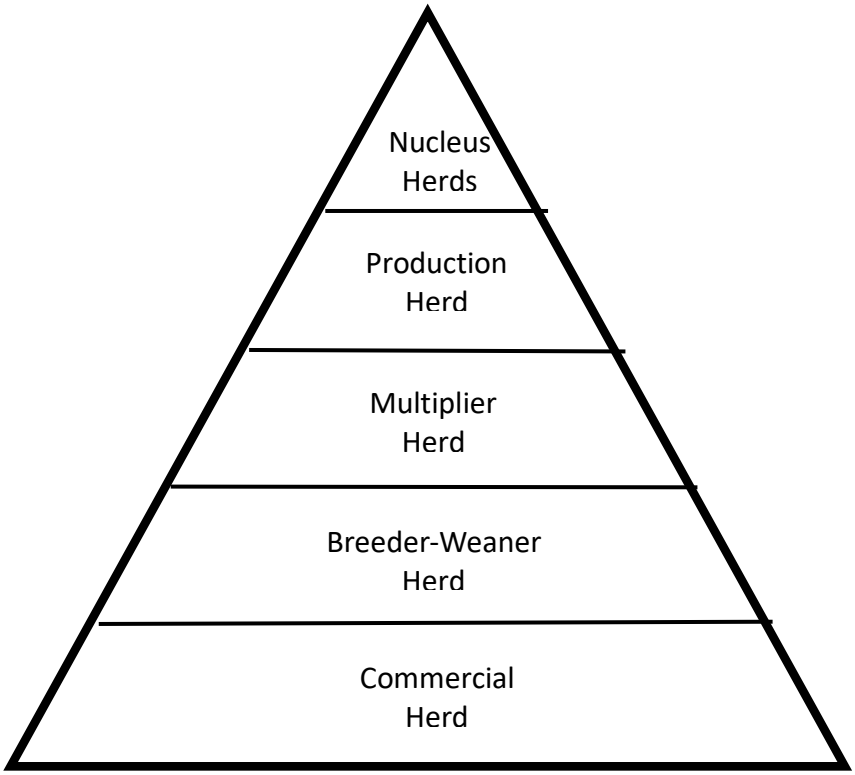


Figure 3-1: Outline of commercial pig breeding systems as designed in the simulations. Schematic representation of the pyramidal structure and the herds and tiers of a commercial pig breeding system as simulated.

3.2.3 Methods Modelled

Our simulation model considered four methods of getting CRISPR/Cas9 gene-editing reagents into zygotes (Figure 3-2A) (McFarlane et al., 2019; Navarro-Serna et al., 2020); 1) microinjection (Hai et al., 2014), 2) electroporation (Tanihara et al., 2016), or transduction of zygotes with recombinant adeno-associated virus (AAV) vectors, performed on zygotes 3) *ex vivo* or 4) *in vivo* (Mizuno et al., 2018; Yoon et al., 2018). These methods have different efficiencies of gene-editing, rates of zygote death, and procedural costs. All simulation parameters are based on CRISPR/Cas9 data for gene-editing by Non-Homolgous End Joining (NHEJ) using one sgRNA for each target gene. We felt it was a relevant decision to base the model on NHEJ and not the more refined HDR approach given that the use of only NHEJ based gene-editing is reaching regulatory approval in several nations.

Microinjection is well established in pigs as a method of introducing gene-editing reagents into zygotes by physically injecting the reagents by needle penetration (Hai et al., 2014). Microinjection requires a skilled technician with specific micromanipulation equipment to perform the procedure. The micromanipulation step necessitates the handling of individual embryos, making it a time consuming and low throughput technique. Swine zygotes have been successfully gene-edited by microinjection of Cas9 mRNA (Burkard et al., 2017; Park et al., 2017; Whitworth et al., 2019), plasmid based Cas9 (Lei et al., 2016) and Cas9:sgRNA ribonucleoprotein (RNP) (Tanihara et al., 2019, 2018) into the zygote cytoplasm on multiple occasions and by direct pronuclear injection of plasmid-based Cas9 (Chuang et al., 2017).

Electroporation works by transiently disrupting the zona pellucida and zygote membrane with electrical impulses, allowing movement of gene-editing reagents from the surrounding solution (Tanihara et al., 2016). Electroporation is less well established in research, but likely to be more commercially attractive due to its capacity for high-throughput and generally higher gene-editing efficiency. There are few published electroporation examples, and they are all performed *in vitro* using Cas9 RNP as the gene-editing substrate (Tanihara et al., 2020a, 2020b, 2016; K. Wang et al., 2015).

AAV are diverse viruses from the Parvoviridae family that can infect a range of mammalian host species and transiently deliver genes by expression of their ssDNA genome (Salganik et al., 2015). Transduction of zygotes with recombinant adeno-associated virus (AAV) vectors, performed on zygotes *ex vivo* or *in vivo*, has to date only been performed in rodent species (Mizuno et al., 2018; Yoon et al., 2018). The difference between the AAV procedures is addition of the viral vector either directly into the oviduct on the day of mating for *in vivo*, whereas AAV *ex vivo* can be performed on IVF-derived or flushed embryos before reimplantation into a surrogate mother. If AAV reagents can be optimised for use on pig zygotes, the relatively low skill and cost requirements alongside its capacity to be scaled up could make it particularly appealing commercially (McFarlane et al., 2019). Furthermore, *in vivo* AAV could be implemented alongside artificial insemination (AI) procedures, making it a

seamless procedural change for current breeding programs. Given that experimental results for all gene-editing methods in zygotes are highly variable, the values identified from literature and assigned to parameters in this simulation model are illustrative.

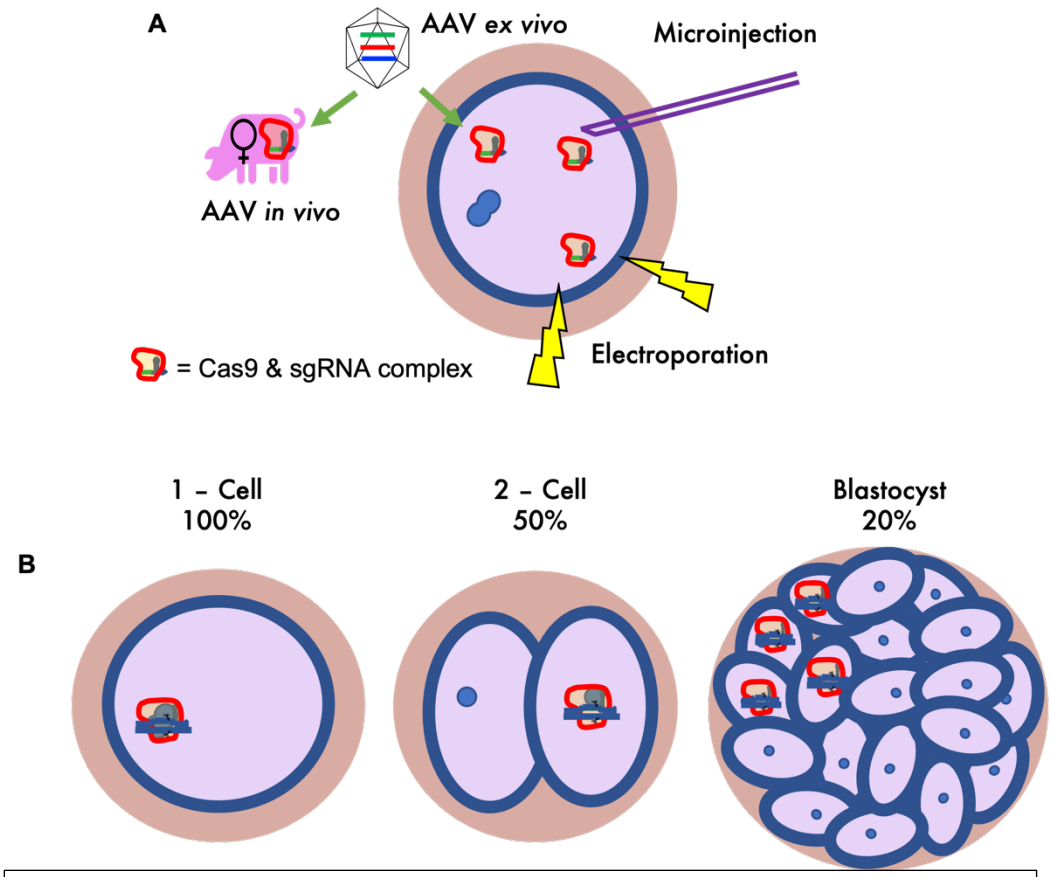


Figure 3-2: Schematic representations of gene-editing techniques considered for commercial applications and gene-editing introduced mosaicism. A) Gene-editing methods applied to porcine zygotes. B) The stochastic distribution of gene-editing reagents during embryonic division or delayed and asymmetrical CRISPR/Cas9 activity can lead to a reduced likelihood of germline transmission as a result of mosaicism.

3.2.4 Mosaicism

An important factor not included in previous livestock gene-editing simulation models is the efficiency at which the gene-edited alleles are introduced in the germline and are therefore permanently heritable (Mehravar et al., 2019; Navarro-Serna et al., 2020; Hennig et al., 2020). The distribution of different alleles in a single organism is known as genetic mosaicism. Several livestock species have reported zygote gene-editing to have mosaicism, and its

presence limits the effective application of gene-editing (Navarro-Serna et al., 2020). Mosaicism occurs during embryogenesis when a mutation happens to alleles asymmetrically or after the first cell division, leading to cellular descendants having different genotypes to their ancestors (Burkard et al., 2017; Park et al., 2017) (Figure 3-2B). As a natural phenomenon, if a *de novo* mutation (DNM) arises during embryogenesis, the developed organism will be genetically mosaic. With gene-editing, genomic variability will arise from variable enzymatic activity of CRISPR reagents and their stochastic distribution following the first cell division. The level of mosaicism is relevant in breeding programs because the heritability of alleles is disrupted if the changes in the DNA are not present in the germline stem cells. Here, mosaicism is referred to specifically in the context of describing the heritability of alleles and is distinguished as germline transmission.

Assumed within these simulations, that are using an NHEJ approach, the genetic indels introduced will be variable on each allele. It is preferential to have symmetrical alleles for biological consistency when studying impacts, but if changes in DNA sequence cause a functional knockout but have different alleles, there are unlikely to be unintended consequences provided there are not large deletions, insertions or inversions at the target site. It is therefore important that gene-editing reagents are introduced into a single cell zygote to allow immediate editing of only two alleles, or as has been previously demonstrated, into the oocyte prior to IVF, which means they are present immediately after gamete fusion or if targeting an X chromosome DNA sequence, the Cas9 activity can occur in the oocyte (Su et al., 2019). The presence of multiple alleles arising from the gene-editing process at the target site during zygote gene-editing, without sequencing for genotype before uterine implantation, is a prevalent issue observed in all mammalian livestock species. Simulating the level of germline transmission in this modelling will help understand the impacts of mosaicism on different gene-editing schemes.

3.2.5 Previous Modelling of Gene-Editing

There has been previous modelling of integrating gene-edited alleles into livestock herds. Two non-specific breed models have assessed the Promotion of Alleles by Genome Editing (PAGE) (Jenko et al., 2015) and the Removal of Alleles by Genome Editing (RAGE) (Johnsson et al., 2019). PAGE observed that inclusion of gene-editing alongside genomic selection could achieve substantial improvements in genetic progress, provided that the quantitative trait nucleotides have a large effect. The RAGE study observed that removal of deleterious variants required the targeting of multiple alleles in a multiplexed genome-editing strategy to be effective.

Modelling in Cattle has been performed with a specific focus on the POLLED allele, that causes cattle to be born with a hornless phenotype. These simulations were performed on US dairy cattle populations and northern Australian beef cattle (Mueller et al., 2021, 2019). In both studies it was concluded that gene-editing alongside genomic selection maintains the highest rates of genetic gain as well as introgression of the desired allele. The use of somatic cell nuclear transfer (SCNT) means that the efficiency and technique of gene-editing used has little relevance to the final outcome. Continuing with a focus on the POLLED allele in dairy cattle, but performing gene-editing on zygotes, modelling observed that reducing the gene-editing efficiency increased the time to allele fixation and lowered the selection response achieved (Bastiaansen et al., 2018).

3.2.6 Chapter Aims

The aims of this thesis chapter were to model how alleles introduced by gene-editing into commercial pig herds would flow from the nucleus herds down to the commercial breeders. The simulation models recorded the level of gene-editing required to reach genotypic and phenotypic fixation in the Finisher herd of a commercial pig breeding system. To compare prevailing gene-editing methods we assessed varying gene-editing efficiencies and zygote death rates under different levels of germline transmission. A comparative economic

analysis was carried out to assess trade-offs and the financial capacity required to deploy a gene-editing program in a commercial pig breeding system.

3.3 Methods

This simulation model was designed to assess the flow of gene-edited alleles through a multi-tiered commercial pig breeding pyramid based upon a three-breed and five-tiered pyramid breeding structure (Figure 3-3) (Knox, 2016; Visscher et al., 2000). Selected methods of gene-editing were assessed with variable levels of germline transmission. The model was developed using R software (R Core Team, Austria). The code is available in the GitHub repository (https://github.com/hamishsalvy/SwineFluGene-Editing). All data visualisations were created using the *plotly* package (R Studio) with the mean values taken from 10 iterations for each gene-editing method with independent germline transmission levels and selection accuracies.

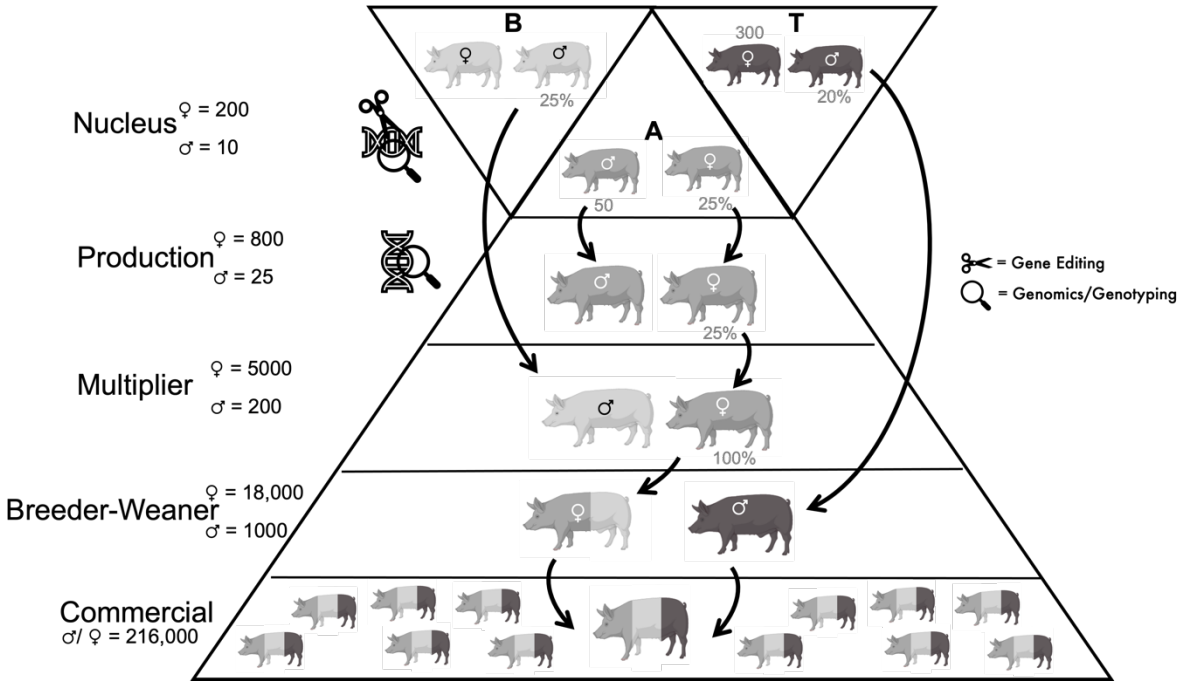


Figure 3-3: Breeding population structure and dynamics used in our simulation model. Numbers above the pigs indicate the number of boars/dams used for breeding in each batch. Percentages indicate the proportion of available females from the tier above that are transferred down a tier.

3.3.1 Base Population

Initially, a population of Nucleus pigs without swIAV resistance alleles was created and split into 3 breeds, “A”, “B” and “T” (Figure 3-3). Simulations were performed assuming herd management in batches. Each batch was 28 days, which allowed for the assumption of 4 batches (112 days) to be a dam pregnancy length and 1 batch to be the lactation period of piglets and the return to oestrus period (Soede et al., 2011). These periods will vary by farm and system but consistent modelling meant dams could be selected for breeding every 5 batches and representative of breeding swine cycles (Visscher et al., 2000). All sows selected for breeding were assumed to be successfully impregnated in the first oestrus after weaning and piglet litters were standardised at 12. Each batch was distinct, with mating only occurring on day one. Breeding age boars and gilts (>8 batches old (Soede et al., 2011)) were made available for selection every batch and culled after 38 and 42 batches, respectively. Random mortality of all pigs over 1 month of age was applied at 2.5% every batch. A summary of the breeding parameters used are presented in Table 3-1.

Parameter	**Value** (in batches)
Sow gestation length	4
Farrowing interval	5
Gilt age at first mating	8
Boar age at first mating	8
Litter size (No of piglets)	12

Table 3-1: Summary of the parameters used for breeding functions in the simulation model. All age and time values are reported in 28-day batches.

Mating pairs were selected according to their genetic merit, determined in a nested design by sorting eligible boars and females in descending order of their genetic merit value. For example, in the “A” Nucleus population, 200 females were selected for mating in each generation (Figure 3-4). The 10 top boars were crossed with the top 10 females with each ordered by descending genetic merit, each subsequent group of 10 females was bred with the same 10 boars. This is known as a nested breeding design (Rutten et al., 2002). The “T” Nucleus population supported 300 females to ensure enough boars are available for natural breeding with the Breeder-Weaner tier. Selection parameters of breeding animals and numbers/proportion of pigs moving down the pyramid are described in Figure 3-3B.

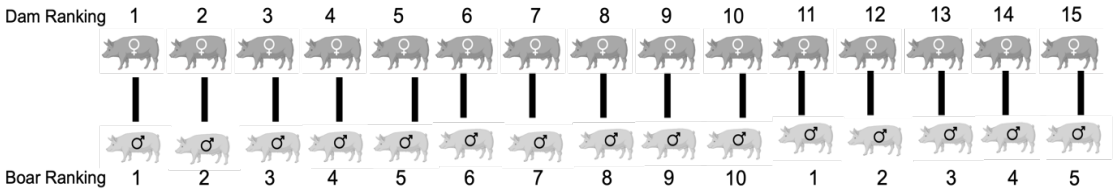


Figure 3-4: Schematic of the nested breeding design used in this modelling. This is an example of the first 15 animals selected and would continue until all breeding animals are selected.

Piglets had an equal probability for sex assignment and alleles were inherited according to Mendelian principles. Founder pigs created for the Base Population pigs were assigned a Breeding Value (BV) by drawing a random variate from a normal distribution with a mean of 0 and standard deviation of 10 (CCSI, 2012). This breeding value was assigned as an aggregated ‘genetic merit’ and not by specific trait indexing. Each piglet was assigned a BV from half of the combined maternal and paternal value plus a Mendelian sampling term. Selection was based on a genomic prediction of these BVs, where the genomic prediction had a heritability of 1 (Dekkers, 2007) and the accuracy of the genomic prediction was set at 1, 0.8 or 0.5 by scaling the genetic standard deviation (indexSD - 10) used in the EBV estimation by the genomic prediction accuracy.

To establish the pyramidal structure, breeding within the Nucleus tier was simulated for 20 batches before the Production tier was initiated. After 45 batches, flow down to the Multiplier tier began, followed by the Breeder-Weaner tier after 55 batches. After 100 batches the pyramidal structured base population used for all forward simulations was established. Piglets were born into their parental tier and could only be present in a single tier. Mating of pigs in the Nucleus and Production tiers were simulated as artificial insemination (AI), with boars used concurrently in these tiers, whilst the Multiplier and Breeder-Weaner tiers were mated by conventional breeding, meaning boars could only be available for selection in a single tier for each batch.

3.3.2 Forward Simulations

Using the established base population, four gene-editing methods were applied to confer monogenic or digenic resistance to swIAV. The inheritance mode of digenic resistance was either linked (with no meiotic recombination) or unlinked to inheritance of resistance genes (Figure 3-5). Each simulation ran for 120 batches (~10 years).

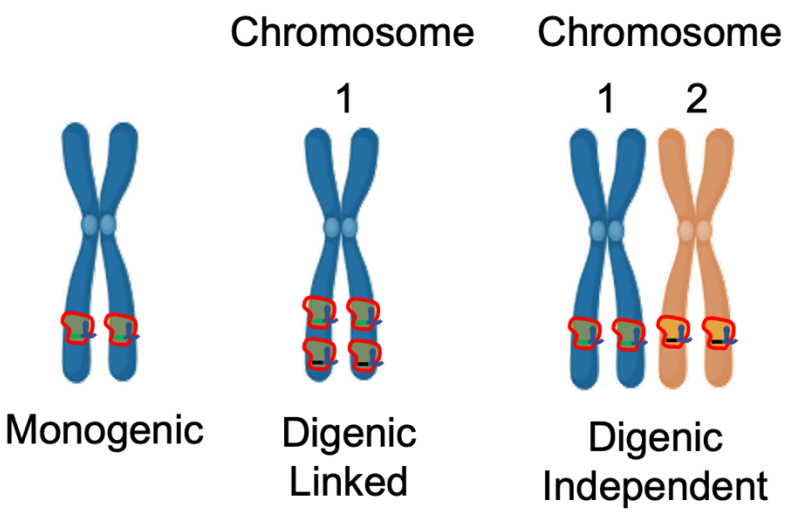


Figure 3-5: The topography and linkage of genes for the simulations. Digenic linked gene targets are found on the same chromosome and therefore to not segregate independently during meiosis. Digenic independent gene targets are on distinct chromosome and are therefore inherited independently of each other.

Selection in the Nucleus and Production tiers was based on a point being assigned to each allele, creating an individual genotype score for each pig. Wildtype animals equalled 0 and digenic resistant animals equalled 4. Breeding animals were primarily selected according to their allele score, followed by selecting the top fraction of eligible mating boars and sows by ranking on genetic merit to the designated percentage or number of each tier. Resistance alleles were only selected for in the Nucleus and Production tiers where genotyping is carried out. In the Multiplier and Breeder-Weaner tiers only the genetic merit values from pedigree geneflow were considered to determine breeding females. The Finisher herd was included for forward simulations.

3.3.3 Gene-editing and Genetic Mosaicism

Gene-editing was applied to zygotes with wildtype alleles in the Nucleus A, B and T populations. The relevant parameters for each gene-editing method are outlined in Table 3-2. The estimated costs of gene-editing include pricing of reagents, embryo transfer, labour and animal husbandry to the point of piglet birth. For AAV based techniques, murine data was used as gene-editing efficiencies and zygote survival data was unavailable for porcine zygotes.

Gene-editing was performed to all zygotes from mating pairs with at least one swIAV resistance allele, with the editing efficiency being applied to zygote alleles individually and the death rate applied post editing and implantation. The phenomena of genetic mosaicism associated with gene-editing was modelled by reducing the proportion of successfully gene-edited alleles that are present in each animals' germline. By example, if gene-editing is successful in introducing the targeted edits in 20% of cells, the blastocysts are 20% mosaic and there was a 20% chance that the germline cells would be correctly edited (20%, 50% or 100%) (Figure 3-3). Zygote survival was assumed using the expected litter size of 12 piglets, with post implantation zygotes perishing to result in a reduced average litter size.

Gene-Editing Method	Editing Efficiency	Zygote Survival	Cost per Zygote	Sources
Microinjection (MI)	37.5%	40%	$100	(Hai et al., 2014)
Electroporation (EP)	60%	25%	$80	(Tanihara et al., 2020a, 2016)
Adeno-associated Virus *ex vivo*	90%	15%	$80	(Yoon et al., 2018)
Adeno-associated Virus *in vivo*	20%	75%	$10	(Yoon et al., 2018)

Table 3-2: Parameters for gene-editing functions used in simulation models. Gene-editing costs based are based on research lab data (personal communication from Dr Chris Proudfoot).

3.3.4 Economic Analysis

The economic analysis was built on selected cost and benefit components associated with implementing gene-editing to generate swIAV resistant pigs. This included the direct costs of gene-editing (such as having fewer pigs reaching slaughter due to zygote deaths) and a reduction in genetic progress arising from diverted selection pressure, against the financial benefit derived from improved productivity and reduced veterinary costs. The parameters used in the economic analysis are described in Table 3-3, with all $ values described herein being United States Dollars (USD).

The annual cost of editing was determined by multiplying the number of attempted zygote gene-edits by the cost of gene-editing per zygote. Costs of gene-editing were extrapolated from research lab data on gene-editing of porcine zygotes (personal communication, Chris Proudfoot). Each zygote death is a pig that can no longer be reared for slaughter and was therefore counted as lost revenue. The price of a finished pig was determined as $109.5, a ten-year mean of whole hog value in the USA (2010 – 2019) (Statista.com, 2019). The cost of swIAV in pigs, accounting for the co-morbidities of Porcine Respiratory Disease Complex (PRDC), has been estimated to be $10.31 (Haden et al., 2002). The reduction in the genetic merit of the Finisher herd from biased selection towards swIAV resistance alleles was determined as a monetary value using

$$Lost\ Merit\ (\$) = Z * Base(t) * number\ of\ commercial\ pigs\ slaughtered$$

(*Z* = proportion of genetic gain compared to control, Base = Annual genetic improvement in profit per pig, *t* = year). It was assumed that the potential for an annual genetic gain of $4 remained consistent over the entire simulation period.

Parameter	Value
swIAV Productivity Loss/Pig(Haden et al., 2002)	$6.60
swIAV Vaccination Cost/Pig (Haden et al., 2002)	$3.71
Annual Genetic Improvement/Pig (Statista.com, 2019)	$4
Herd Immunity(Romagosa et al., 2011)	90%
Interest Rate/Annum (df)	5% (0.05)
Editing Efficiency	Variable for gene-editing method (Table 3-2)
Zygote Death Rate	Variable for gene-editing method (Table 3-2)
Cost per Zygote	Variable for gene-editing method (Table 3-2)
Pig Market Value$_4$	$109.5

Table 3-3: A summary of the parameters relevant to the economic analysis of the simulation results. All monetary values are quoted in US dollars (USD).

The financial benefit derived from having swIAV resistant pigs was termed *health benefit*. For farms with vaccination, prior to gene-editing these farms still achieve an IAV-free productivity boost through the vaccination program. Here, the *health benefit* is the difference between the productivity boost and vaccination cost, which is applied only after the threshold of Herd Immunity (HI) is reached and vaccination can be stopped. For systems without vaccination, improved productivity was added for all phenotypically swIAV resistant pigs, and subsequently to all pigs after the HI threshold was reached. HI was calculated as 90% using $HI = (R0 - 1)/R0$ (Fine et al., 2011). R_0 of

swIAV transmission in unvaccinated pigs calculated to be 10.66 (Romagosa et al., 2011).

Annual costs were summed to generate a Real Value. The Real Value was multiplied by a discount factor (based on inflation of 5% (*r*)) to account for the financial opportunity cost and interest payments to determine a Present Value for each year (*t*) (Hermesch et al., 2013). The present value was captured over the ten years to produce a cumulative Net Present Value (NPV), as:

$$NPV = \sum_{t=1}^{n} \text{x} \ \frac{1}{(1+r)^t}$$

3.4 Results

Our results illustrate how different gene-editing parameters and gene-editing associated mosaicism will affect the flow of gene-edited alleles and genetic progression in a multi-tiered pig breeding pyramid. Further to the genetic facet of these simulations, our economic analysis outlines the considerations breeders should consider when determining whether it is effective to implement a gene-editing program for swIAV resistance.

3.4.1 Monogenic Modelling

When targeting a single gene, the proportion of phenotypically swIAV resistant pigs in the Finisher herd reached the HI threshold (90%) within 120 batches for all gene-editing methods at differing levels of germline transmission and had a delay associated with 20% compared to 100% germline transmission (Figure 3-6). For 50% germline transmission the delay was intermediary (Additional File 1). Monogenic data displayed is for simulations applying a the moderate-high selection accuracy of 0.8. Only the trend of genetic merit, and not the dissemination of alleles through the tiers of the breeding pyramid or the amount of gene-editing required was affected when adjusting selection accuracy (Additional File 2).

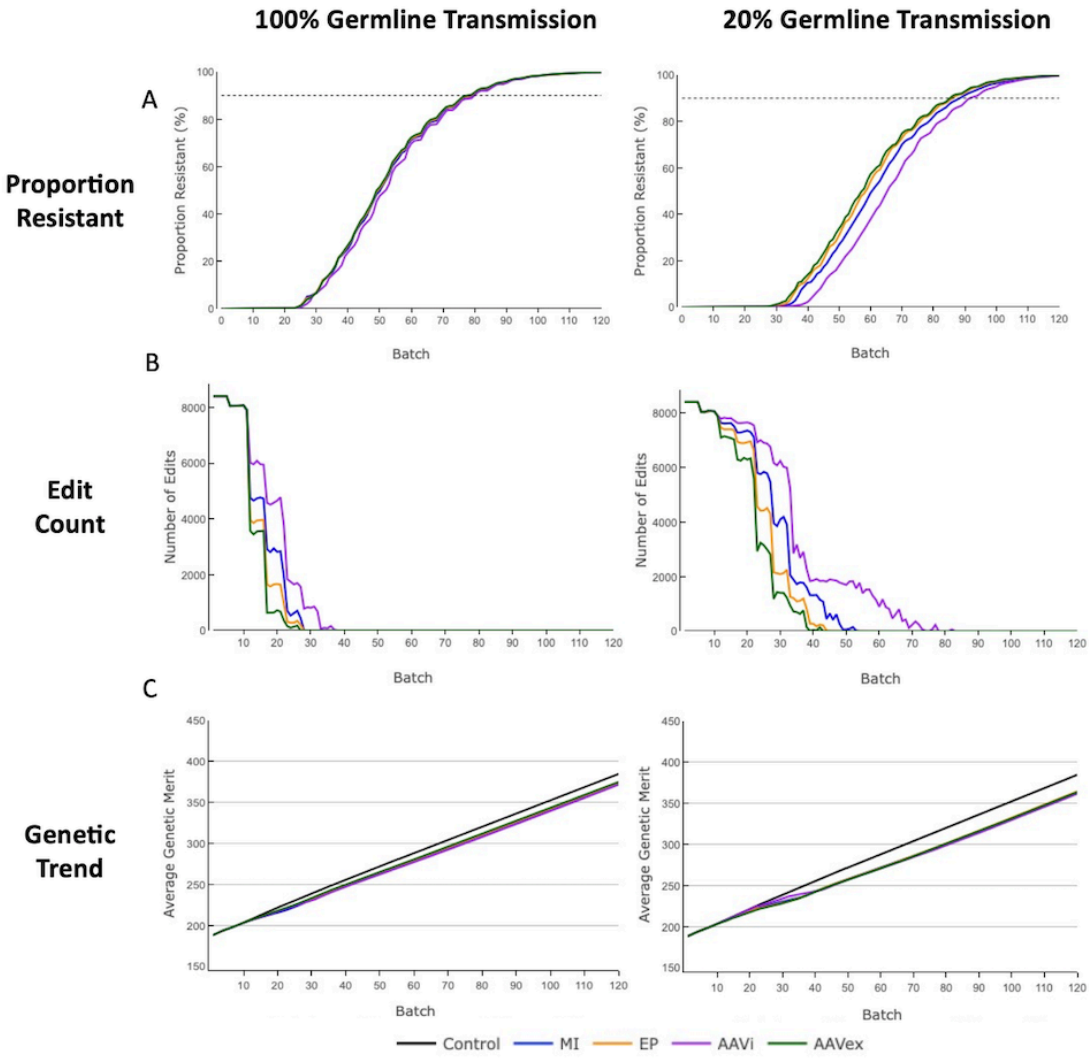


Figure 3-6: Monogenic swIAV resistance with 100% or 20% germline transmission with a selection accuracy of 0.8. MI = Microinjection. EP = Electroporation. AAVi = AAV *in vivo*. AAVex = AAV *ex vivo*. A) Proportion of pigs with phenotypic resistance to swIAV in the Finisher herd. The dashed horizontal line at 90% represents the herd immunity threshold. B) The number of zygotes that were attempted to be gene-edited in all Nucleus tiers per batch. C) The mean genetic merit of pigs in the Finisher herd.

The proportion of swIAV resistant pigs in the Finisher herd aligned by decreasing efficiency of gene-editing; AAV *ex vivo*, electroporation, microinjection, AAV *in vivo*. For 100% germline transmission there were only small differences in time to reach HI between each gene-editing method (<2%), with outcomes becoming more divergent with only 20% of blastocysts having their germline successfully gene-edited (<6%) (Figure 3-7A). AAV *in vivo* had the

largest increase in the time taken to reach HI when changing from 100% down to 20% germline gene-editing, with an increase to the mean of 11 batches (14%), whereas the mean number of batches for AAV *ex vivo* increased by 6 (8%).

The attempted zygote gene-edits also aligned according to decreasing gene-editing efficiency (Figure 3-6B). For lower efficiency gene-editing methods, introducing gene-editing associated mosaicism had a more pronounced impact on the amount of gene-editing required to reach HI and allele fixation. Moving from 100% to 20% germline transmission there was an increase to the mean number of zygotes gene-edited of 68% for AAV *ex* vivo, 74% for electroporation, 80% for microinjection and 89% for AAV *in vivo*. For AAV *in vivo* there was an increase of 44 to the mean number of batches that gene-editing was performed for when reducing germline transmission from 100% to 20%, whereas the mean number of batches that gene-editing was performed for was increased by 16 with the more efficient AAV *ex vivo* method.

For all gene-editing methods there was a greater reduction in genetic progress after 120 batches with 20% as opposed to 100% germline transmission of gene-edited alleles when compared to the control population (Figure 3-6C). With 100% germline transmission there was a 2.5% - 3.1% reduction in the mean genetic merit value across all gene-editing methods compared to the control population after 120 batches and with 20% germline transmission there was a 5.2% - 6% reduction. With a selection accuracy of 0.5, the reduction in mean genetic merit across the gene-editing methods is 2.1% - 3% for 100% germline transmission and 4% - 4.9% for 20% germline transmission, illustrating that a smaller reduction to genetic improvement was observed with lower selection accuracies (Additional File 3).

Digenic Modelling

The digenic model in this simulation requires four resistance alleles to be present for phenotypic resistance and no viral escape mutants were included in the simulation or analyses.

3.4.2 Proportion Resistant

The proportion of resistant animals in the Finisher herd was counted at the end of each batch to observe the time over which resistant animals filtered down to the commercial growers (Figure 3-7). The dissemination of resistance alleles down the breeding pyramid was not affected by changing selection accuracy between 1, 0.8 and 0.5 (Additional File 3).

For all gene-editing methods, the accumulation of swIAV resistant pigs is delayed when resistance alleles were inherited independently compared to when resistance alleles are in complete linkage. With 100% and 50% germline transmission of gene-edited alleles, Finisher herds reached the threshold for HI of 90% within the 120 batches under all gene-editing methods. With germline transmission at 20%, only the more efficient AAV *ex vivo* and electroporation techniques reached the HI threshold for both digenic inheritance modes within 120 batches and swIAV resistant pigs from the lowest efficiency AAV *in vivo* cohort were only just beginning to appear in the Finisher herd. With 100% germline transmission, the most efficient gene-editing method of AAV *ex vivo* reaches the HI threshold 7 batches (10%) later when resistance alleles are independently inherited than when they are in complete linkage, whereas for the least efficient method of AAV *in vivo*, there was a smaller increase of 6 batches (6.5%).

For AAV *in vivo*, the resistance phenotype is just beginning to emerge in the Finisher herd after 120 batches with 20% germline transmission of gene-edited alleles, whilst microinjection will reach HI just beyond simulated timeframe. These results suggest that implementing gene-editing with parameters similar to the AAV *in vivo* values used in these models would make it an unfeasible method in a commercial pig breeding system if the likelihood of gene-editing germline progenitor cells were to be as low as 20%.

3.4.3 Edit Count

The count of zygotes that were gene-edited across all Nucleus populations was recorded per batch. No gene-editing occurred when only swIAV resistance alleles were present in the Nucleus Herd animals that were selected

for breeding. For both linked and independent inheritance across all levels of germline transmission, the number of zygotes gene-edited aligns in order of descending gene-editing efficiency for a selection accuracy of 0.8 (Figure 3-8). There was no observable effect to the level of gene-editing required when changing the level of selection accuracy (Additional File 4).

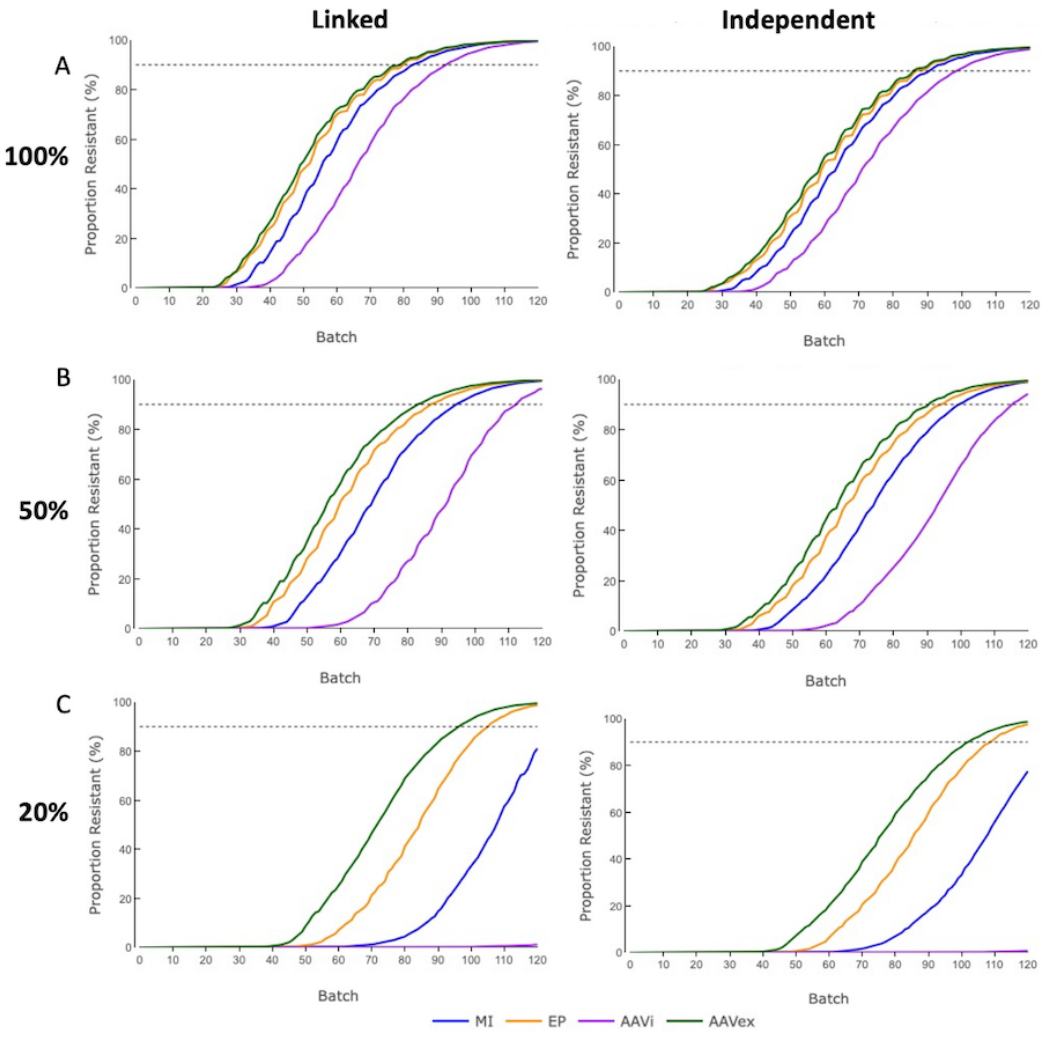


Figure 3-7: The proportion of swIAV resistant pigs in the Finisher herd in a digenic gene-editing program with a selection accuracy of 0.8.
MI = Microinjection. EP = Electroporation. AAVi = AAV *in* vivo. AAVex = AAV *ex* vivo. Influenza resistance alleles were inherited in a completely linked or independent manner. A) 100% germline transmission. B) 50% germline transmission. C) 20% germline transmission.

With 100% germline transmission, for AAV *in vivo* the mean number of zygotes that were attempted to be gene-edited across the 120 batches was 2.7% more for independently inherited alleles than linked alleles, with all other gene-editing methods having <0.2% discrepancy between inheritance modes. Selected Nucleus breeding animals were fixed for swIAV resistance alleles within 27 batches for AAV *ex vivo*, 32 for electroporation and 41 for microinjection at 100% germline transmission for both linked and independent inherited alleles. For AAV *in vivo*, there was a long tail of persistent gene-editing and the Nucleus breeding animals did not reach fixation for swIAV resistance alleles until 87 batches.

With a 20% likelihood of germline progenitor cells being gene-edited , only AAV *ex vivo* and electroporation reach the resistance allele fixation within 120 batches and there is <3% difference in the mean number of zygotes gene-edited over 120 batches between linked or independently inherited alleles. For AAV *ex vivo* and electroporation, moving from 100% to 50% germline transmission resulted in an increase of 61% and 63%, respectively, for both linked and independently inherited alleles. Changing germline transmission from 50% to 20% resulted in the mean number of zygotes being gene-edited increasing by 74% for AAV *ex vivo* with linked alleles and 80% for independently inherited alleles. These results highlight the challenges presented by high levels of gene-editing associated mosaicism.

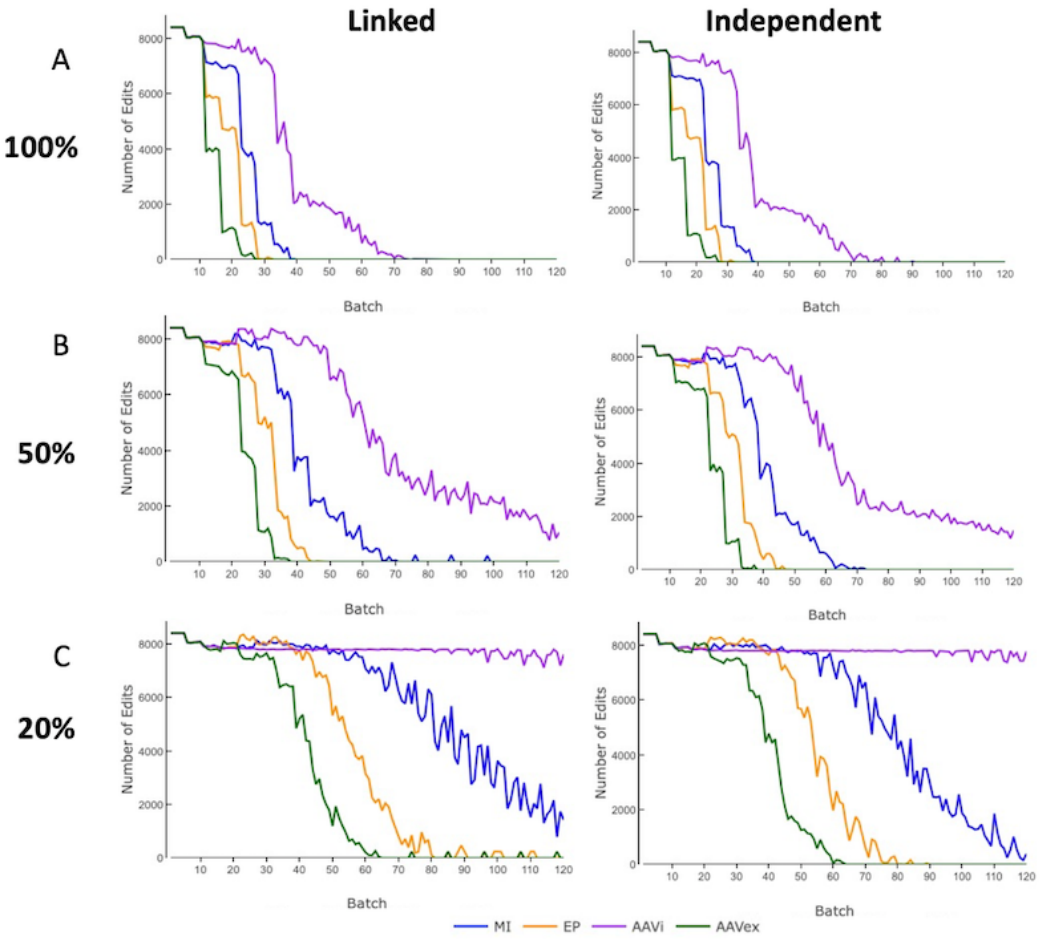


Figure 3-8: Number of zygotes attempted to be gene-edited in the Nucleus tiers in a digenic gene-editing program with a selection accuracy of 0.8. MI = Microinjection. EP = Electroporation. AAVi = AAV *in vivo*. AAVex = AAV *ex vivo*. Influenza resistance alleles were inherited either in a completely linked or independent manner with a selection accuracy of 0.8. A) 100% germline transmission. B) 50% germline transmission. C) 20% germline transmission.

3.4.4 Genetic Merit Trend

The trend in genetic merit in the Finisher herd was measured to assess the impact of prioritising the selection of resistance alleles over an index of genetic merit for the Nucleus and Production tiers (Figure 3-9). The mode of inheritance did not affect the genetic merit index value after 120 batches as observed by alleles inherited in complete linkage being within 2 index points of independently inherited alleles after 120 batches for 100% and 50% germline transmission of gene-edited alleles and 5 points for 20% germline transmission

(Additional File 5). For all selection accuracies, the mean genetic merit after 120 batches was reduced as compared to the unedited control population in alignment with decreasing gene-editing efficiency (except for AAV *in vivo* at 20% germline transmission).

This result was hypothesised because when resistance alleles are more prevalent in breeding animals, selection can be more focused on genetic merit index values. The AAV *in vivo* exception with only 20% of blastocysts having gene-edited alleles in germline progenitor cells occurs because so few swIAV resistance alleles are present in breeding animals after 120 batches, and therefore the rate of improvement in index genetic merit will continue to reduce beyond the endpoint of these simulations as bias towards swIAV resistance allele selection increases in accordance with their allele frequency. As selection accuracy was decreased the difference in index genetic merit values between each gene-editing method after 120 batches was reduced (Figure 3-9).

Across all selection accuracies, the reduction in genetic merit after 120 batches increased when compared to the control population as the level of germline progenitor cells being successfully gene-edited was reduced. For example, under a selection accuracy of 1, AAV *ex vivo* had a 2.6% reduction in mean genetic merit with 100% germline transmission, 5.9% with 50% germline transmission and 11.2% with germline transmission at 20%, whilst microinjection had a 5.2%, 8.6% and 17% reduction with germline transmission at 100%, 50% or 20%, respectively. Electroporation reported values intermediate to those of AAV *ex vivo* and microinjection for all selection accuracies and rates of germline transmission of gene-edited alleles and AAV *in vivo* was an exception to this pattern with 20% germline transmission retaining a higher genetic merit value above when 50% of gene-edited alleles are in germline progenitor cells due to the low level of swIAV resistance alleles being introduced throughout the 120 batches simulated not conferring selection bias towards swIAV resistant pigs.

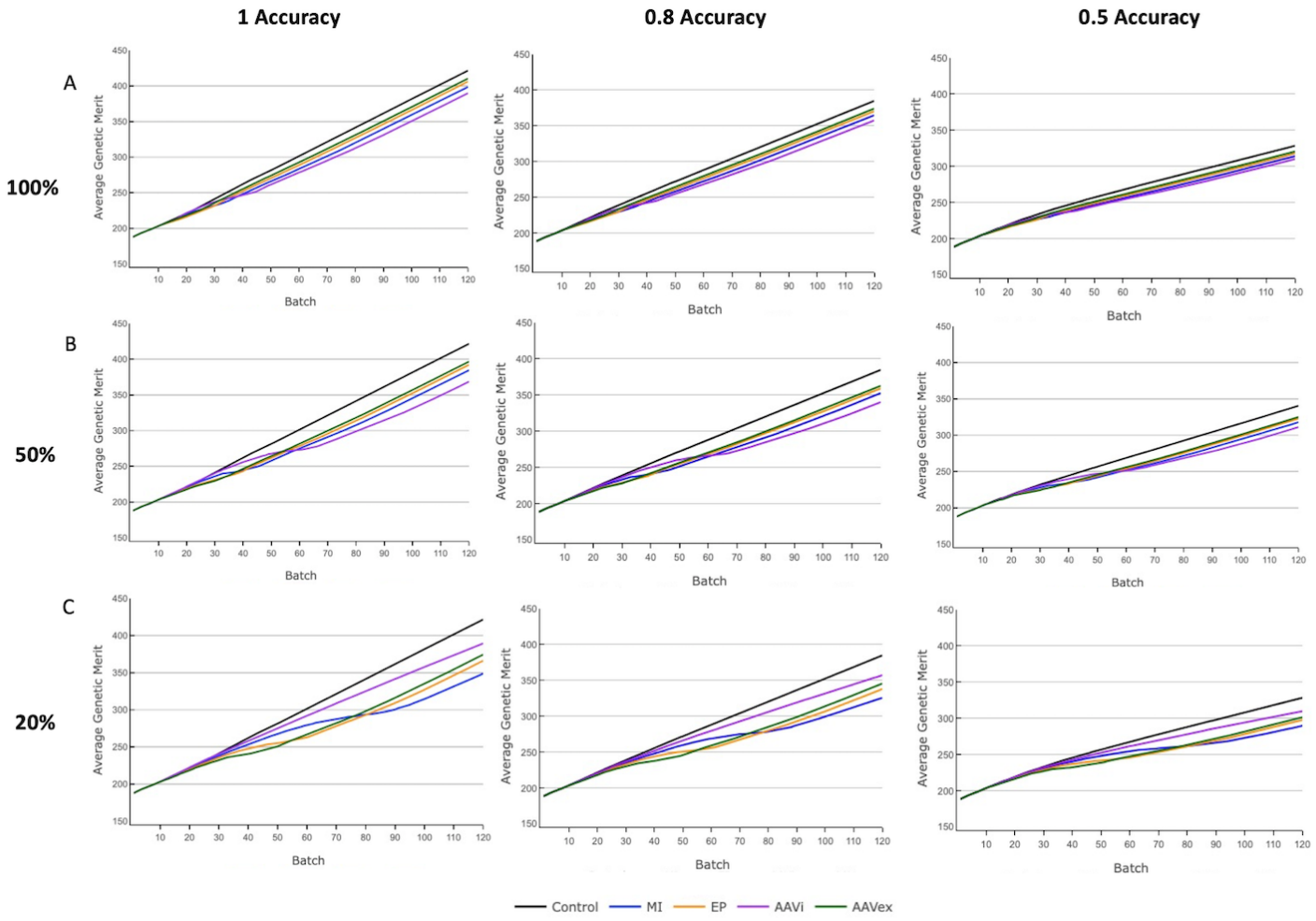


Figure 3-9: Genetic merit trend of piglets in the Finisher herd in a digenic gene-editing program with varying selection accuracies.
MI = Microinjection. EP = Electroporation. AAVi = AAV *in vivo*. AAVex = AAV *ex vivo*. Influenza resistance alleles were inherited in an independent manner. A) 100% germline transmission. B) 50% germline transmission. C) 20% germline transmission.

3.4.5 Economic Analysis

The economic analysis was designed to illustrate how the biological process of gene-editing and economic factors intertwine to influence decision making and the value proposition surrounding the implementation of a commercial gene-editing program. Decisions regarding the utilisation of gene-edited pigs will be affected by the swIAV control measures in place, so the analysis was split into systems with vaccination programs (Figure 3-10) that assumes ubiquitous and effective vaccination, and those with minimal swIAV control measures in place (Figure 3-11). The output for a selection accuracy of 0.8 and independent inheritance of digenic target alleles is shown to represent a moderate-high selection index accuracy in a discrete digenic model. Adjusting selection accuracy did not have a large effect on the economic analysis with the parameters used for these simulations (Additional File 8 and 9).

With vaccination, the economic benefits accrue when 90% of pigs are swIAV resistant and vaccination is no longer required. Farm systems without vaccination benefit prior to this from improved productivity in individually swIAV resistant pigs, and subsequently through productivity improvements to the entire herd once HI is achieved (Donovan, 2005).

For production systems with robust vaccination schemes, only a monogenic target with gene-editing by AAV *in vivo* achieving 100% germline transmission reached a positive cumulative NPV within 120 batches (Figure 3-10A). In no other scenarios was a positive cumulative NPV reached. As the germline transmission of gene-edited alleles was reduced, the cumulative costs from extended gene-editing programs increased the projected time to reach a return on the initial capital investment under all scenarios. When gene-editing digenic targets, AAV *ex vivo* with 100% germline transmission had the smallest negative cumulative NPV and was projected to reach positivity the soonest (Figure 3-10A). The introduction of a second swIAV resistance gene to the gene-editing scheme necessitated a much greater capital investment for all gene-editing methods and levels of germline transmission.

In farming systems that were simulated to have endemic swIAV and do not implement effective control measures, in the instance of monogenic

resistance, all methods except microinjection with germline transmission of gene-edited alleles at 20% reach a positive cumulative NPV within the 10 years simulated (Figure 3-11). In order of time to reach a positive cumulative NPV, AAV *in vivo* was the fastest, followed by AAV *ex vivo* and electroporation with similar projections, and finally microinjection. With 100% germline transmission, AAV *in vivo*, AAV *ex vivo* and electroporation reach a positive cumulative NPV within 6 years, which increased to 7 years for AAV *in vivo* and 9 years for AAV *ex vivo* and electroporation with germline transmission reduced to 20%.

For the digenic models in farm systems with endemic swIAV and no effective control measures, with 100% of cells carrying the desired gene-edit, all methods of gene-editing were simulated to reach a positive cumulative NPV within the 10 years simulated. AAV *ex vivo* was the most cost effective, followed by electroporation, AAV *in vivo* and microinjection. With germline transmission of 50%, only AAV *ex vivo* reached a positive cumulative NPV within the 10 years simulated. For 20% mosaicism, negative cumulative NPVs were reported over the 10 years for all gene-editing methods simulated, with only AAV *ex vivo* and electroporation beginning to trend towards a positive value. These economic analyses outline some of the considerations outwith biological optimisation of gene-editing protocols that should be taken into account when looking to integrate gene-editing into commercial pig breeding system.

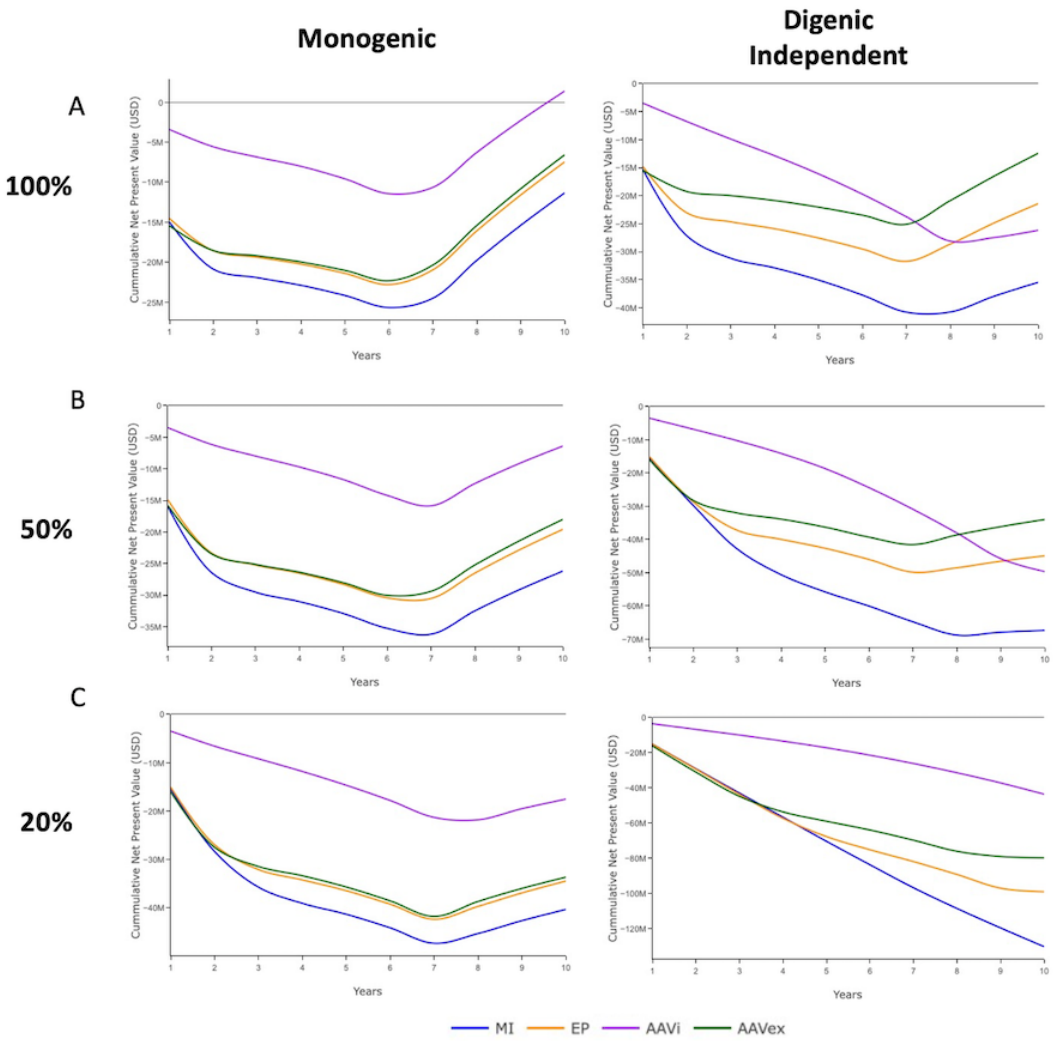


Figure 3-10: Economic analysis of farm systems with vaccination programs for monogenic and independently inherited digenic swIAV resistance alleles with a selection accuracy of 0.8.
MI = Microinjection. EP = Electroporation. AAVi = AAV *in vivo*. AAVex = AAV *ex vivo*. The cumulative financial benefits of resistance outweigh the cumulative costs in USD of implementation once the line is above 0. A) 100% germline transmission. B) 50% germline transmission. C) 20% germline transmission.
Figure 3-10: Economic analysis of farm systems with vaccination programs for monogenic and independently inherited digenic swIAV resistance alleles with a selection accuracy of 0.8. MI = Microinjection. EP = Electroporation. AAVi = AAV *in vivo*. AAVex = AAV *ex vivo*. The cumulative financial benefits of resistance outweigh the cumulative costs in USD of implementation once the line is above 0. A) 100% germline transmission. B) 50% germline transmission. C) 20% germline transmission.

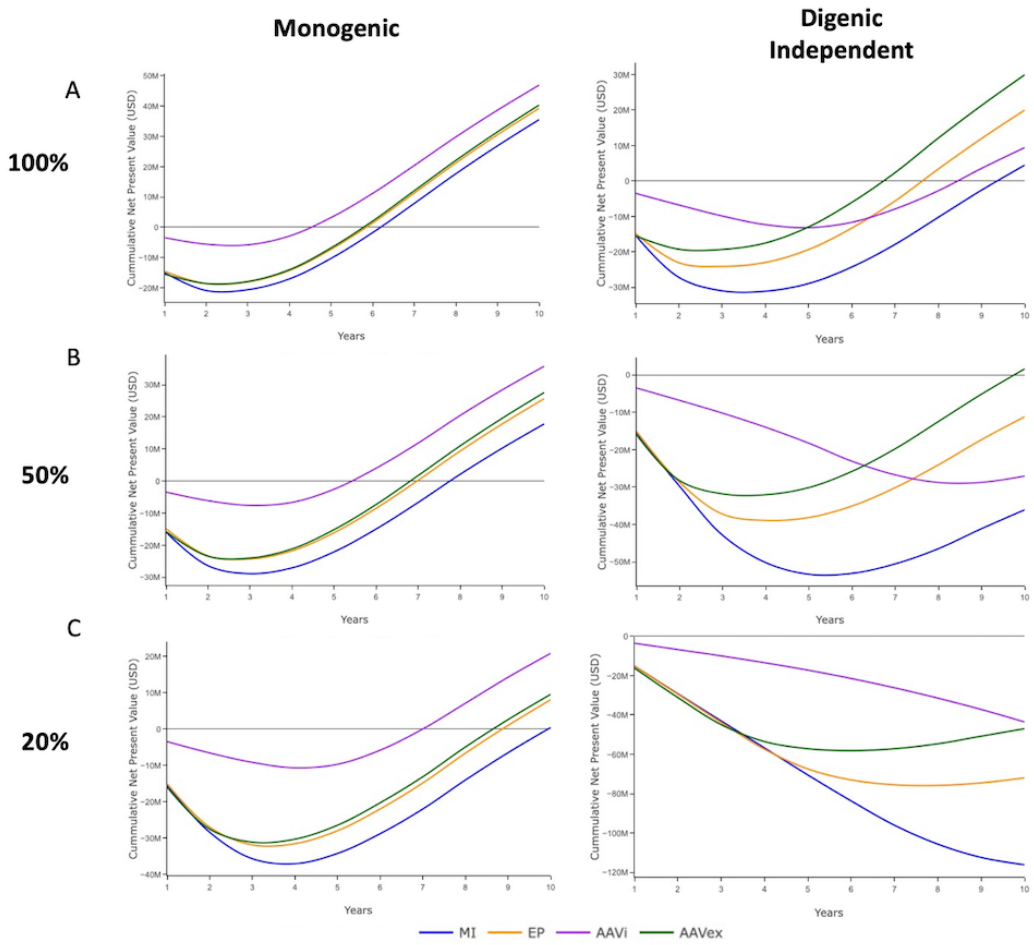


Figure 3-11: Economic analysis of farm systems with no vaccination program present for monogenic and independently inherited digenic swIAV resistance alleles with 0.8 selection accuracy.
MI = Microinjection. EP = Electroporation. AAVi = AAV *in vivo*. AAVex = AAV *ex vivo*. The cumulative financial benefits of resistance outweigh the cumulative costs in USD of implementation once the line is above 0. A) 100% germline transmission. B) 50% germline transmission. C) 20% germline transmission.

Figure 3-11: Economic analysis of farm systems with no vaccination program present for monogenic and independently inherited digenic swIAV resistance alleles with 0.8 selection accuracy. MI = Microinjection. EP = Electroporation. AAVi = AAV *in vivo*. AAVex = AAV *ex vivo*. The cumulative financial benefits of resistance outweigh the cumulative costs in USD of implementation once the line is above 0. A) 100% germline transmission. B) 50% germline transmission. C) 20% germline transmission.

3.5 Discussion

The simulation models presented here provide a novel analysis of the genetic and economic considerations when implementing a gene-editing program in a commercial pig breeding. system. The inclusion of digenic resistance and mosaicism provides further insight into the flow of resistance alleles that adheres to the biological reality of gene-editing in mammalian livestock for viral resistance that has not previously been published.

3.5.1 Monogenic Modelling

In the genetic analysis of the monogenic modelling there are only small changes in the time to reach fixation and in the progression of genetic merit between the methods of gene-editing. Reducing the occurence of the gene-edited alleles being present in germline cells had a much larger effect on extending time to allele fixation than gene-editing efficiencies and zygote survival rates, therefore the output of these models suggests that in order to optimise gene-editing programs, reducing the occurrence of mosaicism should be the primary concern (Lamas-Toranzo et al,, 2019). Although a single genotype can confer resistance, given the high rate of IAV mutation and its adaptative ability, targeting only a single gene could be a strategy with higher risk, due to the potential of mutations arising that circumvent host resistance mechanisms (Hussain et al., 2017). These would need to be assessed on a case-by-case basis, as some single gene modifications would provide more robust resistance mechanisms than other digenic strategies.

3.5.2 Digenic Modelling

For the ANP32 gene family swIAV resistance targets in pigs, both mutant genes are recruited in the same process by swIAV for improving genome replication efficiency. Therefore, in our simulations all four recessive alleles were necessary for phenotypic resistance to swIAV infection. In an ideal scenario, editing of two host genes encoding proteins that are exploited by discrete steps in the viral replication cycle, such as a cell surface receptor (Sialic Acid) and a protein that is recruited to assist viral genome replication (such as

ANP32A in chickens) would create two distinct barriers to reinfection (Burkard et al., 2017; Long et al., 2016).

In our digenic modelling the efficiency of gene-editing had a greater effect on the model outputs than when only a single gene was targeted however, as with a monogenic target, to maximise economic and genetic benefits, ensuring gene-editing at least occurs in germline progenitor cells should be prioritised over improving the efficiency of gene-editing. The chromosomal location of the target genes was observed to have only minor effects on the genetic progress of commercial pigs and the time to fixation of resistance alleles in breeding animals between linked or independent inheritance of resistance alleles. Notably, for the lower efficiency gene-editing techniques, the impact of gene-edited alleles not being present in germline cells was more pronounced.

3.5.3 Gene-Editing Techniques

For all gene-editing methods described, it is important to emphasise that illustrative parameters are used, and that these may vary widely between target sites and protocols. Data available on gene-editing in porcine zygotes is limited and highly variable, with continual optimisation being performed to what are still relatively novel techniques (Sato et al., 2020b; Yang and Wu, 2018). Not only are the gene-editing efficiency and zygote death rates considered here, but differences in cost between the methods of gene-editing are included in our analysis. In large animal research it is important to be able to realise a scientific aim with the lowest possible use of animals with the intention of adhering to the 3R's of animal research. These are Replacement, Reduction and Refinement of animals for scientific purposes. These tenets are considered in industrial practice and should be accounted for in industrial gene-editing as well, however as well as these there is the unavoidable matter of cost. Not to find the most affordable outcome, but if something is beyond financial reach it cannot be considered to be a viable approach.

Being a technically demanding and low-throughput technique, it is likely that microinjection could not be practically implemented for use in a scale of

program such as the one we have simulated. If the breeding program focused on a small number of boars and used these for crossing over offspring, despite the obvious inbreeding implications it could become financially viable.

Using murine models there has been progress to performing electroporation *in vivo* (Iwata et al., 2019; Sato et al., 2020a). Optimisation of this technique would allow natural mating of pigs and only minor surgery for access to fertilised zygotes in the oviducts. Electroporation *in vivo* is similar to the pipeline for AAV *in vivo* with the addition of surgically exposing the oviducts for access of electrodes. Further benefits of AAV techniques are the ability it offers to introduce larger templates for homologous recombination, and that the vectors can be designed using online tools and delivered as packaged vectors, reducing the time and facilities to carry out the gene-editing procedures. A drawback to these *in vivo* strategies is the huge volume of RNP or AAV virions required which could result in immune stimulation that is counterproductive to successful gene-editing.

The AAV based systems in particular are likely to require significant optimisation to be translated from rodent zygotes and porcine somatic cells to porcine zygotes in order to be feasible and practical in a commercial setting (Mussolino et al., 2011; Steines et al., 2016; Yoon et al., 2018). With reflection of the 3Rs, AAV *in vivo* and AAV *ex vivo*, with the parameters used here, provide contrasting benefits. AAV *ex* vivo requires the lowest input for gene-editing, and therefore would rely on the fewest surgical procedures for embryo transfer and IVF. If these are already implemented this would not be a detraction, but if they are supplementing the normal breeding protocols, the surgery required would be considered detrimental for animal welfare. AAV *in vivo* circumvents these concerns of additive surgery as it could be performed alongside AI. Further research is required to know if this is a plausible approach.

Hurdles in development of AAV *in vivo* may arise from repeated application in dams due to the potential immune response elicited after the first attempt due to the significant number of AAV virions needed in a porcine oviduct for the technique to be effective. As well as an immune response from repeated vector use being elicited, data from mice suggests that immunity

against Cas9 can be elicited, which is unsurprising given it is a non-native bacterial origin protein (A. Li et al., 2020). While it may not be AAV *in vivo* that becomes the primary intrauterine gene-editing method in livestock, it is likely that a technique whereby CRISPR/Cas9 can be assimilated into the AI protocols would be popular due to ease of integration into a platform that has AI but does not rely on ET.

3.5.4 Alternative ways of generating gene-edited progeny

Previous gene-editing models have included Somatic Cell Nuclear Transfer (SCNT) as a method. However, the technical expertise, time and limitations in its scalability led to it not being considered a viable commercial strategy in pigs. However, there are significant benefits of SCNT, including no genetic mosaicism associated with the target alleles in progeny, which we have described as the major limiting factor to commercial gene-editing success (Portela and Digard, 2002). Microinjection also requires highly trained personnel, specific micromanipulation equipment and a trained operator for gene-editing reagents to be injected into each zygote individually, making it less suitable for the scale required in commercial pig breeding.

Creating gene-edited zygotes allows for the offspring to immediately carry the genotype and pass it on, provided germline progenitor cells carry the desired genetic edits, to their progeny in an F1 generation. If low zygote gene-editing efficiencies are present, editing of oocytes or spermatogonia could be an alternative strategy (Onuma et al., 2017; Webster et al., 2021). Gene-editing of sex specific genes, particularly in oocytes as they can be individually handled and microinjected, is an approach that has been found to generate non-mosaic embryos (Su et al., 2019).

Gene-editing of spermatogonial stem cells (SSCs) could also be tied into another breeding technology called Surrogate Sire Technology (SST). SST describes the sires that are lacking the capacity to produce mature spermatozoa. These have been created in pigs by disruption of the *NANOS2* using CRIPSR/Cas technology (Park et al., 2017). The germline ablated boars can then have SSCs from wildtype sires, isolated from testicular tissue and amplified

with or without gene-editing applied, transplanted into their own testicular tissue. The transplanted SSCs will establish functional and regenerating spermatogonia cell lineage, and through natural mating can disperse the genotype of the make from which the original testicular tissue was isolated (Giassetti et al., 2019; Oatley, 2018).

A pipeline measure that has yet to be established in pigs is the gene-editing of stem cells, whether they are induced (iPSCs) or embryonic derived. The nature of embryo formation following exogenous stem cells being incorporated into a blastocyst would mean that genetic mosaicism of the full organism is inevitable. However, there would be benefits in being able to sequence the genotype of the stem cells implanted into the blastocyst, as any resulting genetic mosaicism would be between only the alleles from the introduced cells and the wildtype (Mclean et al., 2020).

In a research setting, whether in an inducible or constitutive manner, there is a role for the use of Cas9 expressing pigs (Rieblinger et al., 2021; Wang et al., 2017). For commercial application the introduction of a non-native protein would not be a viable approach due to the regulatory, safety and marketability flaws of Cas9 being present. This is not a comprehensive list of current or conceptual methods for gene-editing in livestock, but serves to be illustrative of the diverse factors that ought to be taken into account, depending on the regulatory landscape, facilities, gene targets and expertise available.

For zygote gene-editing to develop into a breeding technology, regardless of the selected technique, that can be reliably deployed by livestock breeders in a cost-effective manner, the experimental shortcomings of gene-editing in zygotes must be resolved. With single-step zygote gene-editing, as it stands, variable alleles being introduced during gene-editing is the expected and not the exceptional outcome. The occurrence of multiple alleles being introduced can be minimised by optimising the timing, concentration of reagents or utilising of molecular enhancers (Bischoff et al., 2020; Tanihara et al., 2019). It is promising that there are strategies currently being researched that are progressing on this issue. Improving the reliability of germline transmission would improve the capacity for gene-editing to be integrated as a

high-throughput technique alongside current artificial breeding technologies such as *In Vitro* Fertilisation (IVF) Embryo Transfer (ET) and Artificial Insemination (AI)) (McFarlane et al., 2019).

3.5.5 Pig Breeding Limitations of Gene-Editing

Despite being one of the most populous domesticated mammalian species, the reproductive biology of pigs has made the application of modern breeding technologies more difficult than with other species and a wide margin for improvement remains. IVF, ET and AI are the three main advanced breeding technologies available, of which at least one will almost always be a component of a gene-editing program. Because porcine oocytes are most often obtained from pigs post slaughter, the multiple use of sows in our model necessitates that the embryos are collected post insemination. This means that not all zygotes would be at the single cell stage and the isolation of single cell zygotes would require on-site expertise. Ovum pick up technologies may be developed in sows in the future, but because of the anatomical obstacles of swine genitalia, it is currently unavailable.

AI is beneficial by allowing the widespread of sires to be used without a reliance on mating, meaning boars can be in many places at once and not be limited in the number of dams they can service. Genetically high value boars can be used at higher rates to improve genetic gains. Issues remain with the cryopreservation of boar semen, however, limiting the distribution and efficacy of the fertilisation following defrosting. Uptake of AI is improving as the technology improves and in high genetic value herds AI is now standard practice (Knox, 2016). AI is mostly relevant for gene-editing techniques that can be performed *in vivo*.

Following the flushing and isolation of fertilised single-cell zygotes, the gene-editing protocols and *in vitro* maturation to embryos, there remains the need to successfully implant the embryos into a surrogate dam. Embryo implanting can be done non-surgically but piglet litter sizes are still lower than from naturally mated sows (Martinez et al., 2015; Yoshioka et al., 2012). A further issue of ET is that cryopreservation of porcine embryos has limited

success due to their high lipid content (Zhou and Li, 2009). ET is growing in the pig industry, but with the anatomical and biological complexities of swine embryology, there is still a way to go yet before a pipeline would be comparable to success rates of natural matings, and the for gene-editing of a large number of animals to be attractive, improvements in ART will be necessary (Martinez et al., 2019).

3.5.6 Pig Breeding Outcomes

The multi-nucleus pyramid structure of pig breeding makes it particularly attractive for gene-editing programs, as alleles can efficiently flow down by selection to the Finisher herd, reducing the number of genome-edited animals required. The model was designed to be adaptable to other species with pyramid breeding systems such as chickens. Without genotyping, gene-editing would not be viable at the scale necessitated by commercial pig farming. Given that the use of genomic technologies and genotyping is already standard practice in the Nucleus and Production tiers of breeding pigs (Knol et al., 2016), additional genotyping of swIAV resistance alleles could be readily incorporated with current breeding practices.

Although there was no direct measurement of inbreeding, the population structure and selection criteria applied (nested breeding) can result in lower levels of inbreeding (Rutten et al., 2002). Bastiaansen *et al*, 2018 observed that the continual introduction of novel alleles by gene-editing reduced the repetitive use of dams and sires when simulating gene-editing in dairy cattle, whereby gene-editing herds had lower inbreeding rates compared to when only genomic selection was applied, due to the expanding pool of animals available for selection with a genotype of interest (Bastiaansen et al., 2018; Mueller et al., 2019).

This modelling was intentionally designed to be illustrative of how genetic progress is impacted by prioritisation of resistance allele selection over an aggregated genetic index and how this will affect the economic outcomes of each gene-editing strategy, as opposed to being a genuine reflection of gene-editing in a specific industry herd. Breeding programs are all unique to each

breed and company with focus on distinct traits in specific herds. Despite being generalised and not designed around industrial information we do not consider this to affect the relevance of the data. The modelling code is adaptable to different breeding herds for more relevant data to a particular business if more accurate advice were to be required.

3.5.7 Economic Perspectives

The financial outlay required to gene-edit pigs at a commercial scale will be high, particularly if the strategy involves targeting multiple genes. Our model determined the greatest costs of a gene-editing program to be not from the gene-editing procedure itself, but from unrealised gains including the loss of genetic progress compared to a herd breeding under status quo conditions and from fewer pigs reaching slaughter because of gene-editing procedure associated zygote death causing smaller litters.

The economic analysis uses data from an experimental setting for the R_0 value (Romagosa et al., 2011), fixed gene-editing costs extrapolated from application in research and a specific value for the annualised financial benefit of genetic improvement. These parameters will vary according to the farm region and system of interest. As a result, it may be quicker to reach herd immunity at a lower cost, which would affect the final decision-making process and not be directly replicated by the data presented here. However, this analysis still provides a preliminary basis for identifying the method of optimal financial efficiency when implementing a gene-editing program in commercial pigs.

The selection accuracies simulated reflect the accuracy of EBV index selection in real farming systems (Badke et al., 2014). The implications observed regarding accuracy when considering the practical implementation of a gene-editing program are that as selection accuracy increases, there will be a marginal reduction in the improvement of genetic merit compared to an un-edited herd. These marginal changes are contained within the economic analysis but do not alter the time by which the gene-editing methods reach a positive financial return.

In farm systems with vaccination programs, the cost of gene-editing must be low and the rate of gene-editing in germline progenitor cells high for even a monogenic target to reach a positive return on investment. For digenic targets, due to the longevity of the gene-editing programs, the benefits of high gene-editing efficiency outweighed the benefit of the low cost but lower efficiency. The slower dissemination of swIAV resistance associated with low gene-editing efficiency was also observed when modelling the implementation of gene-editing in dairy cattle herds (Bastiaansen et al., 2018; Mueller et al., 2019). The results from the digenic modelling suggest that reaching fixation of the resistance alleles in breeding animals as quickly as possible and then continuing selection based upon genetic merit provides a better value proposition than persistent low efficiency editing that was observed to be associated with a prolonged reduction in genetic progress. To assess the economic situation relevant to a specific real-life situation for swIAV resistance, we would recommend running the simulation model with user defined input data for gene-editing efficiency, zygote death and costs specific to the target sites and experimental protocols in place as well as interest rates and further economic factors relevant only to specific cases.

A benefit of swIAV resistant pigs in a herd that was not included in our economic analysis is the fact that their presence is likely to reduce the prevalence of other infectious agents of PRDC (Detmer et al., 2012; Rose et al., 2013). This will lead to indirect reductions in veterinary costs and improvements in animal welfare standards and productivity. Another factor not included in the simulations was regulatory and bureaucratic hurdles that will be faced when creating gene-edited swIAV resistant pigs for the first time, which are likely to be a significant exclusion (United States Food and Drug Administration (FDA), 2017; Whelan et al., 2020; Hallerman et al., 2022). Our analysis does not encompass every factor, but the data provides an initial framework for economic considerations.

Separation of herds into vaccinated and non-vaccinated for the economic analysis was effective for demonstrating the different considerations between farming systems, and that different regions such as the USA, with high

vaccine prevalence, and Africa, with lower vaccine prevalence, will not be making decisions for gene-editing implementation from the same context. There were many assumptions inbuilt to this that mean the modelling would vary in the real world, and the code could be adjusted to do this. Vaccine efficacy was assumed to be 100%, which is true of no vaccine. As was described in the introduction, waning of piglet immunity is individual and therefore the timing of vaccination administration affects means that piglets will be variably susceptible, even in a vaccinated herd. Vaccine design is based on circulating strains, but because of viral evolution, vaccine effectiveness varies according to when it is in use and what type of vaccine is used. If a novel variant emerged, which cannot be accurately anticipated, there would be an immediate change to the paradigm of vaccination, and the economic considerations would shift to reflecting an unvaccinated herd.

Gene-editing or vaccination is not a binary decision, and alongside some basic and easy to action control measures outlined in Chapter 1, could work together to reduce the risk and burden of swIAV. Control strategies should be multifactorial to be the most effective, and forgoing all control will never be an option in the face of a virus that has a persistent reservoir in migratory birds.

The benefits of controlling swIAV should not be considered in isolation to pig farming, due to the zoonotic implications for human health and other IAV affected species (Chastagner et al., 2019b; Long et al., 2019b). Each pig that is swIAV resistant is removed from the ecosystem as a potential "mixing vessel" and therefore reduces the likelihood of a new IAV strain emerging by genomic reassortment and becoming a pandemic strain after transmission to humans. Although it is a difficult to define due to the unpredictability of pandemic emergence and severity, it could be of great value to public health and macroeconomic performance in the instance that an event such as the 2009 swine influenza zoonoses is mitigated. It would be an unfortunate scenario for inertia to be given to gene-editing of agricultural species for viral resistance to only arise in the wake of a zoonotic incident that resulted in a human pandemic.

3.6 Conclusions

The results of our simulation model have highlighted the challenges of gene-editing two targets in a commercial pig breeding population. Monogenic resistance had considerably fewer negative genetic and economic impacts but will be more likely to be rendered ineffective by viral mutation. For all scenarios, low rates of gene-editing in germline progenitor cells and lower gene-editing efficiencies had a negative effect on the genetic merit value of pigs received by producers and increased the time to reach the HI threshold. The translation of gene-editing from a research environment to commercial livestock breeding could be transformative for animal welfare and production, and the opportunity to control the spread of IAV by reducing the role of pigs as a zoonotic transmission node could greatly benefit human health. These results highlight the need for protocol optimisation and further work to be done in improving gene-editing protocols for economically viable translation to livestock zygotes.

3.7 List of Abbreviations

AAV – Adeno-Associated Virus

AI – Artificial Insemination

ANP32A – Acidic Nuclear Phosphoprotein 32 A

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

DNA – Deoxyribonucleic Acid

HI – Herd Immunity

IAV – Influenza A Virus

NPV – Net Present Value

PRDC – Porcine Respiratory Disease Complex

PRRSV – Porcine Reproductive and Respiratory Syndrome virus

RNA – Ribonucleic Acid

SCNT – Somatic Cell Nuclear Transfer

swIAV – Swine Influenza A Virus

TGEV – Transmissible Gastroenteritis Virus

USA – United States of America

USD – United States Dollar

3.8 Declarations

Ethics approval and consent to participate

Not Applicable

3.8.1 Consent for publication

Not Applicable

3.8.2 Availability of data and materials

The simulation code available online at https://github.com/hamishsalvy/SwineFluGene-Editing. The datasets analysed during the current study are available from the corresponding author on reasonable request.

3.8.3 Competing interests

HAS and CBAW have funding from Genus Pig Improvement Company (PIC). This work was performed entirely independently from this body of funding.

3.8.4 Funding

HAS was funded to work with AbacusBio Ltd by a Flexible Talent Mobility Account 2 grant from the BBSRC (BB/S50791X/1). CBAW receives funding from BBSRC (BBSRC Institute Strategic Programme award BB/P013759/1).

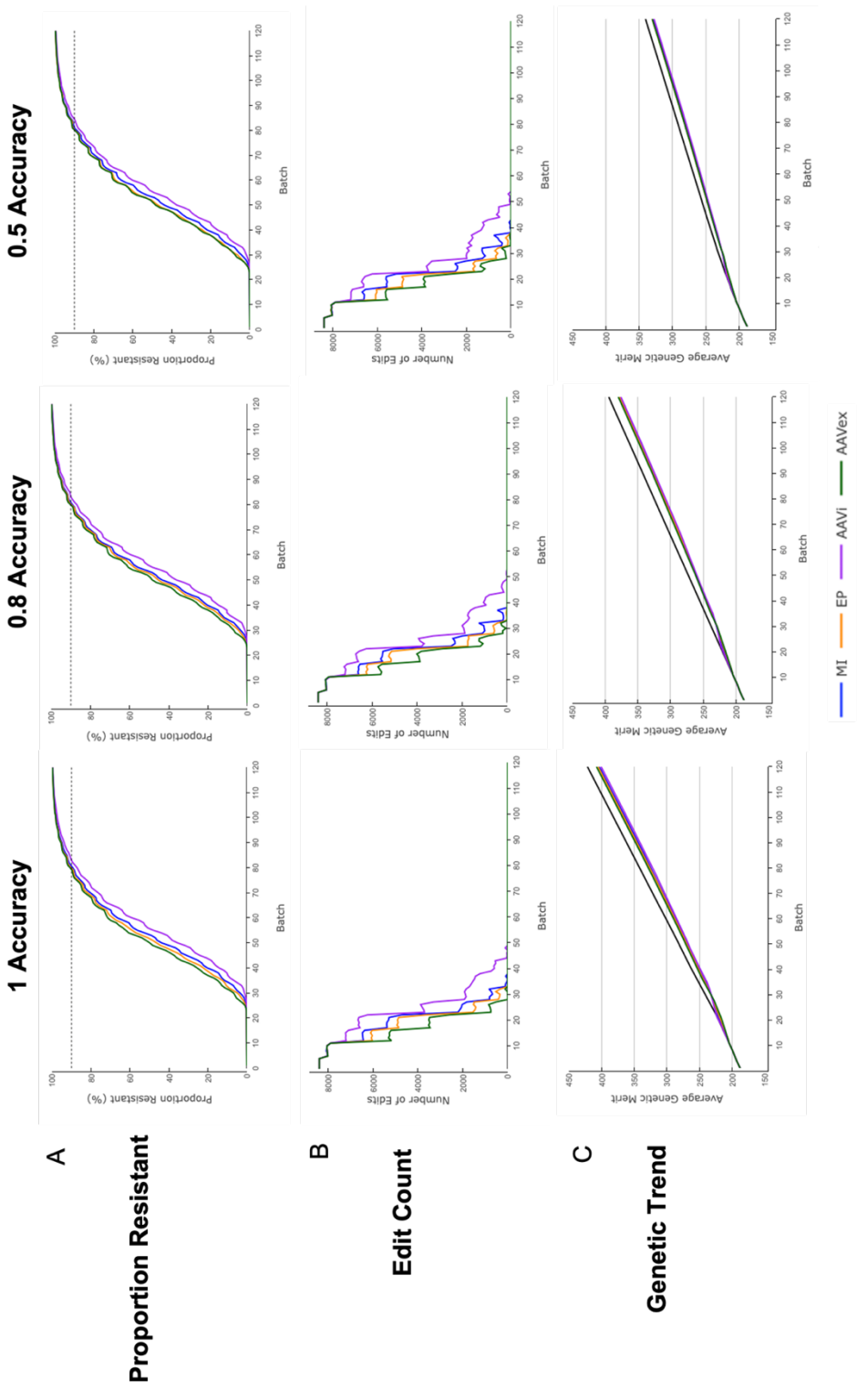
3.8.5 Authors' Contributions

HAS wrote the manuscript, developed the simulation model and analysed the data. FH assisted with model design and editing of the manuscript. CBAW and TJB conceptualised the project and assisted with editing and reviewing the manuscript.

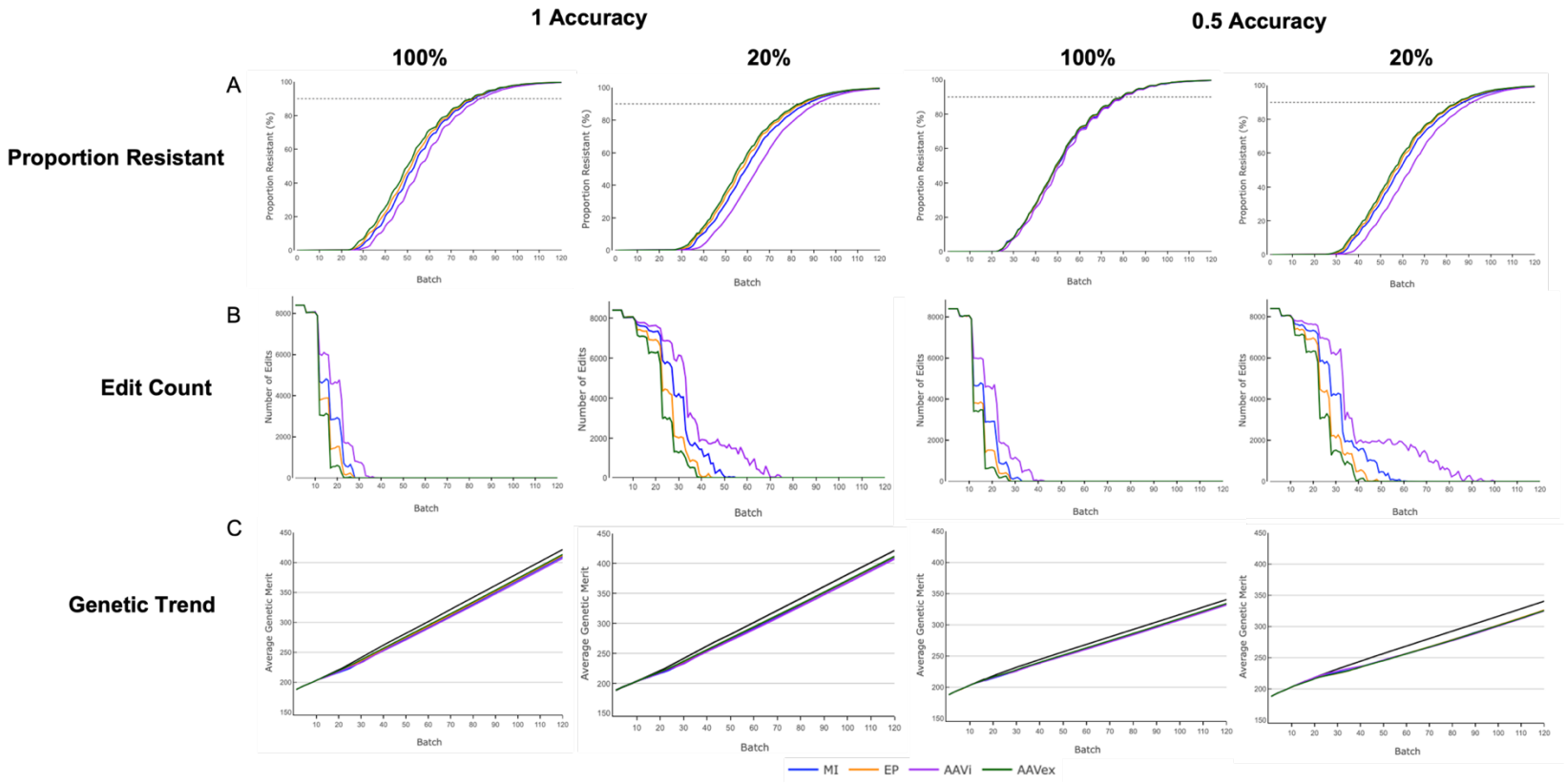
3.8.6 Acknowledgments

Support in developing the simulation model was provided by several members of the AbacusBio team, in particular to Jonah Duckles, Dr. Peter Amer and Dr. Gertje Petersen.

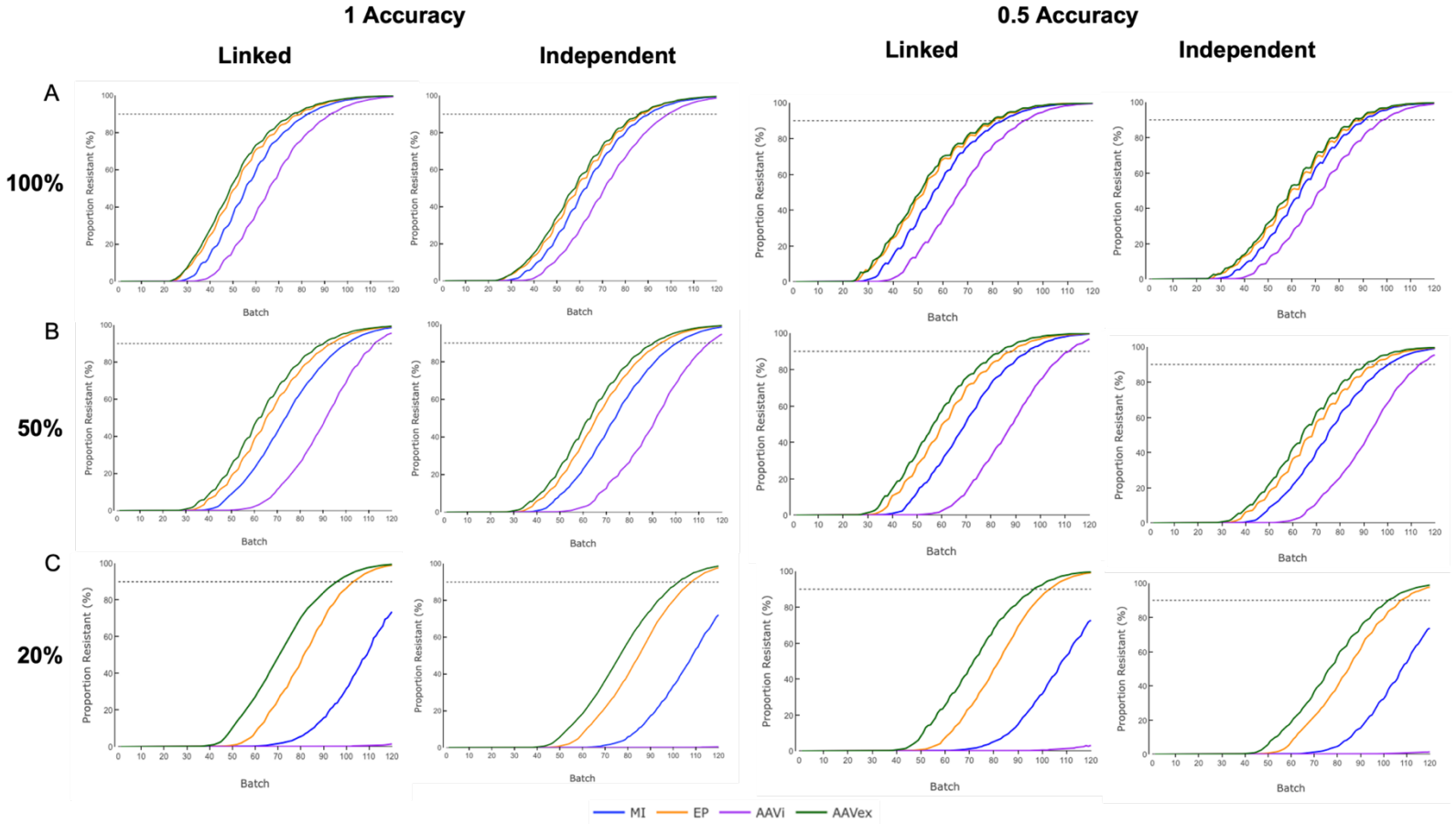
3.9 Additional Figures



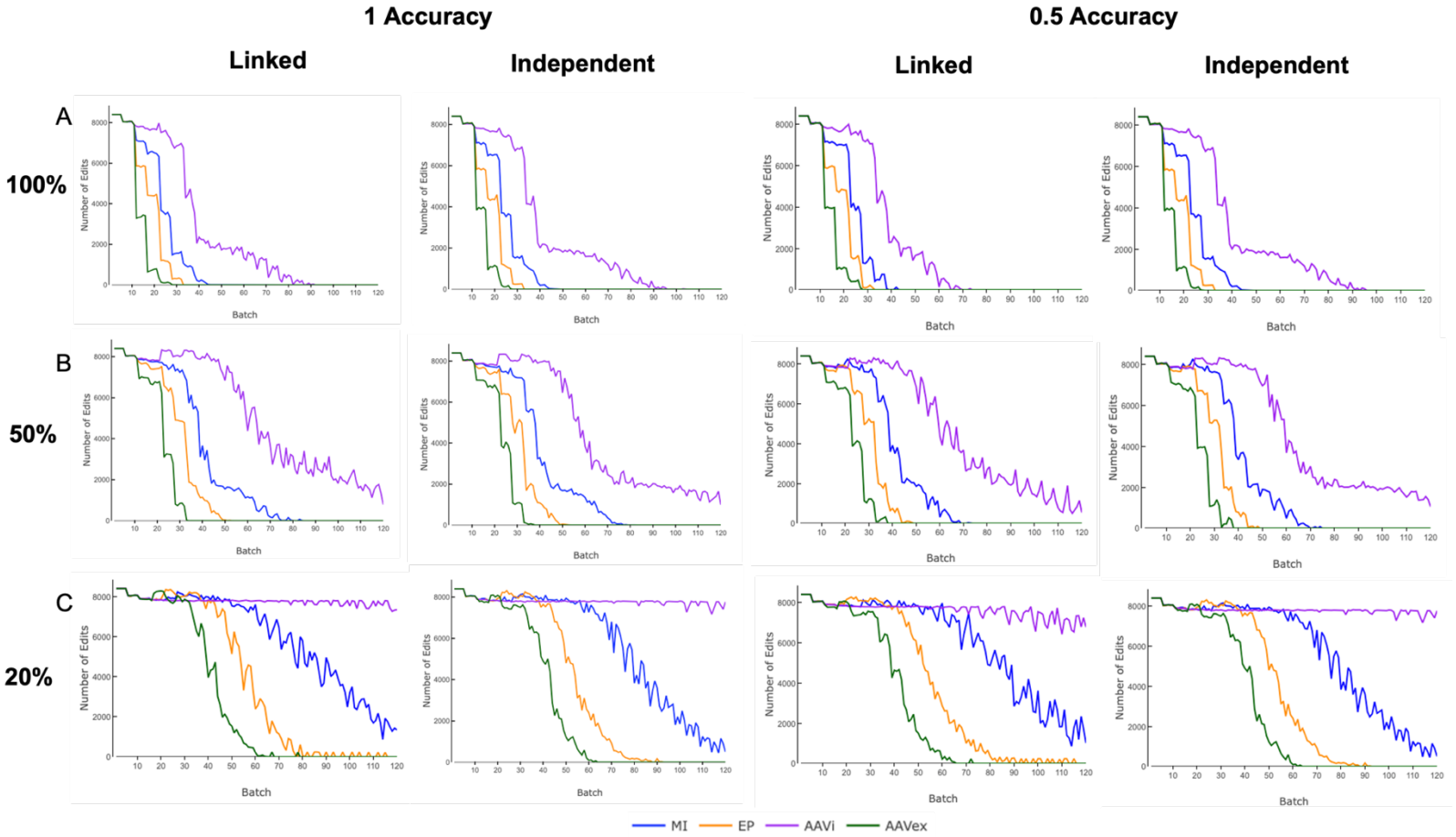
Additional File 1: Monogenic resistance with 50% germline transmission. MI = Microinjection. EP = Electroporation. AAVi = AAV *in* vivo. AAVex = AAV *ex* vivo. A) Proportion of pigs with phenotypic resistance to swIAV in the Finisher Herd. B) Total count of zygotes attempted to be gene-edited across all Nucleus herds per batch. C) Mean genetic merit of pigs in the Finisher Herd.



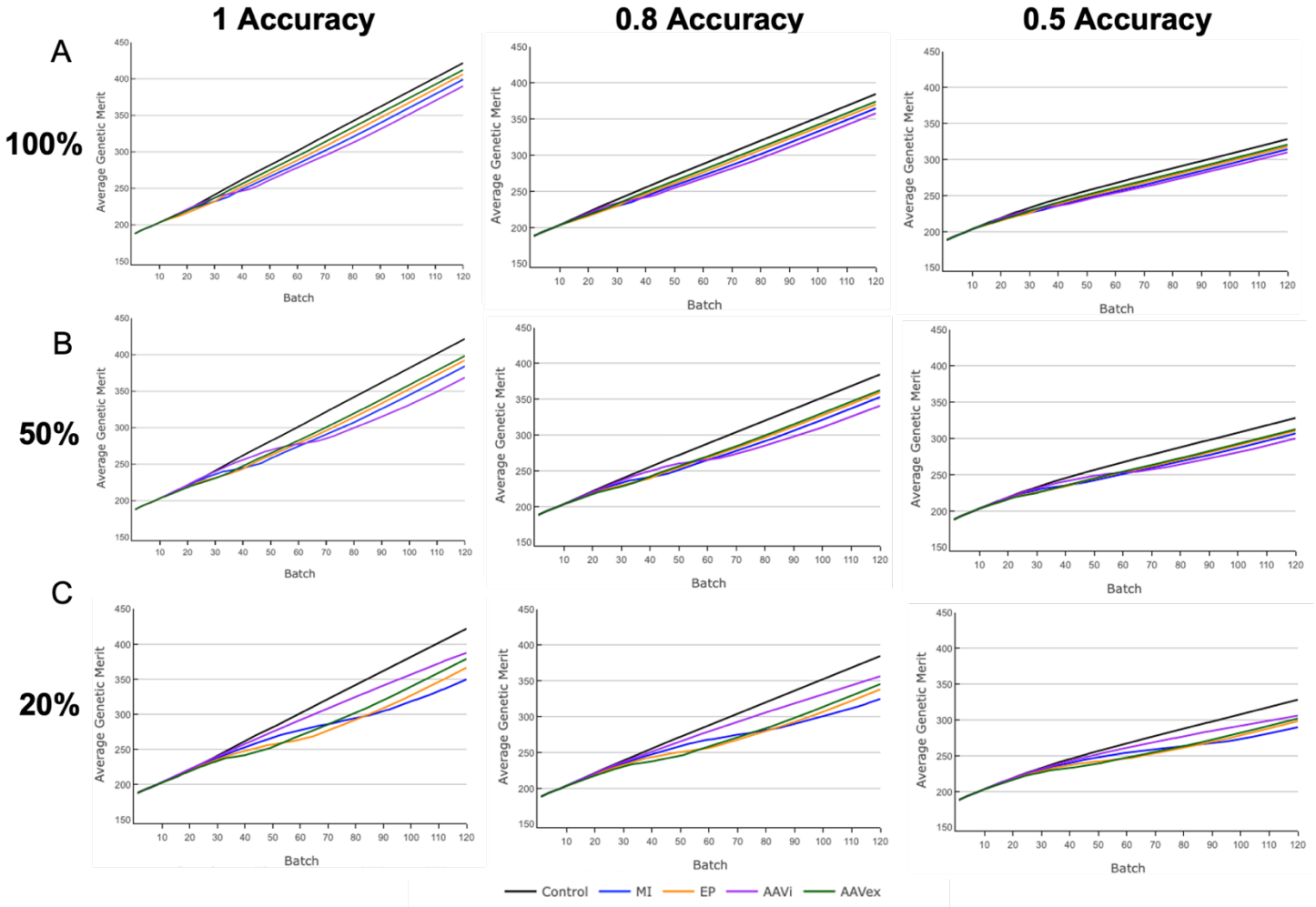
Additional File 2: Monogenic resistance with 1 and 0.5 accuracy and 100 or 20% germline transmission. MI = Microinjection. EP = Electroporation. AAVi = AAV *in* vivo. AAVex = AAV *ex* vivo. A) Proportion of pigs with phenotypic resistance to swIAV in the Finisher Herd. B) Total count of zygotes attempted to be gene-edited across all Nucleus herds per batch. C) Mean genetic merit of pigs in the Finisher Herd.



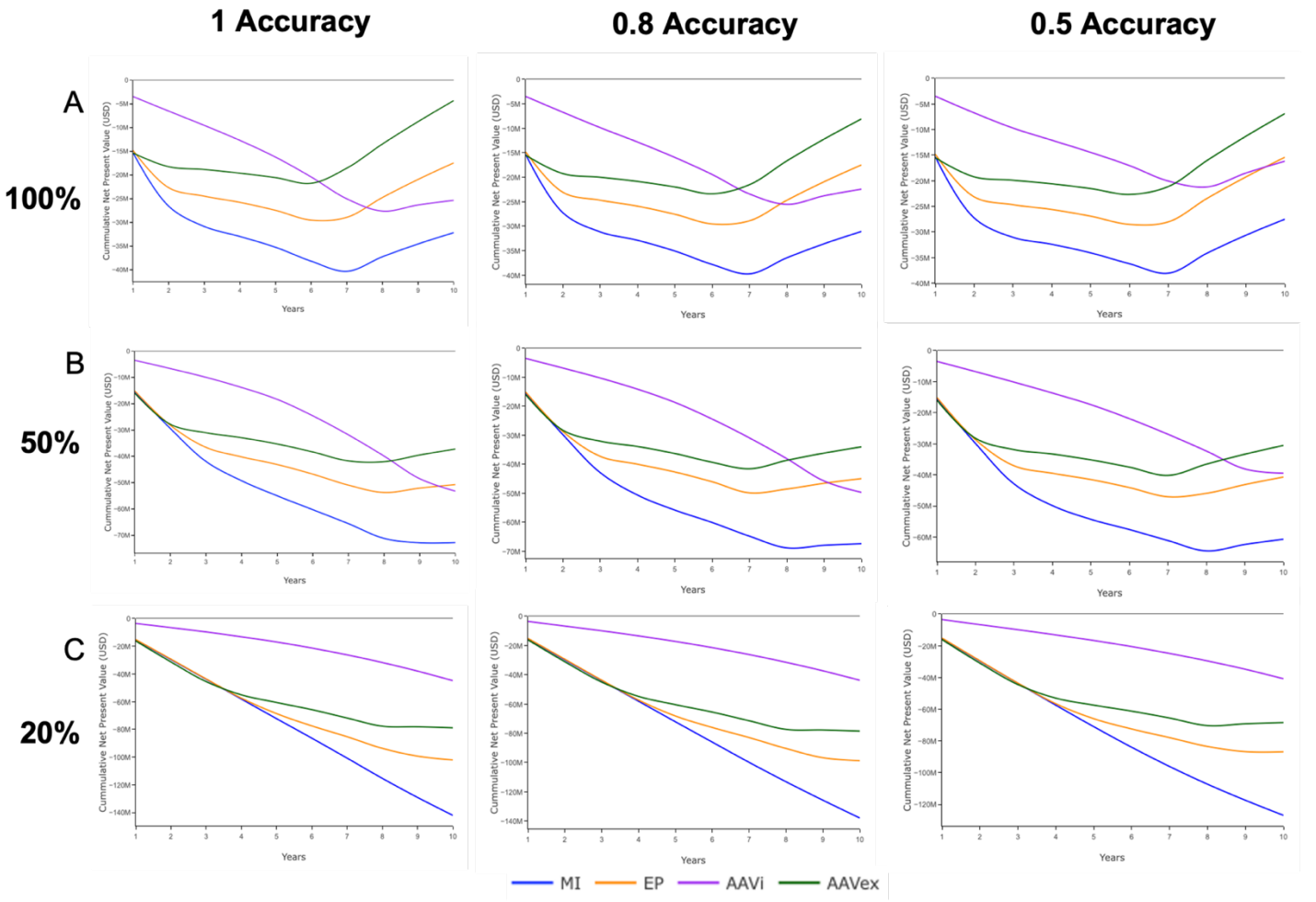
Additional File 3: The proportion of phenotypically resistant pigs in the Finisher herd in a gene-editing scenario of digenic swIAV resistance with 1 and 0.5 selection accuracy. Changing of selection accuracy did not affect the dissemination of alleles. MI = Microinjection. EP = Electroporation. AAVi = AAV *in* vivo. AAVex = AAV *ex* vivo. Alleles were inherited in a completely linked or independent manner. A) 100% germline transmission. B) 50% germline transmission. C) 20% germline transmission.



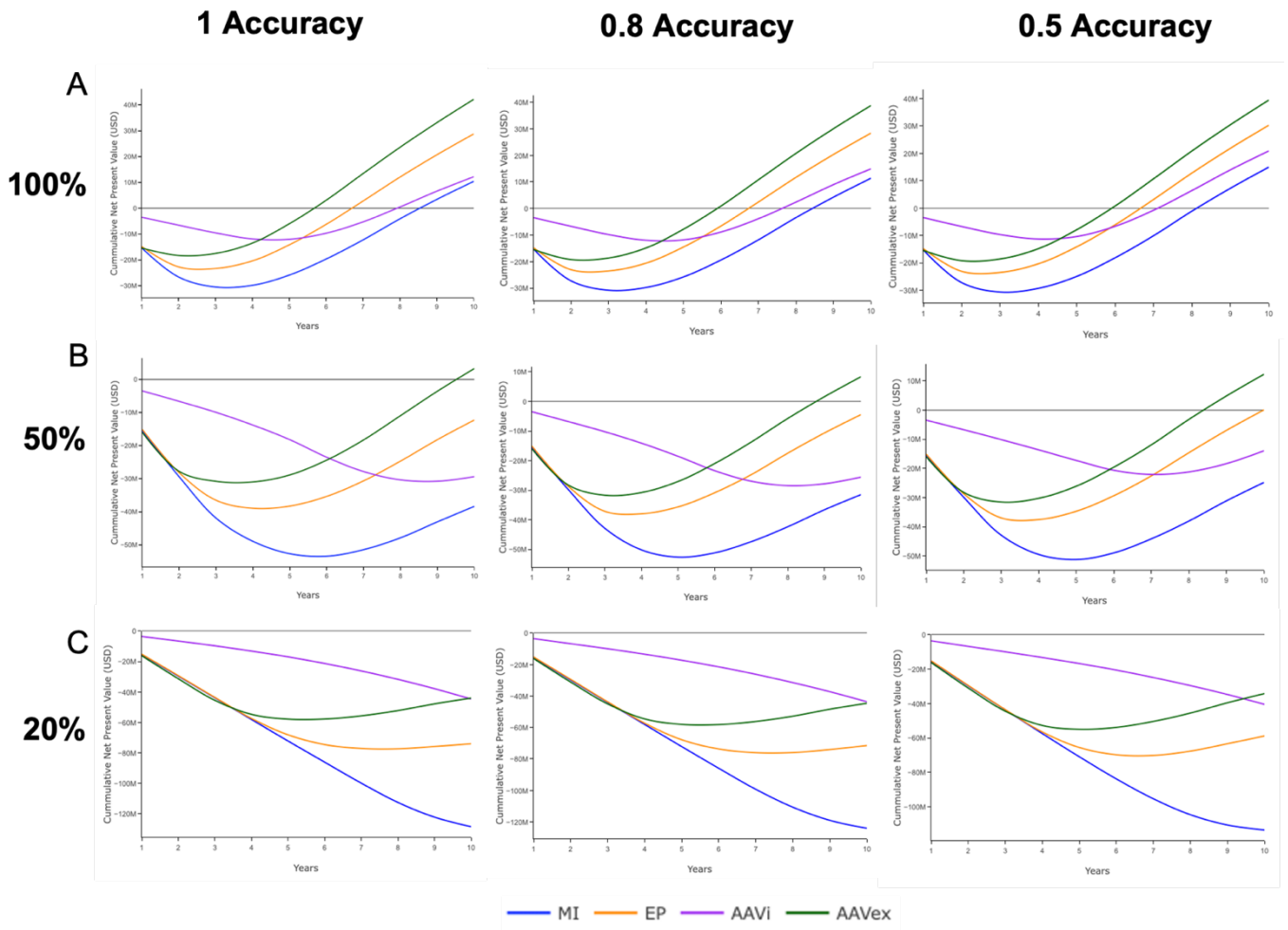
Additional File 4: Number of zygotes attempted to be gene-edited in the Nucleus tier in a digenic gene-editing program for linked and independent inheritance. MI = Microinjection. EP = Electroporation. AAVi = AAV *in* vivo. AAVex = AAV *ex* vivo. A) 100% germline transmission. B) 50% germline transmission. C) 20% germline transmission.



Additional File 5: Genetic merit trend of piglets in the Finisher herd in a digenic gene-editing program with linked allele inheritance. MI = Microinjection. EP = Electroporation. AAVi = AAV *in* vivo. AAVex = AAV *ex* vivo. A) 100% germline transmission. B) 50% germline transmission. C) 20% germline transmission.



Additional File 6: Economic analysis of farm systems with vaccination programs for linked swIAV resistance alleles for varied selection selection accuracies. MI = Microinjection. EP = Electroporation. AAVi = AAV *in* vivo. AAVex = AAV *ex* vivo. A) 100% germline transmission. B) 50% germline transmission. C) 20% germline transmission.



Additional File 7: Economic analysis of farm systems with no vaccination program for linked swIAV resistance alleles for varied selection selection accuracies. MI = Microinjection. EP = Electroporation. AAVi = AAV *in* vivo. AAVex = AAV *ex* vivo. A) 100% germline transmission. B) 50% germline transmission. C) 20% germline transmission.

4 Developing a relevant in vitro model to assess the impacts of targeted gene-editing on swine IAV infection

4.1 Introduction

The use of model systems to investigate the biology of higher organisms has been an essential tool in genetics becoming established as a scientific endeavour. From Thomas Morgan's *Drosophila* screens (Morgan, 1910) to Shinya Yamanaka's induced pluripotent stem cells (Takahashi and Yamanaka, 2006), by using model systems researchers have been able to disentangle molecular networks that are embedded within more complex biological systems. Investigating the genetics of what makes pigs susceptible to IAVs from avian species and humans in an *in* vitro system could contribute to our understanding of the evolution and molecular kinetics of IAV replication. The better we understand how and why swine have a central role in IAV ecology, the more we can do to control its endemic and zoonotic transmission, which will ultimately contribute to improvements in animal welfare standards and economic performance, as well as reducing the potential for pigs to be an intermediate mixing vessel for novel IAV strain generation. In this chapter we developed an *in vitro* model to investigate the role of specific host co-factors hypothesised to support IAV replication in swine.

4.1.1 Tools for Gene-Editing

The principle of mutating genes to investigate gene function was originally performed in animals through random genome-wide mutagenesis induced by chemical mutagens (Nüsslein-volhard and Wieschaus, 1980). By breaking DNA throughout the genome and allowing error-prone host repair systems to introduce or delete nucleotides at the cut site, changes in phenotype could be mapped to the genetic aberrations. From genome-wide mutagenesis screens, there was a graduation in specificity to using restriction enzymes, proteins that cut DNA at specific target sequences. This allowed more accurate

targeting of DNA sequences of that were of particular interest, however with restriction enzymes rarely having homology with a single site in a genome, the confounding presence of DSBs being introduced at all the recognition sequences of the restriction enzyme and not just at the intended sequence remained. A major development through site specific DNA digestion was that it allows for homologous recombination (HR) (Smithies et al., 1985). HR is the exchange of genetic material between two strands of DNA and it can be recruited as a strategy for genetic modification. The inclusion of mutations in the integrated DNA strand results in a non-native nucleic acid sequence replacing the original DNA at the DSB site.

The life science communities' growth in knowledge and technological improvements has culminated in our present ability to alter nucleic acids in an accurate and site-specific manner. The current toolkit, described as 'genome editors', defines a group of reagents that can be used for gene-editing. The current genome editors in use are two facet systems. There is an endonuclease component, which metabolises the DNA, and a nucleic acid recognition mechanism. Gene-editing processes rely on host repair mechanisms, such as HR, that resolve naturally occurring or environmentally induced DNA breakages.

The three primary pathways of repairing DSBs are via host processes termed non-homologous end-joining (NHEJ), homology directed repair (HDR) and microhomology-mediated end-joining (MMEJ). NHEJ is the canonical homology-independent repair pathway. The Ku protein complex recruits nucleases or polymerases to chew-back or extend the DSB to form blunt ends, which are then ligated back together without a DNA template (Davis and Chen, 2013). This often causes small indels to be introduced. The loss or insertion of extra nucleotides can disrupt the open reading frame (ORF). If the indel number of DNA base pairs is not a factor of three, the gene transcript is likely to a contain a premature stop codon or be rendered as nonsense for codon reading. MMEJ, also known as alternative end joining, occurs in a Ku and DNA ligase IV independent manner when short microhomologies are present either side of the cut site. The short regions of complementarity result in annealing and a small deletion or inversion (Wang and Xu, 2017). The most common form of

HDR is HR, a process that uses sequence homology in either a dsDNA or ssDNA template to repair the DSB present at the target locus (Chen et al., 2008; Liang et al., 1998). This process has minimal residual errors if the template is identical to the original sequence.

ZFNs and TALENS

The innovation of techniques that could accurately create a DSB at a user defined nucleic acid sequence was pioneering for genome editing. The first edition of genome editors being applied in mammalian cells. was meganucleases (Rouet et al., 1994). Meganucleases have a fixed DNA recognition site. This lack of flexibility in where the DSB is introduced is a limiting factor as few target DNA sequences are exactly the sequence of a discovered meganuclease. Subsequently, the development of customisable tools such as Zinc-Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) quickly superseded meganucleases due to their superior versatility (Kim and Kim, 2014).

ZFNs are typically comprised of between three and six zinc fingers that each have specific affinity to 3 nucleotides. A zinc finger is a 30 amino acid peptide originally isolated as a transcription factor from *Xenopus laevis* (Miller et al., 1985). The α-helix amino acids of ZFNs are programmable to recognise specific nucleotide interactions (Gaj et al., 2013). Zinc-finger arrays are complemented with a *trans*-binding pair, resulting in binding upstream and downstream of the target locus. The DNA-binding zinc-fingers are fused with the catalytic domain of an endonuclease, typically *FokI*, that only has catalytic activity upon dimerisation. Thus, the requirement for ZFNs to bind upstream and downstream of the target locus, and endonuclease activity only being instigated upon dimerisation, ZFNs offer a highly specific genome editing tool.

TALENs functionally operate in a similar manner to ZFNs in that they are a *trans*-binding pair of DNA binding elements which are fused with a non-specific restriction enzyme activated by dimerisation (Kim and Kini, 2017). Conventionally, it is also the *FokI* catalytic domain that provides the endonuclease activity. TALENS are derived from Transcription Activator-Like

proteins. The TAL effector region can be designed to bind almost any DNA sequence through engineering of a repeat variable diresidue (RVD). The RVD is a conserved 33-34 amino acid sequence which has binding affinity for a particular nucleotide. Altering combinations of the 12th and 13th amino acids in the RVD alters which nucleotide the binding specificity is for, and so by engineering of the RVD in multiple aligned TAL effectors, virtually any DNA sequence can be targeted. The paired nature of ZFNs and TALENs makes them highly specific in where they create DSBs. However, the requirement that a novel protein pair is created for every locus that is to be edited means that design and production has substantial barriers of complexity and molecular engineering. These factors limited the widespread application of ZFNs or TALENs.

CRISPR

Whilst ZFNs and TALENs are effective genome editing methods, their use has largely been usurped by the popularity of CRISPR/Cas editing. CRISPR reagents are a reengineered bacterial antiviral immune mechanism The system was first identified through the presence of repetitive genomic elements interspersed with non-repetitive sequences in *Escherichia coli* (Ishino et al., 1987). Shortly after these repetitive elements were classified, nearby genes encoding nucleases were identified, which were named as CRISPR associated (Cas) proteins. With the nuclease sequences being proximal to the identified repetitive elements, and the fact that in CRISPR-negative prokaryotes the nuclease sequence was absent, a collaborative role between the repetitive elements and the nuclease was hypothesised to be involved in the targeted metabolism of DNA (Jansen et al., 2002).

The CRISPR system operates through a guiding RNA sequence that targets nucleic acids through Watson-Crick base paring, and an endonuclease enzyme that digests the targeted nucleic acid. In the originally discovered prokaryotic context, the targeting guide RNA (gRNA) is comprised of a CRISPR RNA (crRNA) and a *trans*-activating CRISPR RNA (tracrRNA) (Gross et al., 2015; Jinek et al., 2012; Ran et al., 2013b) (Figure 4-1). The crRNA is an 18-20 nucleotide sequence that confers the guiding capacity. The tracrRNA hybridises

with the crRNA and supports binding between the gRNA and a Cas protein (Jinek et al., 2012). With the addition of intervening nucleotides between the crRNA and tracrRNA, they can be fused as a chimeric single guide RNA (sgRNA). Through modification of the crRNA region of the guideRNA sequence, almost any nucleic acid sequence can be targeted for endonuclease activity. For endonuclease activity to occur at a target site it is essential that a protospacer adjacent motif (PAM) is present. For *Streptococcus pyogenes* Cas9 (*sp*Cas9) the PAM is a 5'-NGG-3' recognition motif proximal to the 3' end of the gRNA sequence. The PAM sequence is variable with Cas proteins from different bacterial species (Kleinstiver et al., 2019). The DSB created by Cas9 occurs 3 nucleotides downstream from the PAM recognition motif.

Moving from understanding the original bacterial response to the extrapolation of CRISPR/Cas9 into an improved and adaptable gene-editing technology that allows specific changes to be made to eukaryotic genomes was a seminal moment across biological studies (Jinek et al., 2012). There are three major CRISPR/Cas systems that have been discovered. Type I and III feature a multitude of Cas genes to carry out the process, whereas Type II requires only a single nuclease (Jinek et al., 2012). Cas9 is a type II system. It is the most widely used endonuclease and was used in this research project. Unless otherwise specified, the Cas protein of reference herein is *sp*Cas9.

Through having a programmable RNA sequence as opposed to a large peptide sequence conferring the DNA targeting capacity, CRISPR reagents can be designed and tested across multiple sites relatively rapidly and at a fraction of the cost of the preceding technologies (Adli, 2018). This has led to is widespread adoption as the preferred gene-editing methodology. With high cutting efficiency at loci throughout the genome and low off-target endonuclease activity, CRISPR has driven the dissemination of genome-editing. Through biological research the CRISPR toolkit has become diversified and optimised. Engineering of the Cas9 amino acid sequence has reduced the level of off-target effects (Kleinstiver et al., 2016; Slaymaker et al., 2016) as well as to create Cas9 variants that nick only one strand of DNA (Ran et al., 2013b). Further discoveries and modifications within the CRIPSR/Cas genome editing

toolbox have led to capabilities that include but are not limited to substitution of a single base pair, larger scale genome engineering, epigenetic modifications and diagnostic detection of DNA or RNA (Anzalone et al., 2020; Gootenberg et al., 2017). Because the variable region of a gRNA consists of only 20 nucleotides, testing of multiple gRNAs is achievable and CRISPR can be deployed for targeting single genes *in vitro* and *in vivo*, or scaled up to perform genome-wide CRISPR knockout screens (Han et al., 2018; Yau and Rana, 2018).

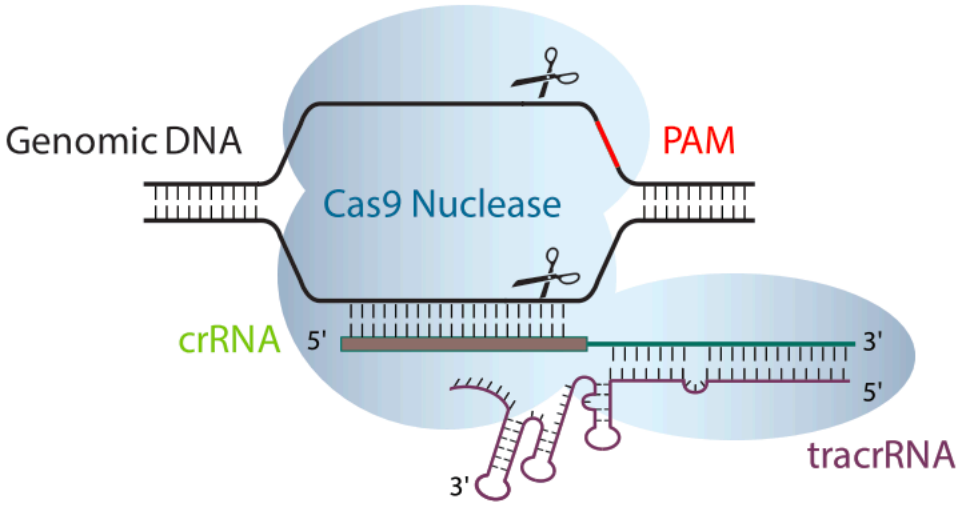


Figure 4-1: The CRISPR/Cas9 complex. The cRNA (green) confers the nucleic acid targeting capacity and the tracrRNA (purple) hybridises with the crRNA and supports the gRNA interaction with the Cas9 enzyme. The presence of a PAM (red) is required for endonuclease activity to occur. Figure adapted from Gross et al., 2015.

4.1.2 Host Exploitation by IAV

Being an intracellular parasite with a core proteome of only 10 proteins (Pinto et al., 2020) means that IAV is not able to complete its replicative cycle without support from host factors. It relies on host proteins for cell entry, endosome processing, nuclear import of viral RNA, transcription, nuclear export of viral mRNA, translation, viral packaging and budding (Figure 1-8) (Karlas et al., 2010; König et al., 2010; York et al., 2014). Cap-snatching of 5’ caps from host

mRNAs to prime the initiation of vRNA transcription, the role that Nuclear Export Factor1 (NXF1) has in translocating IAV mRNAs, and host ribosomes performing protein translation are primary examples of host factors that are exploited to support IAV propagation.

Identifying Host Co-Factors

Host co-factors that promote or are in some way beneficial to IAV propagation have been discovered through a range of biochemical and genetic investigation methods (Figure 4-2) (Schaack and Mehle, 2020). Biological screens that disrupt an entire proteome or genome to observe protein or nucleic acid interactions between hosts and IAV have been critical in providing insights into viral manipulation of a host and potential vulnerabilities that could be focused on for therapeutic interference. The screening approaches outlined here, as with all investigations, have different objectives in what type of interaction they can expose, the stage of the viral replication cycle they are relevant to, and whether they can uncover proviral or antiviral factors. For gene-editing applications towards IAV resistance in animals, the most informative outcomes are the proviral factors identified. The alteration of host proviral components which increase IAVs ability to propagate can result in defective IAV replication. Methods that have been used to identify putative gene-editing targets have largely been performed in human cell lines with human-origin IAVs, however the results are still informative when considering investigations of other IAV subtypes and host species, including pigs.

Genome-wide association studies (GWAS) can be used to identify Single Nucleotide Polymorphisms (SNPs) that affect IAV susceptibility. They have been effective at identifying SNPs with small effects associated with the susceptibility of a host to severe disease by comparing control populations to a population with a severe disease phenotype. GWAS have not been performed for pigs with swine influenza, but in human studies there have been no factors identified that could be suitable targets for IAV resistance to date (Allen et al., 2017; Clohisey and Baillie, 2019; Garcia-Etxebarria et al., 2015; Zhou et al., 2012).

Prior to CRISPR/Cas9 being developed, random mutagenesis was the predominant genome-wide *in vitro* screening method. Through a gene-trap screen in haploid cells the host genes SLC35A2 and CMAS were identified. These are genes whose products are important in the pathway for glycans to be modified with sialic acids (Carette et al., 2009). Because haploinsufficiency is less likely to affect viral replication significantly, the use of haploid cell lines negates the issue of needing homozygous edits for gene knockouts as is necessary in diploid or polyploid cell lines. The paucity of antiviral genes identified from this haploid screen led to the authors suggesting that antiviral responses are multifactorial and that the loss of single genes has limited effects on viral replication.

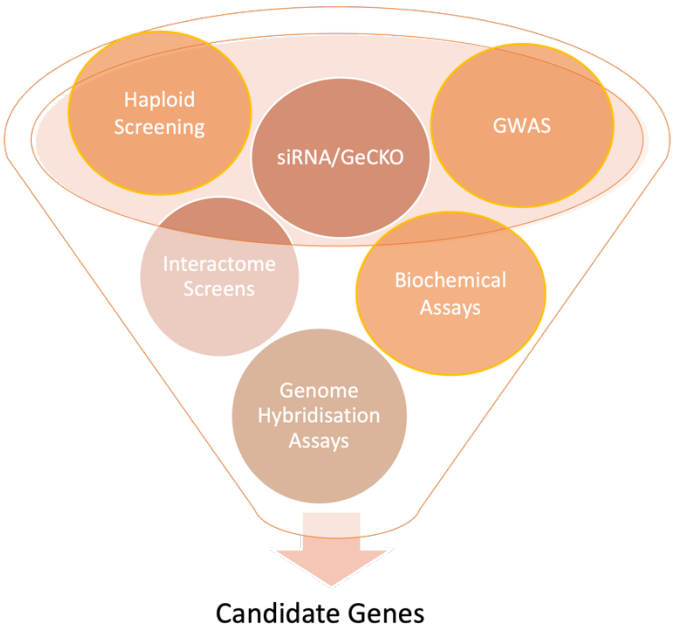


Figure 4-2: By sorting and filtering biological data candidate genes for introducing IAV resistance to pigs can be identified.

An alternative genome-wide screening method that is functional in diploid cell lines is RNAi. Genome-wide screens with RNAi have assessed *in vitro* susceptibility to IAV through different experimental readouts, all with the aim of identifying host factors that are exploited during the IAV replication cycle. The RNAi screen by Konig *et al*. 2010 assayed viral gene expression by proxy through luminescence detection. A screen by Karlas *et al*. 2010 measured whole virions and nucleoprotein production. Brass *et al*. 2009 measured HA transport to the cell surface as a proxy for IAV replication, whilst Su *et al*, 2013 sequenced DNA of transduced cells to detect which of the RNAi expressing constructs were enriched after several days of viral infection. Across these screens, 4.6% of protein-coding genes in the human genome have been associated with affecting IAV infection (Capitanio and Wozniak, 2012). However, in this meta-analysis, no genes were identified across all studies and 92% of the gene hits were found in a single screen. These variable outcomes are likely due to disparities In the design, IAV subtypes and the cell lines used across the studies. Further research has suggested that the discrepancy is caused by false-negatives rather than false-positives (Hao et al., 2013). A premise supported by the fact that many of the gene hits not present across multiple screens are from similar protein complexes or proteins that overlap in biological function (Capitanio and Wozniak, 2012; Hao et al., 2013). A drawback of RNAi experiments, especially those using only one interfering RNA molecule per gene as in some of these studies, is that gene knockdown can be partial, and if IAV exploitation of the gene is not dose dependent, the presence of a minimal amount may not affect susceptibility. The capacity to stop gene expression rather than instigate degradation of mRNA would solve this issue at the genetic source.

CRISPR Screens

The scaling up of CRISPR/Cas9 allows for gene disruption at a genome-wide scale (Han et al., 2018; B. Li et al., 2020; Sanjana et al., 2014; Shalem et al., 2014). Known as a genome-wide CRISPR knockout (GeCKO) screen, these lentivirus libraries target all protein-coding genes in the human with multiple sgRNAs to increase the reliability that knockout will be effective. GeCKO screens

have been used to identify host factors that are essential for many different viruses (Puschnik et al., 2017; Wang et al., 2021; Wei et al., 2021). The transduction of cassettes expressing Cas9 and a sgRNA at a low MOI is calculated so that only 1 gene is likely to be targeted in each cell.

There have been two reported GeCKO screens performed with IAV infection (Han et al., 2018; B. Li et al., 2020). These screens have corroborated the findings from the haploid screens that identified genes involved in sialic acid production and modification pathways, several V-type ATPase family proteins (important for endosome acidification), COP-I proteins that are required for retrograde transport of host contents between the Golgi apparatus and endoplasmic reticulum and recruited by IAV for assembly as well as NXF1 that supports nuclear export of mRNAs. The Han *et al*. 2018 screen selected cells after multiple rounds of viral infection and found sgRNAs targeting 501 genes were enriched after infection. The Li *et al*, 2020 screen measured surface expression of viral HA by antibody staining as the phenotypic readout. Infected cells were sorted into low and high bins according to surface viral HA and compared to an uninfected wildtype control population, with the relative abundance of transduced sgRNAs sequenced in each bin indicating whether gene targets enhance or inhibit IAV propagation. From the low HA expressing cells, there were 121 genes enriched, indicative of a proviral function. Only six genes were identified across at least four RNAi screens, and these were all identified in this GeCKO screen.

CRISPR screening has also been applied with dead Cas9 (dCas9), an inactivated Cas9 protein, fused with a synergistic activation mediator, a complex that recruits transcription factors to target sequences in promoters (Konermann et al., 2015). Through overexpression, host factors that can reduce infection of IAV have been identified (Heaton et al., 2017). These gene activation screens are important studies in improving our understanding IAV infection dynamics in the host and could play a role in the development of transgenic animals, however gene-editing could only be applied through site specific modifications aiming to alter expression or epigenetic regulation.

Genome-wide screening is very effective at identifying proviral host factors that could be effective gene-editing targets. Remove the IAV exploited host factor, and IAV propagation is reduced. But as a downside they do not provide a mechanistic understanding of how these identified factors actually affect viral propagation and they do not distinguish between factors that interact directly with viral proteins and those that indirectly affect IAV replication. Complementing the knockout screens with interaction screens does offer further insights into whether the gene target is directly or indirectly affecting IAV propagation. Two interactome screens that used mass spectrometry have been performed to reveal interactions specifically with the IAV RNP complex and each protein individually (Watanabe et al., 2014; York et al., 2014).

The approach taken by York *et al*., 2014 was to use purified strep-tagged vRNP complex to reveal host proteins that interact with vRNP as a complex as opposed to with FluPol's individual components. This screen identified 171 cellular proteins. The Watanabe *et al*., 2014 study individually transfected each core viral protein (and additional accessories) and thus interactions between the host protein and each individual IAV protein were identified. This was a powerful investigation and top hits identified were confirmed through siRNA experiments, minigenome assays and virus-like particle formation measurement. One gene identified from the screen was Acidic Nuclear Phosphatase 32 Family Member B (ANP32B) (Watanabe et al., 2014).

The Discovery of the ANP32 and IAV Association

IAV genome amplification requires that the viral RNA (vRNA) is copied to complementary RNA (cRNA) and then amplified from the cRNA template. If IAV could efficiently replicate its own genome, the minimal requirement for this reaction to occur would be vRNP and vRNA. IAV genome replication was observed to be an inefficient process in a cell-free environment by Sugiyama *et al*, 2015. Using fractionated nuclear extracts they isolated two proteins that drastically improved the efficiency of vRNA synthesis from the cRNA template. These two proteins were identified as pp32 and APRIL, aliases for the better

ascribed Acidic Nuclear Phosphoprotein 32kDa A (ANP32A) and Acidic Nuclear Phosphoprotein kDa B (ANP32B). The mechanism of how they affected viral genome replication was not specifically defined, but this was the first identification of ANP32A and ANP32B as being proviral factors for IAV replication.

In an *in vitro* mammalian system, avian-origin IAVs are restricted in their ability to replicate and adaptive mutations in the viral genome are required to optimise their replicative ability. Part of the biological barrier following zoonotic transmission of IAVs is the suboptimal viral polymerase activity (Arai et al., 2018; Gabriel et al., 2005; Long et al., 2019b; Rodriguez-Frandsen et al., 2015). In mammalian cells, amino acid substitutions at specific sites in the PB2 protein (590/591, 627, 701) of avian-origin IAVs are well documented to significantly affect polymerase activity. For site 627, avian-origin IAVs can be rescued to replicate efficiently in human cell lines by substitution of glutamic acid (E) to lysine (K) at 627 (E627K) (Subbarao et al., 1993).

To isolate host factors that are mechanistically involved in this host adaptation process, Long *et al*. 2016 performed a genome hybridisation assay (Long et al., 2016). This study used a chicken genome radiation hybrid panel, which is a hamster cell line that contained fragments of chicken chromosomes. The hamster cells that supported the highest level of avian origin IAV viral polymerase activity were hypothesised to contain a segment of the chicken genome encoding for a co-factor that was specific to improving the function of avian adapted IAV in the mammalian context. In one of the chicken chromosomes in the hamster genome was ANP32A. Expression of chicken ANP32A rescued activity of 627E avian-origin IAV to the same level as the 627K mutant virus in a human cell line. Avian ANP32A was thus revealed as a co-factor that is recruited by avian-origin IAVs to improve the efficiency of FluPol activity, and thereby support more effective production of progeny virions.

4.1.3 ANP32 Protein Family

The ANP32 family are conserved proteins of early eukaryotic origin that are characterised by N-terminal leucine rich repeats (LRR's) and a C-terminal low

complexity acidic region (LCAR) that are found in species from *Plasmodium* to pigs (Figure 4-3A)(Glass et al., 2018). These small proteins (~29 kDa) are a critical regulatory node with diverse functions (Reilly et al., 2014). The LRR domains form a globular structure of parallel beta-sheets and the uniquely acidic LCAR is a flexible region that is soluble and can interact with positively charged surfaces (Figure 4-3B) (Huyton and Wolberger, 2007; Matilla and Radrizzani, 2005). As the name suggests, ANP32 proteins can be post-translationally phosphorylated (Fries et al., 2007; Hong et al., 2004). ANP32A is variously labelled as LANP, C15orf1, PP32 and I1PP2A to name a few and ANP32B is called APRIL, PHAPI2 and SSP29 (Glass et al., 2018).

Most vertebrates have three conserved ANP32 proteins which have retained bona fide function. These are ANP32A, ANP32B and ANP32E. In pigs, ANP32A and ANP32B are 84% homologous and ANP32A has 75% protein sequence homology with ANP32E (NCBI BLAST). ANP32C and ANP32D are intronless paralogs of ANP32A that have been considered to be retrogenes or pseudogenes with oncogenic activity (Chen et al., 1996; Yuzefovych et al., 2015), however because ANP32C and ANP32D have not been biochemically identified as proteins, and their intronless structure making them susceptible to genomic DNA contamination during reverse transcription, Reilly *et al*., 2014 argue that they should not be considered as genuine members of the ANP32 family. ANP32 family proteins have been implicated in leukaemia and prostate cancer through mutations within the tumour suppressor domain (Agarwal et al., 2014; Kadkol et al., 1998).

The roles of ANP32 proteins are diverse, and despite some functional redundancy being evident, they have discrete roles as well. Their localisation was initially described as being exclusively nuclear, however further investigation has observed nuclear-cytoplasmic shuttling in certain situations. With various reports on their localisation and function, it is clear that the cellular and molecular context they are present in will affect their role. ANP32A and ANP32B are expressed constitutively throughout swine tissues (Freeman et al., 2012) and although they are part of the same gene family their functions differs depending on the tissue and molecular setting they are found in.

Importantly, as the site of IAV infection in swine they are expressed in tracheal and lung tissues of swine as determined by RNA-seq (Figure 4-4).

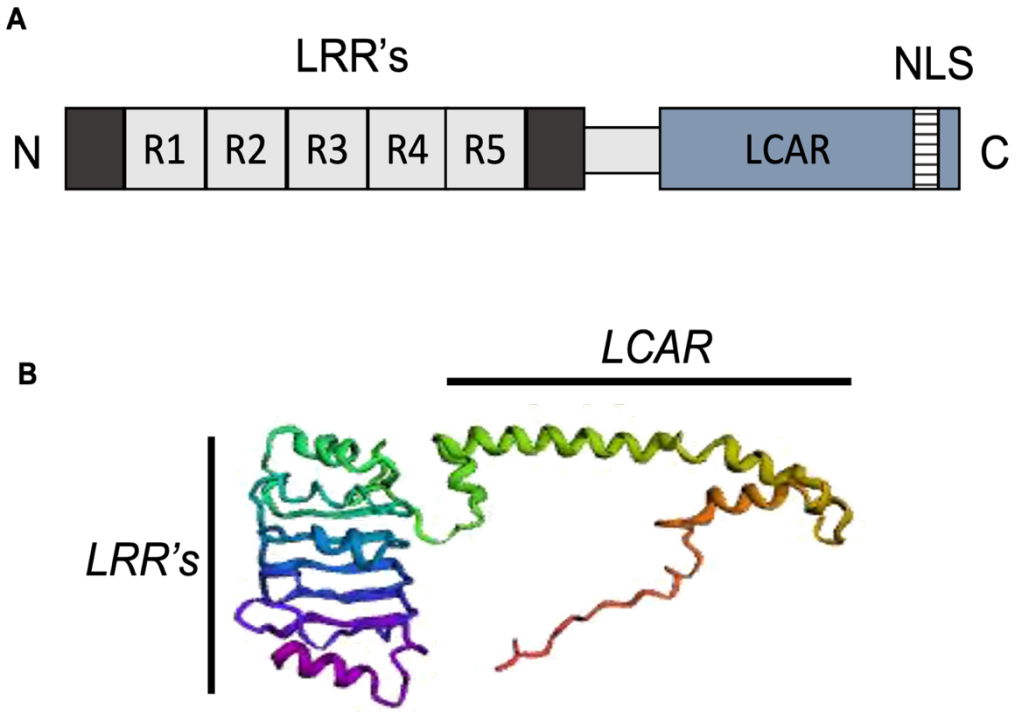


Figure 4-3: ANP32 family proteins. A) A schematic representation of ANP32 family proteins. ANP32 proteins are characterised by Leucine Rich Repeats (LRRs; R1-R5) at the N-terminus and a Low Complexity Acidic Region (LCAR) at the C-terminus, which also contains a Nuclear Localisation Signal (NLS). B) Wild type ANP32A structure from prediction software Robetta (Yang *et al.*, 2020).

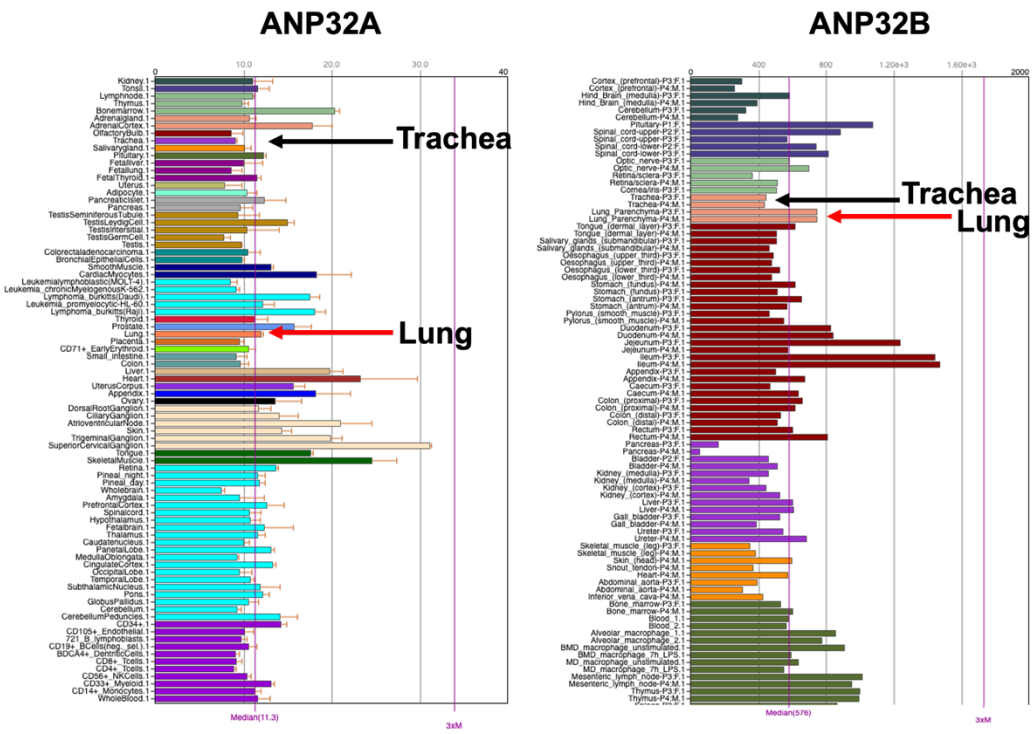


Figure 4-4: Gene expression of ANP32A in swine tissues determined by RNA-seq (http://ds.biogps.org/?dataset=BDS_00012gene=100155298) and ANP32B (http://ds.biogps.org/?dataset=BDS_00012gene=100152263). Black arrows indicate expression in tracheal tissue (purple bars) and red arrows are lung transcripts (orange bars).

ANP32A and ANP32B affect transcription regulation via chromatin remodelling. For ANP32A this is through steric hinderance of histone tails (a member of the Inhibitor of Histone Acetylation (INHAT) complex), and for ANP32B it is through histone chaperone activity (Seo et al., 2001; Tochio et al., 2010). They also both regulate gene expression through altering the affinity that some transcription factors have when binding with particular promoters (Huyton and Wolberger, 2007; Munemasa et al., 2008; Tochio et al., 2010). ANP32A has been established to interact with SET, an oncoprotein and apoptotic regulator (Agarwal et al., 2014). The LRR region of ANP32 family proteins act as adaptors for protein interactions, such as between the nuclear export factor CRM1 (also called XPO1 and identified in RNAi and GeCKO screens as an IAV host factor), the mRNA-binding HuR for export of mRNA to the cytoplasm, SET oncoprotein complex affecting signal transduction and cellular

proliferation, and MAP1B in an Ataxin-1 dependent manner during neurogenesis (Fries et al., 2007; Opal et al., 2003).

Because of the high level of conservation between animal species and the diverse functions of ANP32 proteins, it could be anticipated that their loss of function would have severe consequences. In more primitive metazoans, RNAi of the single ANP32 homolog results in embryonic lethality for *Caenorhabditis elegans*, whilst in *D. melanogaster* there was no observed effect (Mummery-Widmer et al., 2009; Rual et al., 2004). In mammals, only mice have thus far been used as animal models for investigating the role of ANP32 proteins in development and disease. ANP32A null mice, and ANP32E null mice are viable, fertile and were not observed to display abnormal behaviours (Opal et al., 2004; Reilly et al., 2010). Their offspring inherited alleles approximately according to Mendelian inheritance pattens, and thus it was concluded that functional redundancy between at least ANP32A and ANP32E from the ANP32 family proteins was significant.

Knockout mutagenesis of ANP32B in mice demonstrated that this conclusion would be premature. ANP32B is important for murine embryogenesis and its removal substantially reduced embryo viability, whilst mice that were born had a reduced size and showed premature ageing (Reilly et al., 2011). Lethality was not attributable to specific defects as aborted embryos showed abnormalities in diverse organs. Furthermore, introduction of an ANP32A null genotype in the context of ANP32B null exacerbated the nonviability of mice embryos. This effect was not observed for ANP32E, and in a triple mutant background, one functional ANP32B allele was sufficient for survival to weaning age. This study in mice outlines a hierarchical redundancy of ANP32B>ANP32A>ANP32E for development in mice. In other mammals this may not recapitulate faithfully, but it is likely that despite divergence in function, there will be functional redundancy and a loss of ANP32A and ANP32B concurrently would prevent viability. Despite the association of ANP32A and ANP32B with development in mice, there is no record of genetic disorders being associated with any ANP32 family proteins in the Online Mendelian Inheritance

in Man (OMIM) database, which could allude to the fact that loss of function

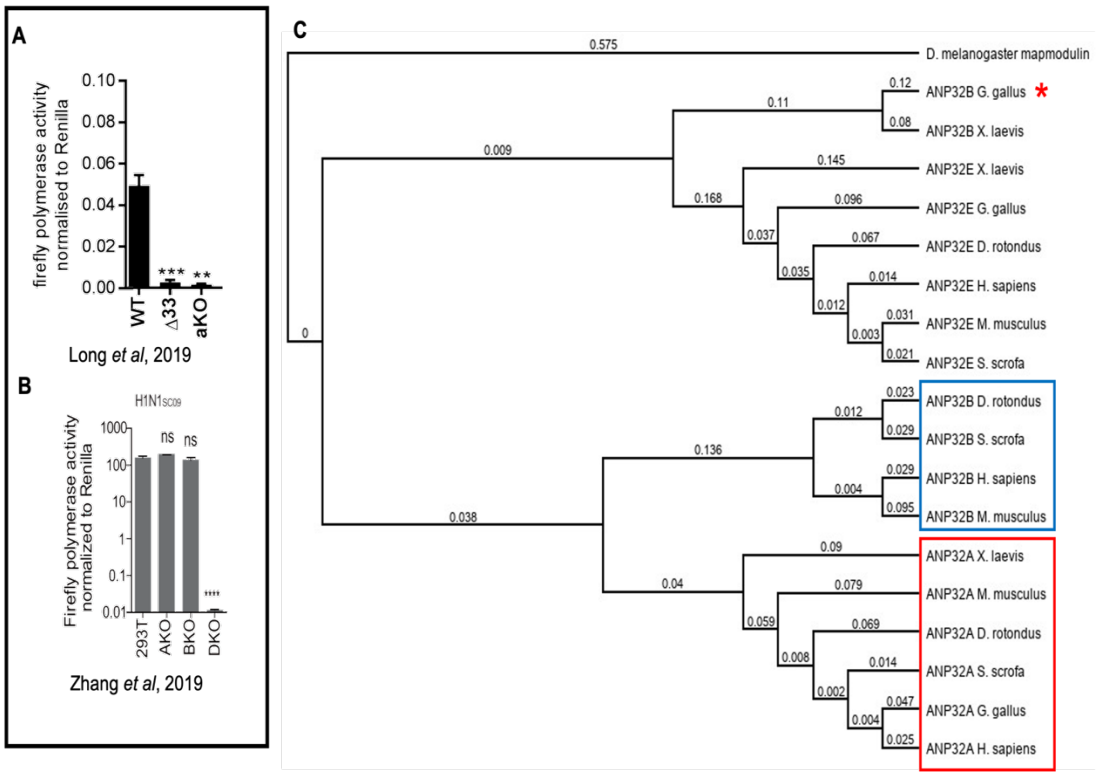


Figure 4-5: ANP32 proteins promoting avian and human IAV polymerase activity. A) Minigenome activity in chicken primordial germ cells with polymerase from the avian origin 50-92 IAV strain. Figure from eLife with permission. B) Minigenome activity in 293T cells (human origin) with polymerase from human origin H1N1 IAV. Figure from Journal of Virology with permission. C) Tree generated of ANP32 family phylogeny using Geneious alignment with a Blosum 90 cost matrix using *Mapmodulin* from *Dl melanogaster* as an outgroup. The red box indicates ANP32A proteins grouping. The blue box indicates mammalian ANP32B proteins. The red asterisk is chicken ANP32B.

mutants in humans causes embryonic failure in humans.

4.1.4 Using CRISPR For targeted Investigation of Swine IAV Host Interactions

This chapter set out to use CRIPSR/Cas reagents to investigate the role of the swine ANP32 family proteins on swIAV propagation. Previous work by Long *et al.* 2019 showed a loss of polymerase activity and significantly reduced viral replication in the absence of avian ANP32A in avian progenitor germ cells (PGCs) (Figure 4-5A), and Zhang *et al.* 2019 demonstrated that in humans, both

ANP32A and ANBP32B are required to be disrupted to achieve the loss of polymerase activity and replication capacity (Figure 4-5B) (Long et al., 2019a; Zhang et al., 2019). It was also observed in the Zhang *et al.* 2019 study that chicken ANP32B and murine ANP32A could not reconstitute activity of avian or human-origin IAV polymerase, likely due to differences at key residues which interact with the vRNP complex.

Because of the role swine have in the ecology of IAV and their underlying biology that allows them to serve as a mixing vessel for avian and mammalian-origin IAVs, it is important to investigate the role that ANP32 family proteins have during IAV infection of swine. Using *D. melanogaster* mapmodulin as an outgroup, we generated a phylogeny tree to interpret the relationship between ANP32A and ANP32B from humans, pigs, chickens, and mice (Figure 4-5C). This showed that swine ANP32A and ANP32B are closely related to human and mice ANP32A and ANP32B. Because chicken ANP32B is distantly related, and more closely related to mammalian ANP32Es, that have not been identified to promote IAV polymerase activity, we selected ANP32A and ANP32B as the only proteins in this family that we would target for disruption in our *in vitro* swine model.

4.1.5 Chapter Aims

The aim of the work undertaken in this chapter was to develop and validate an *in vitro* model for swIAV resistance created using CRISPR technology. The piglet tracheal cell line was selected to provide insight into the role that host factors ANP32A and ANP32B play during IAV infection. NPTr was chosen because in swine, IAV infections are generally restricted to the respiratory tract (Janke, 2013). As the only secondary cell line originating from epithelial cells of the respiratory tract, NPTr represents the most relevant cell culture model for IAV infections. These CRISPR-edited NPTr cells with ANP32A and ANP32B null mutations are now available as a resource for investigating the role that ANP32 family proteins have in swine during infection with avian, human and swine origin IAVs, and could be utilised further for understanding the emergence of zoonotic IAV strains.

4.2 Results

4.2.1 The Localisation of ANP32A and ANP32B in Swine Trachea

To determine the localisation of ANP32A and ANP32B in swine trachea, a site of IAV infections in swine and the origin of the cells used in subsequent *in vitro* work, we performed immunohistochemistry against ANP32A and ANP32B, individually. DAPI was used as a marker for cell nuclei as it binds to AT rich regions of double-stranded DNA. For ANP32A (green) and ANP32B (red), there is colocalisation of fluorescence signal with DAPI, inferring that both proteins are present in the nuclei of cells in piglet trachea (Figure 4-6).

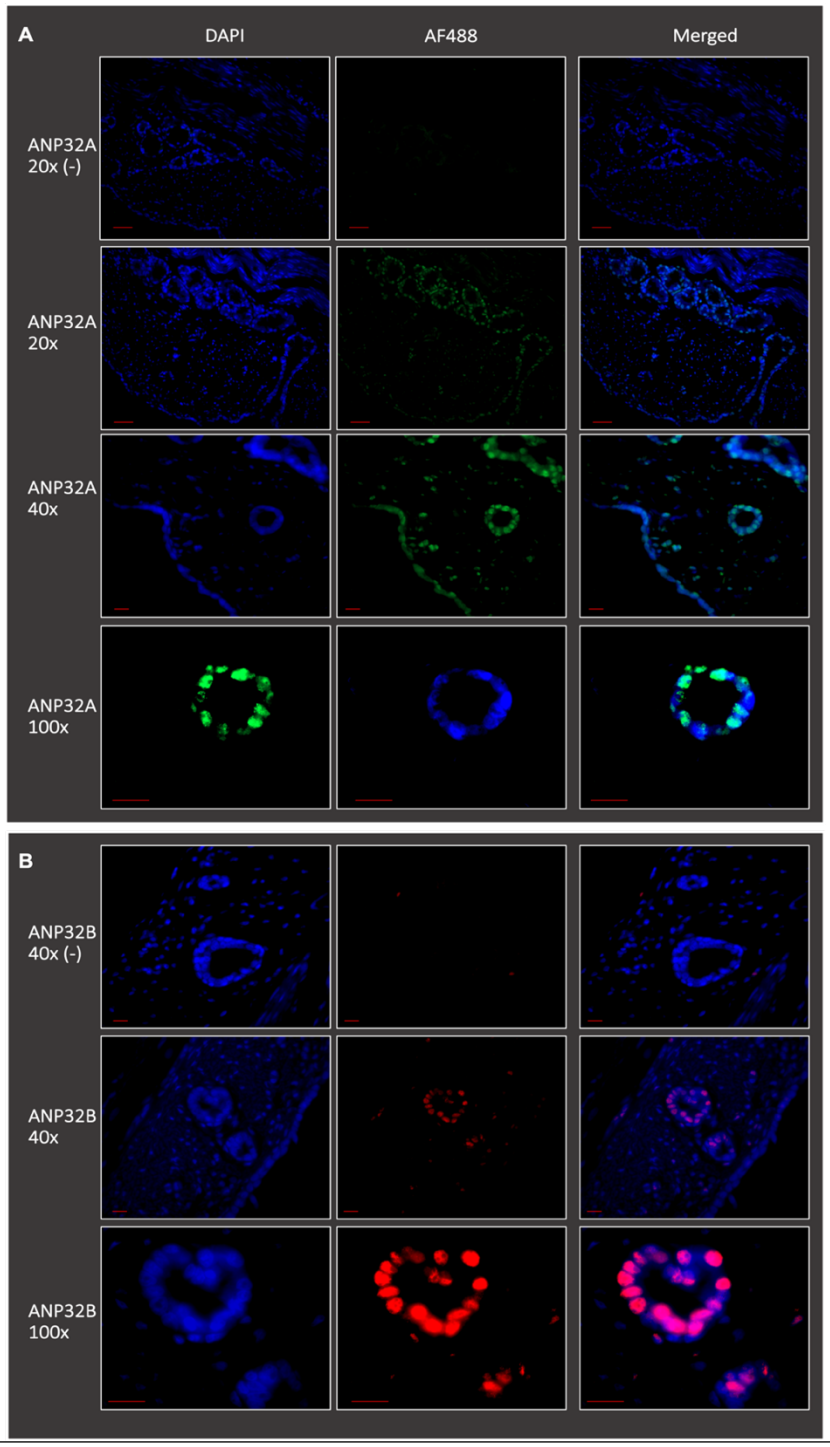


Figure 4-6: Immunohistochemistry to observe the localisation of ANP32A (ab189110) and ANP32B (ab4224) in piglet trachea. Nuclei are counterstained with DAPI (blue). A) Images of ANP32A show a nuclear localisation. B) Images of ANP32B show a nuclear localisation. Scale bars for 20x are 50 µM, 40x are 20 µM, and 100x are 20 µM.

4.2.2 Confirming ANP32A and ANP32B Gene Expression in NPTr

To validate Newborn Pig Tracheal cells (NPTr) as an appropriate model system for investigating whether FluPol recruits ANP32A and ANP32B in pigs, the genes must firstly be expressed. Gene expression can be assessed in a non-quantitative manner by RT-PCR. Primers for the housekeeping gene Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ) were selected for use as a control. The YWAHZ primers were validated for qPCR usage as housekeeping controls in swine by Nygard et al., 2007. Primers for cDNA of ANP32A and ANP3B were specifically designed for testing in this project.

Total RNA was extracted from actively dividing Wildtype (WT) NPTr and confirmed to be uncontaminated with DNA and undegraded by TapeStation, with an RNA Integrity value (RIN) of >9.8 (Fig 4-7A). Negative controls were samples that had no RT included in the RNA to cDNA conversion step. YWHAZ amplified the predicted size product (203 bp) (Figure 4-7B). Primers for ANP32A and ANP32B also amplified a PCR product of the predicted size (127 bp and 106 bp, respectively), confirming that both genes are expressed in NPTr. As this was a non-quantitative assay that was performed as part of optimising for subsequent qPCRs, the faint band observable in the ANP32B (-) lane indicating of contamination was not of concern

4.2.3 The Localisation of ANP32A and ANP32B In NPTr

The localisation of a protein provides information that has a bearing on its potential functionality. To improve the efficiency of IAV polymerase activity, it is essential that ANP32A and ANP32B are present in the nucleus to interact with FluPol during genome replication. By immunofluorescence staining in NPTr and co-staining with DAPI, we aimed to determine the distribution of ANP32A and ANP32B in NPTr. Background fluorescence was accounted for using a negative control that was incubated with only the secondary antibody. ANP32A was observed to colocalise with DAPI in the nucleus (Figure 4-8A). Furthermore, co-staining with the cytoskeletal marker phalloidin emphasised its nuclear localisation (Peacock et al., 2020b) (Appendix 2). Similarly, ANP32B was

observed to have a nuclear localisation (Figure 4-8B). These data confirm the presence of ANP32A and ANP32B in the nucleus of NPTr. To confirm that both ANP32A and ANP32B were concurrently present in nuclei, we co-stained with both antibodies and specific secondary antibodies. In these images, it was observed that ANP32A and ANP32B were predominantly present in the nuclei. The exception was in cells with condensed chromatin, in which both ANP32A and ANP32B were diffuse throughout the cell (Figure 4-8C).

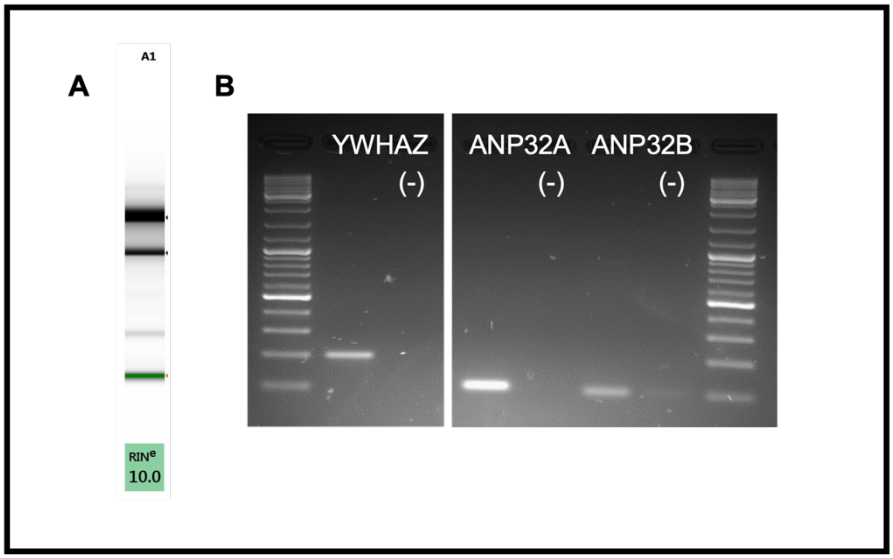


Figure 4-7: Gene expression of ANP32A and ANP32B in NPTr confirmed by RT-PCR. A) As quality control on the extracted RNA, Tapestation was performed. RIN = RNA integrity value, maximum score is 10. B) End-point PCR on cDNA samples following cDNA conversion. (-) control samples had no reverse transcriptase included during cDNA synthesis. Expected fragment sizes; YWHAZ = 203 bp. ANP32A = 127 bp. ANP32B = 106 bp.

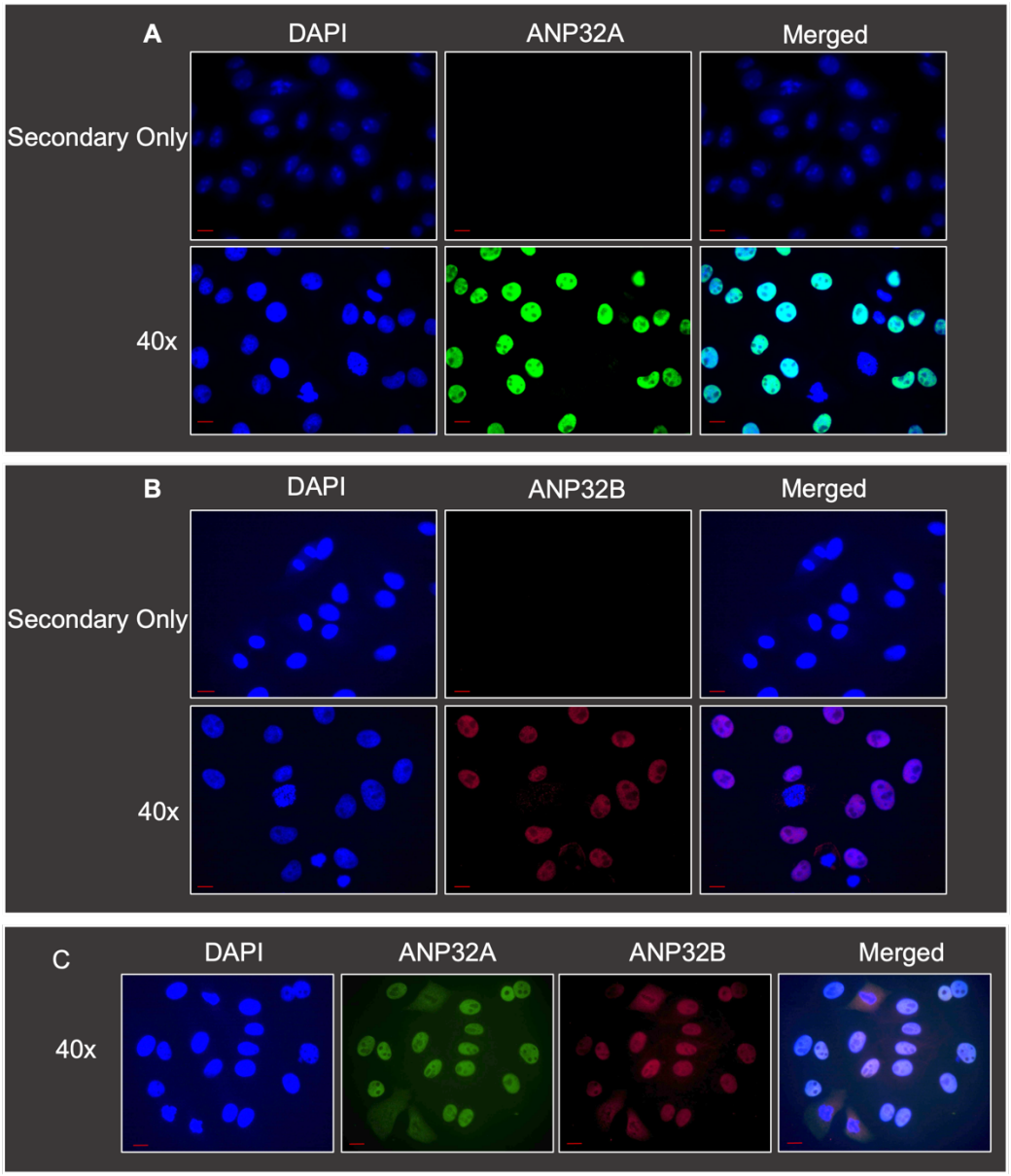


Figure 4-8: Immunocytochemistry of ANP32A (ab189110) and ANP32B (ab4224) to determine their localisation in NPTr. 40x magnification. Nuclei are counterstained with DAPI (blue). A) Images of ANP32A (green) show a nuclear localisation. B) Images of ANP32B (red) show a nuclear localisation. C) Co-staining of ANP32A and ANP32B. Scale bars (red bar) are 20 µM.

4.2.4 Disrupting ANP32A and ANP32B in NPTr with CRISPR/Cas9

gRNA Design

To investigate whether ANP32A and ANP32B are recruited by IAV to improve the efficiency of viral genome replication in porcine cells we set out to disrupt the coding sequence of each gene using CRISPR/Cas9 technology. gRNAs were designed with the intention of introducing DSBs in the DNA sequence at the target site that would result in indels being introduced which disrupt the ORF, and thereby ablate presence of the functional proteins.

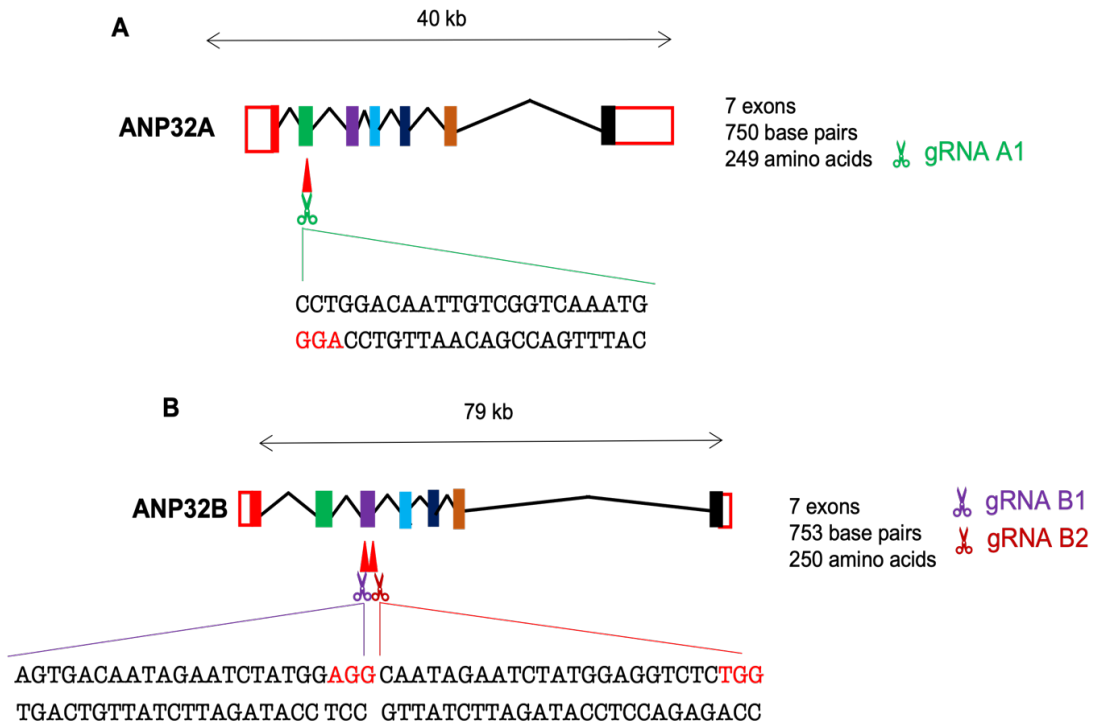


Figure 4-9: Linear schematics of swine ANP32A and ANP32B based on the pig genome annotation *Sscrofa11.1*. The scissors are gRNAs tested for CRISPR gene-editing. The black nucleotides are the gRNA sequences, and the red nucleotides are the PAM site upstream of the gRNA. A) ANP32A transcript and gRNA location. B) ANP32B transcript and gRNA location.

The target sequence of gRNA's was selected using the CRISPOR (Haeussler et al., 2016) online tool designed to the *Sscrofa11.1* genome. Selection of appropriate gRNAs was based on high specificity scores (low off-target effects) and high predicted on-target activity (Doench et al., 2016). A gRNA sequence against ANP32A exon 2 (previously tested by Claire Neil, The Roslin Institute) and two gRNA sequences targeting exon 3 of ANP32B were selected for testing (Figure 4-9).

gRNA Cloning into Expression Vector

For gene-editing of ANP32A and ANP32B we used a plasmid vector (pSL70; modified by Dr Simon Lillico from pX458 and originally gifted from Dr Feng Zhang) containing a CMV driven Cas9-T2A-eGFP and U6 expressed gRNA cassette that could be transfected into NPTr's (Figure 4-10A). The gRNAs were purchased as homologous oligonucleotides with 5' overhangs for sticky-end cloning.

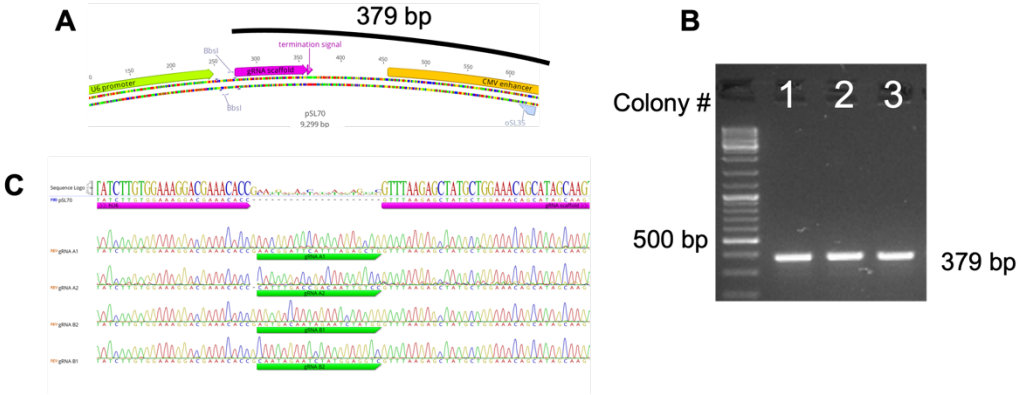


Figure 4-10: Cloning and confirmation of gRNAs into pSL70, the Cas9 and sgRNA expression vector. A) pSL70 contains a U6 promoter upstream and a tracrRNA scaffold downstream of the *BbsI* recognition sites that are used for sticky-end ligation of the oligonucleotides. B) Colony PCRs to confirm oligo integration in the correct orientation was performed using the forward gRNA oligo and oSL35. Bands of 379 bp indicate gRNA oligos are cloned into the vector in the correct orientation. C) An example of Sanger sequencing to confirm gRNA retained fidelity after cloning.

The ligated oligonucleotides were cloned into pSL70 and their orientation was confirmed by colony PCR, using the forward orientated gRNA oligonucleotide as a primer and oSL35 (Table 2-3 and 2-6) as a reverse primer. Amplification of a product of 379 bp was inferred as successful integration of the gRNA oligonucleotides (Figure 4-10B). Sequencing confirmed the gRNA was orientated correctly in relation to the U6 promoter and had maintained sequence integrity during amplification (Figure 4-10C).

Testing of gRNAs against ANP32A and ANP32B

To assess the relative cutting efficiency of gRNA's, NPTr were transfected with the Cas9 and gRNA expressing plasmids and checked for transfection by visual assessment for GFP after 24 hours. Genomic DNA of the regions of ANP32A and ANP32B targeted for gene-editing was amplified by PCR using primers that were designed to amplify the relevant genomic regions and optimised across a gradient of annealing temperatures. The relative cutting efficiency of gRNAs was estimated by T7 assay. T7 endonuclease has cleavage activity at DNA mismatches. T7 assays are a proxy detection method for Cas9 efficiency as the presence of indels detected at the target site indicates Cas9 activity.

For the gRNA targeting exon 2 of ANP32A, the negative control sample transfected with an empty pSL70 vector and selected for GFP positivity had no T7 endonuclease activity observable (Figure 4-11). The secondary band present below the T7 incubated gRNA A1 indicates nucleotide mismatches (indel presence) when incubated with T7 endonuclease. For ANP32B, there was heterozygosity observed in the WT samples incubated with T7 endonuclease by the bands below each positive (T7) lane. For gRNA B1, no further bands were observed outside those already present in the positive control sample. The gRNA B2 sample has bands at the expected sizes for the Cas9 cutting of 285 bp and 228 bp. With Cas9 activity detected for gRNAs A1 and gRNA B2, these were selected for the experiments to generate knockout cell lines by NHEJ.

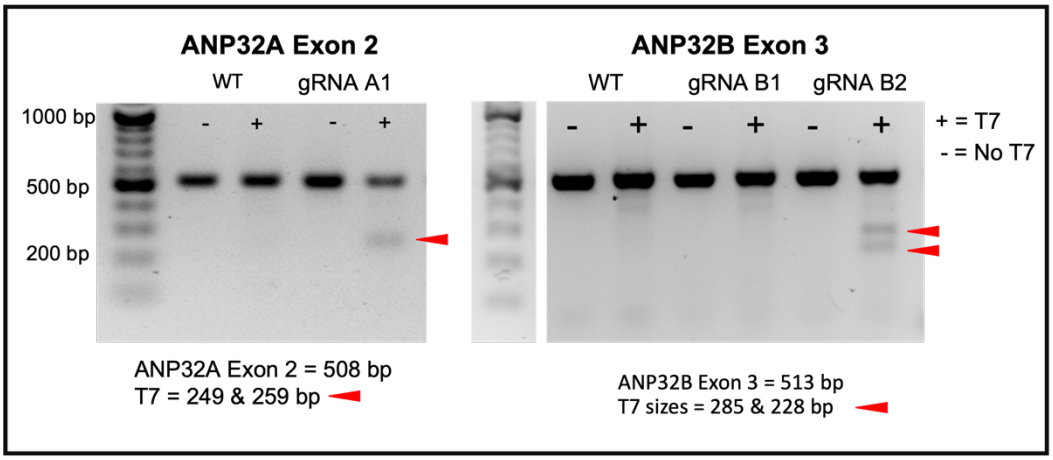


Figure 4-11: T7 endonuclease assays were performed to estimate the cutting efficiency of gRNAs targeting ANP32A and ANP32B in NPTr. PCR amplification of the region containing the CRISPR-targeted loci before incubation with T7 endonuclease that digests mismatched DNA base pairs identifies the presence of indels and is therefore a proxy for gRNA efficiency. Red arrows on the gel indicate cleaved bands that are indicative of double strand breaks and NHEJ occurring at the target site.

Clonal Expansion of Putative ANP32A and ANP32B Knockout Cell Lines

Transfections into NPTr were performed into WT NPTr with gRNA A1 and gRNA B2 individually as well as multiplexed to generate loss-of-function ANP32A and ANP32B knockout cell lines. Cells were sorted by FACS for the presence of GFP, which in pSL70 is expressed as part of the same mRNA transcript as the Cas9 so can be used as a proxy for gene expression (Figure 4-12A). To assess gRNA activity, a pool of GFP positive cells were assayed for editing by T7 assay (Figure 4-12B).

In the ANP32A only transfections (lane 1), Cas9 activity as detected by T7 assay was only present in with the gRNA A1. For the ANP32B gRNA only transfections (lane 2), there was no detection of indels introduced in exon 3 as there were no bands present that are not in the negative control. The heterozygosity observed here was also observed in the gRNA validation assays. In the multiplexed lane (lane M), PCR fragments that are not present in the negative control samples are observed for both ANP32A exon 2 and ANP32B exon 3, suggesting the introduction of indels has occurred in this sample. Using Geneious' *Analyze CRISPR Edits* tool, the proportion of alleles edited was

determined as 80% for monogenic ANP32A targeting and 79% for ANP32A when digenic targeting. ANP32B alleles were edited in 25% of the digenic clonal populations. Alongside the pooled samples, eGFP positive cells were clonally sorted into 96 well plates and cultured until they could be split, with one sample being sent for Sanger sequencing to determine their genotype.

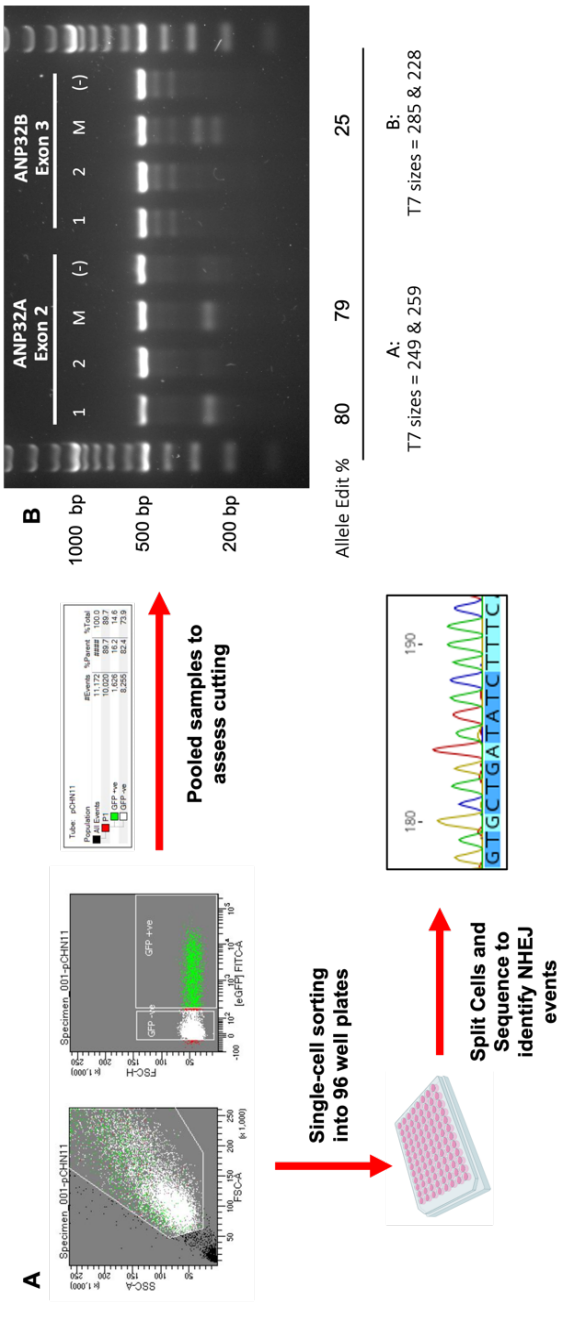


Figure 4-12: Clonal isolation of ANP32A, ANP32B and multiplex genome edited cells and confirmation of CRISPR activity at the target loci by T7 endonuclease assay in pool of GFP positive NPTr's. A) FACS of cells by selecting for expression of GFP. First, GFP positive cells were individually seeded into a 96-well plate for expansion before Sanger sequencing to determine genotype. B) Pooled NPTr with ANP32A targeted (1), ANP32B targeted (2) and ANP32A and ANP32B concomitantly targeted in multiplex gene-editing (M). The percentage of alleles edited in the clonally isolated cells was determined using Geneious *Analyze CRISPR editing tool*.

Sequence Results of ANP32A and ANP32B Targeting

Genomic DNA was extracted from the clonally isolated cells and ANP32A and/or ANP32B target regions were amplified and sent for Sanger sequencing to identify cells with homogenous genotypes that have mutated the ORF of either ANP32A, ANP32B, or both together (Figure 4-13). The indels that were introduced at the gRNA target sites in the selected cell lines that were used onwards in this project are displayed in Table 4-1. Cell line AKO is selected from AKO3 and BKO is selected from BKO1.

Interpretation of Sanger sequence chromatograms cannot be done visually if alleles are edited asymmetrically. To discriminate how indels were distributed in each clonal cell population, Sanger sequence data was input into Inference of CRISPR Edits (ICE; Synthego) online software. ICE deconvolutes Sanger sequence chromatograms with multiple variations at a target site to identify what genotypes are present (Figure 4-14A). An example of the asymmetrical sequences detected in AKO is shown in Figure 4-14B. The bases in figure 4-13B that are *N* are unidentified nucleotides, as ICE software only has the capability to align sequences and not to identify the nucleotides at the indel sites.

	ANP32A		**ANP32B**	
Allele	**#1**	**#2**	**#1**	**#2**
AKO	+1	-1	*	*
BKO	*	*	-8	-2
DKO1	+1	-1	+1	-2
DKO2	+2	-1	+1	-1
DKO3	-1	-12	+1	+1

Table 4-1: Table of alleles for the ANP32A and ANP32B knockout cell lines used throughout this project. * denotes a WT allele.

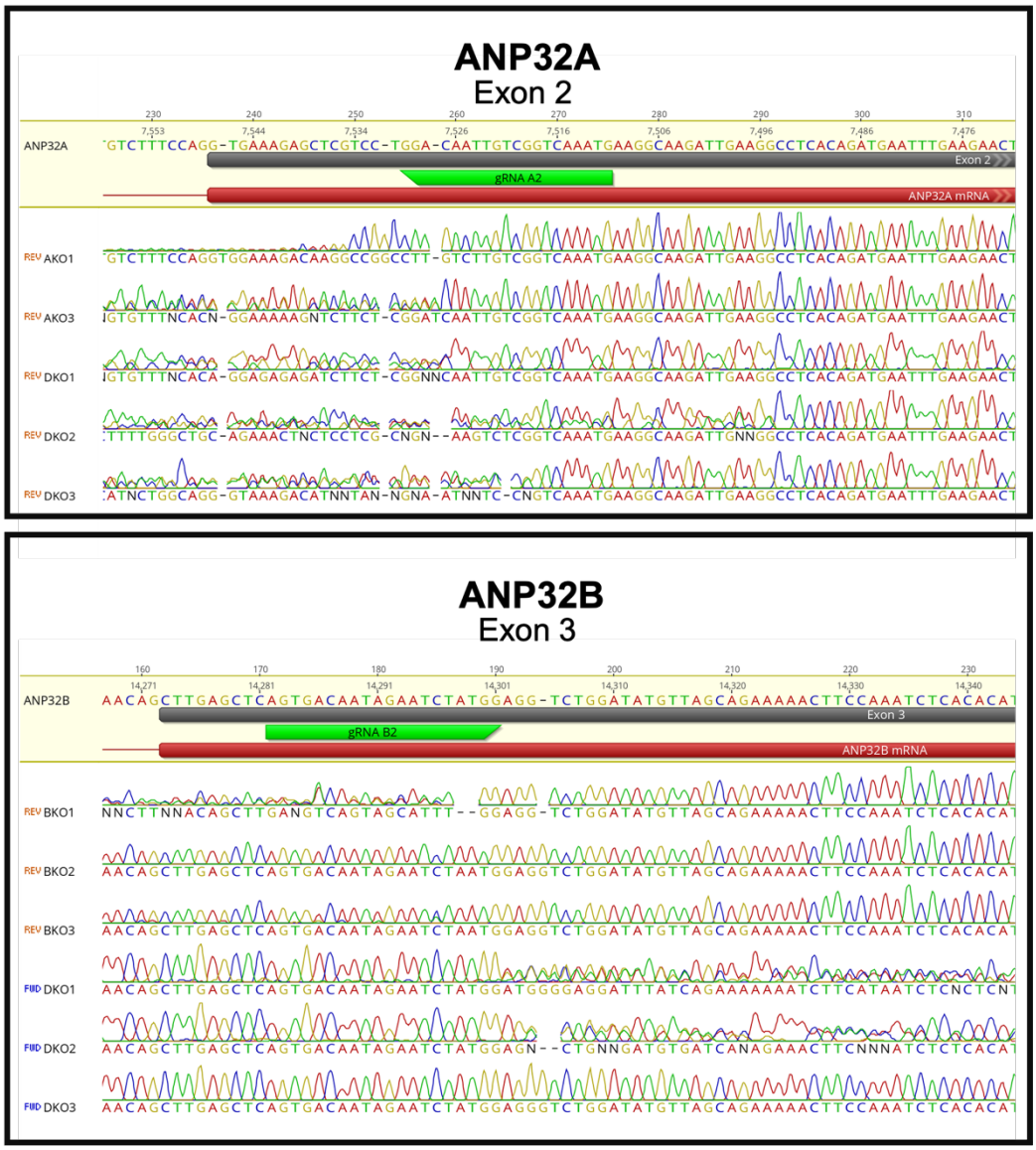


Figure 4-13: Sanger sequencing data from the CRISPR-edited NPTr's isolated for downstream analyses. The wildtype sequence is displayed in brown at the top of each annotated gene. The DSB is introduced 3bp downstream from the 5'gRNA terminus (green). With asymmetrical indels introduced, the chromatogram shows multiple peaks, for symmetrical indels there is a loss of alignment with the WT reference sequence and only a single peak observed in Sanger sequencing.

Changes to the ORF will differ according to the edit that has been introduced. All edited alleles were manually transferred into Geneious genome annotation software to determine whether the changes in the coding sequence resulted in changes to the translated peptide sequence that will introduce an early stop codon (Figure 4-15A). Stop codons are depicted by the black amino acids and are denoted with *. For all the cell lines with the genotype of ANP32A

disrupted there are premature stop codons introduced within exon 2. For ANP32B there are premature stop codons introduced in exon 3 (translation data not shown, represented by ICE plot with matching indel% and knockout scores).

For the DKO3 cell line, one of the edited alleles is a -1 bp disruption to the ORF that disrupts the coding sequence and introduces a premature stop codon. The other allele is a 12 bp deletion that keeps the ORF in-frame and is predicted to introduce the loss of four amino acids and not a nonsense transcript. To gain insight into how the loss of four amino acids could affect protein folding, the amino acid sequence was input into two protein structure predictions models, Robetta (Yang et al., 2020) and I-TASSER (Roy et al., 2010) (Figure 4-15B). From I-TASSER, there was little difference in tertiary structure to WT ANP32A, with the globular leucine rich head region and soluble acid tail structure retained. According to the Robetta prediction software, the loss of 4 amino acids had a significant effect on the tertiary folding of ANP32A. This cell line was retained for further analysis despite the potential it would not be a true ANP32A loss of function knockout.

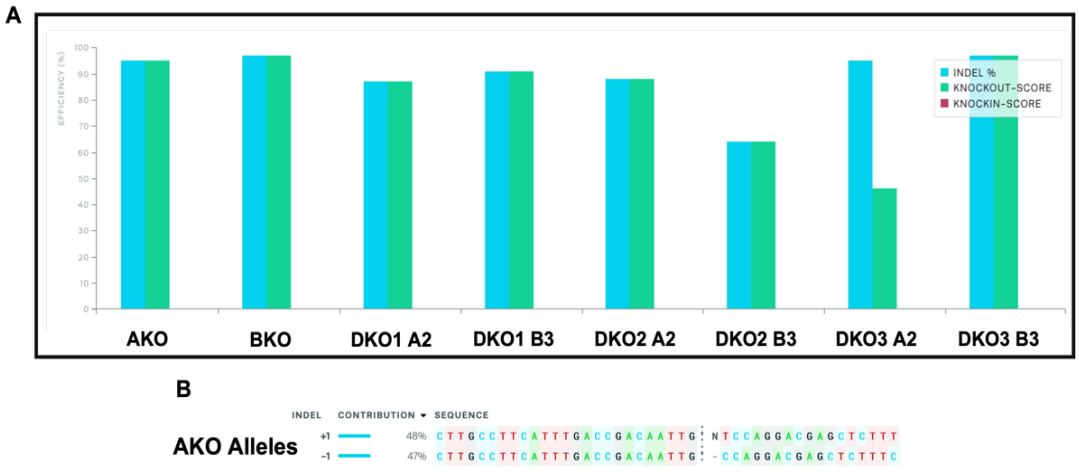


Figure 4-14: To analyse how CRISPR-targeting affected the genotype at target loci delineation of Sanger sequence data by ICE software was performed. A) ICE discriminates the Sanger sequencing chromatograms and compares edited sequences to wildtype sequences. Blue bars indicate the indel % detected (differs to WT at cut site) and green bars indicate the predicted proportion that will cause open-reading frame shifts that result in gene knockouts. A knockout score equal to the indel score indicates that all indels will result in an ORF shift that is likely to introduce a premature stop codon. B) Example image from the AKO cell line of the alleles present. The N is denoting any nucleotide as ICE software does not call nucleotides at insertion sites.

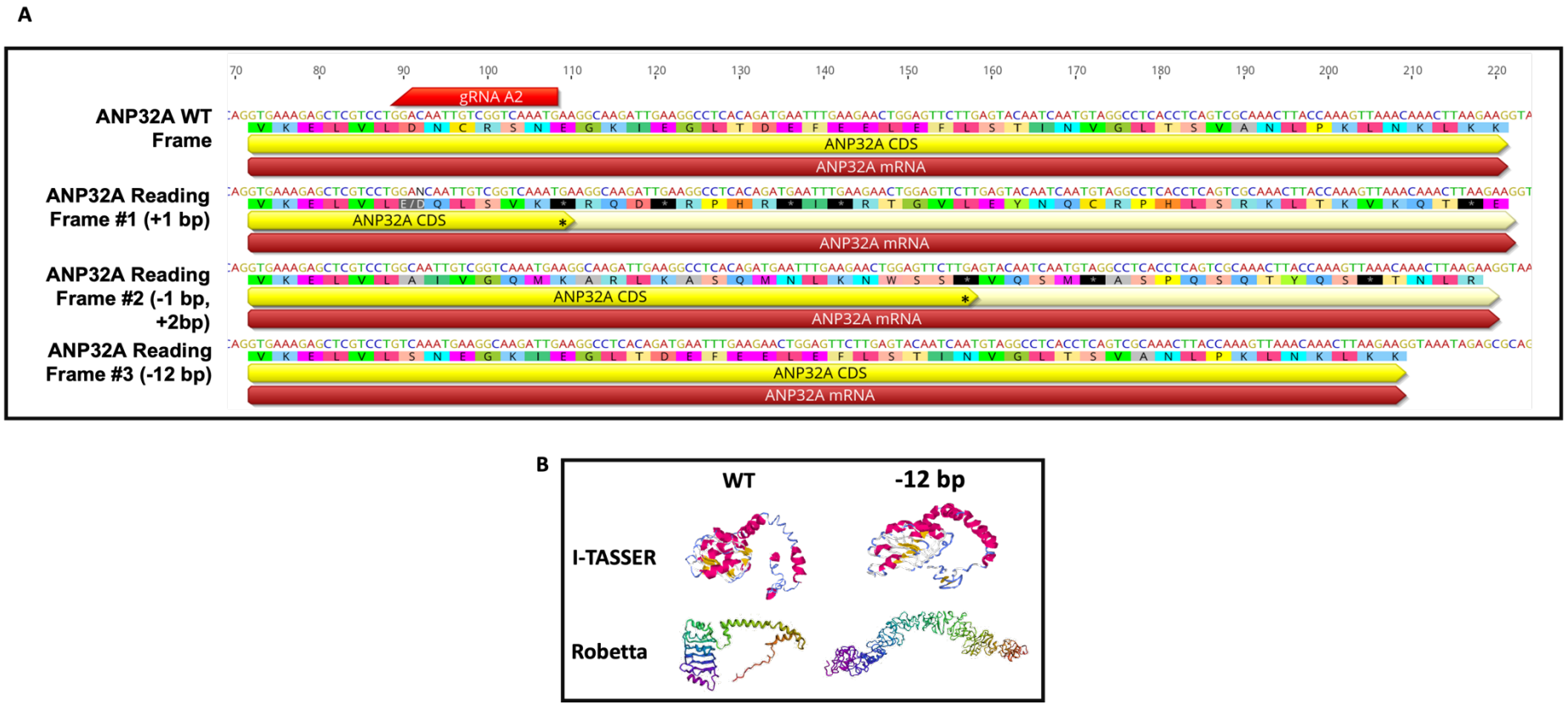


Figure 4-15: Changes to the genotype of ANP32A in the CRISPR-edited NPTr's altered the peptide sequence and introduced a premature stop codon in the open-reading frame. A) The different alleles for ANP32A that were introduced. The black amino acids indicated with * are indicative of stop codons. Different indels affect the ORF differently, but all have introduced the premature stop codon. B) Protein structure predictions of ANP32A wildtype and the ANP32A allele that has a 4 amino acid deletion using I-TASSER and Robetta software.

4.2.5 Validation of ANP32 Knockout Cell Lines

With the genotype of our gene-edited cell lines indicating disruptions to their ORF would result in a premature stop codon being introduced, we set out to assess whether this aberration led to definitive biological consequences through a loss of mRNA expression and protein translation.

Gene Expression of ANP32A and ANP32B Knockout Cell Lines

The disruption of the ORF leads to a transcript that will not be recognised as a host mRNA sequence. As a novel transcript that does not have protein coding capacity, it may be degraded through a process known as nonsense mediated decay (NMD) (Schweingruber et al., 2013). Using the primers established previously for RT-PCR of ANP32A and ANP32B, we performed qPCR on total RNA extracted from our selected loss of function cell lines. The primers (silver) were designed for this experiment to cover a region after the gRNA (green) target locus and to be exon spanning to reduce the potential to detect contamination from genomic DNA (Figure 4-16A).

YWHAZ was used as the house keeping gene for normalisation of the expression of our genes of interest in all NPTr cell lines. Gene expression is given as the relative abundance of mRNA compared to YWAHZ, as opposed being measured as the absolute abundance of transcripts present. For ANP32A, there was a large reduction in mRNA detected in the AKO and DKO cell lines relative to the YWHAZ mRNA detected (Figure 4-16B). In the AKO cell line, the abundance of ANP32A transcripts was reduced, whilst expression of ANP32B mRNA remained static relative to the abundance of YWHAZ (Figure 4-16C). In the BKO and DKO cell lines, there were drastic reductions in the presence of ANP32B mRNA, whilst gene expression of ANP32A appeared unaffected. These changes in the relative abundance of the CRISPR-edited genes relative to a housekeeping gene demonstrated that levels of mRNA for ANP32A and ANP32B are reduced when the open reading frame is disrupted.

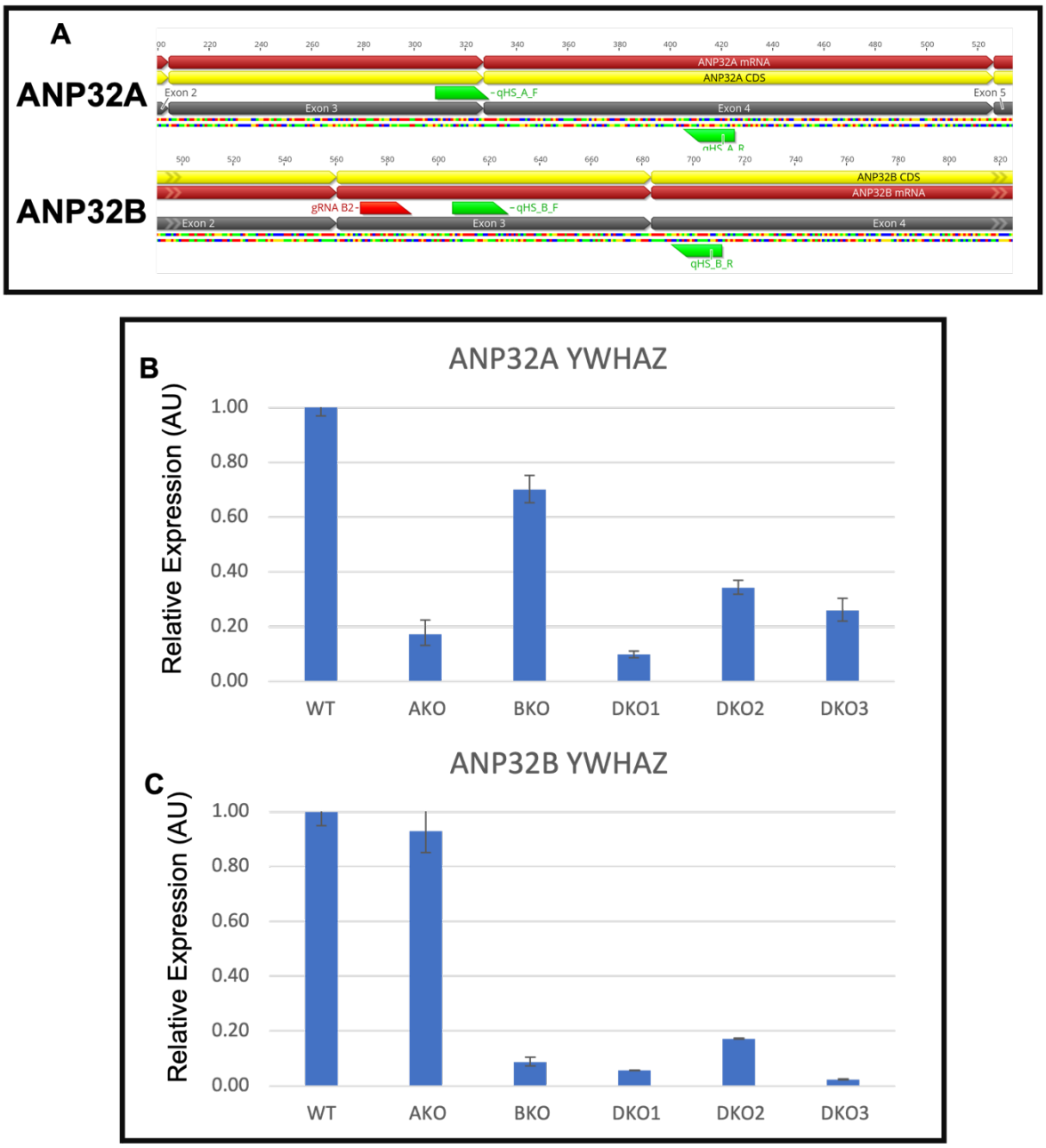


Figure 4-16: To detect if disruption of the ANP32A and ANP32B coding sequence affected gene expression qPCR was performed. Gene expression of ANP32A and ANP32B were measured relative to the expression of the housekeeping gene YWHAZ. A) Schematic representations of primers (green) in the target genes. B and C) Relative expression of ANP32A and ANP32B compared to the housekeeping gene YWHAZ.

Gene expression does not necessarily equate to protein presence, and as Zhang *et al*., 2019 observed, the effect of ANP32 proteins on polymerase activity is dose dependent and low quantities can still improve FluPol efficiency (Zhang et al., 2019), meaning a total loss of detectable protein is likely to be necessary. To confirm that ANP32A and ANP32B transcripts are translated from mRNA into proteins, and in the edited cells the presence of protein is ablated, antibodies with predicted immunogenic reactivity to synthetic human peptides situated near the C-terminal were selected for testing against the swine proteins. For ANP32A, the antigenic site in pigs has 98% sequence homology with the synthetic human epitope the antibody was raised against (Abcam correspondence), providing confidence that an antibody raised against the human peptide was likely to work.

Translation of ANP32A at the predicted size of ~29 kDa was confirmed by Western blot of Human Embryonic Kidney cell lysate, as a positive control for the antibody, homogenised pig spleen, homogenised pig tracheal tissue and NPTr lysate (Figure 4-17A). In all samples Western blotted the control protein beta-actin was detected. Observation of ANP32A from the tracheal lysate required longer exposure as the samples were loaded at ½ the concentration of other samples due to the cartilaginous nature of trachea making protein extraction from the tissue more problematic. There was no detection of ANP32A protein in the ANP32A knockout cell line or the double knockout cell lines. ANP32A was translated in the ANP32B knockout cells. The presence of ANP32A in only the BKO cell lines affirmed that the disruption to the ORF and introduction of a premature stop codon of ANP32A exon 2 was preventing translation of canonical swine ANP32A transcripts that are associated with IAV to a level detectable by Western blotting.

Translation of ANP32B in NPTr

To assess translation of ANP32B, Western blots were performed. HEK cells were used as the positive control as the antibody was raised against a human ANP32B peptide sequence. The swine ANP32B region was 100% homologous to the human peptide that the antibody was raised against. The beta-actin loading controls were present in all samples Western blotted. For ANP32B, the protein was detectable in HEK, NPTr and AKO (Figure 4-17B). In the BKO, DKO1 and DKO2 NPTr's there was no observable detection of ANP32B protein. For the DKO3 cell line, there was no clear band, but due to exposure issues on the blot this was not entirely conclusive. These Western blots suggest that ANP32B is translated in the ANP32A knockout cell lines to a similar level as observed in the NPTr control, whilst in the BKO and DKO cell lines translation was no longer detectable.

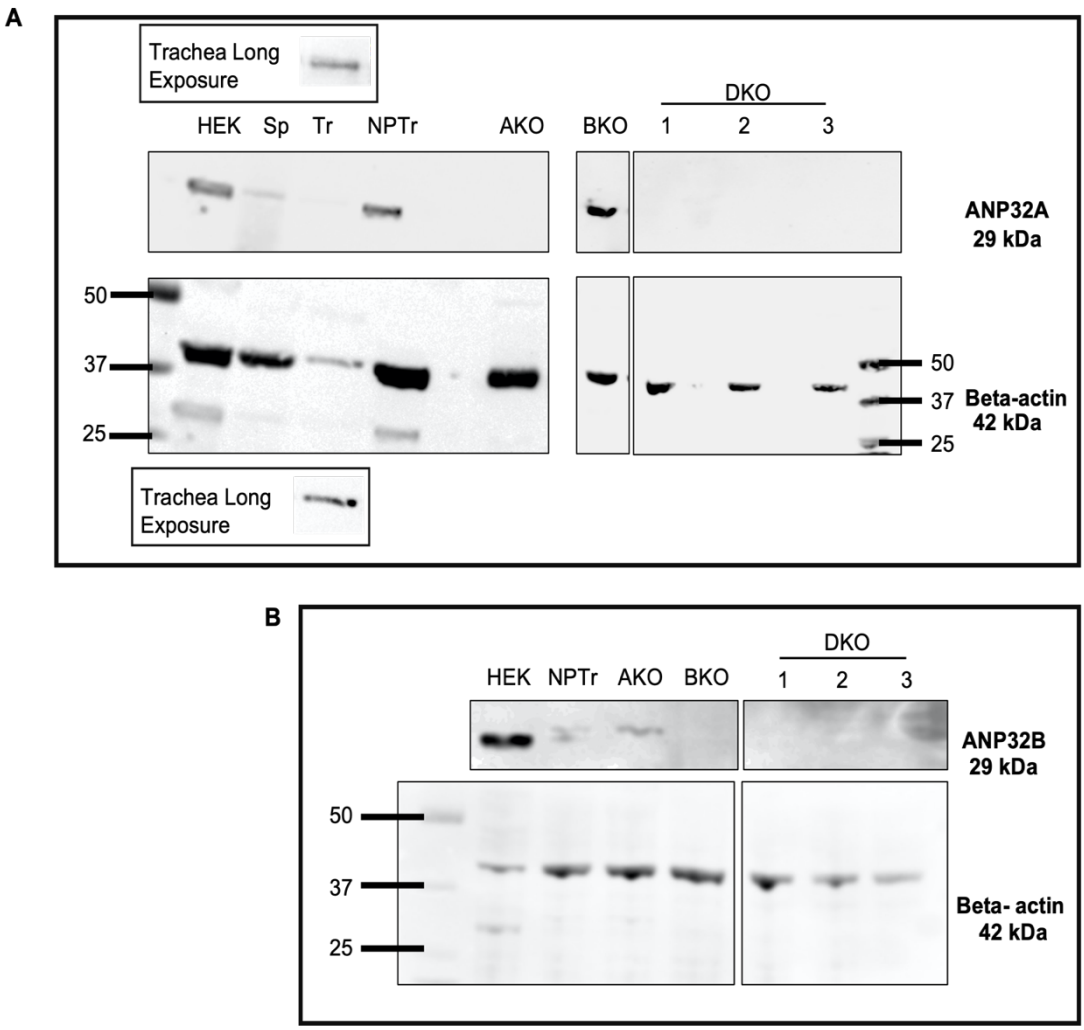


Figure 4-17: Protein translation as detected by Western blot for ANP32A and ANP32B in NPTr WT, CRISPR-edited NPTr's and control cell lines or tissues. For all samples, ANP32A and ANP32B were expected to be 29 kDa in size and Beta-actin was expected to be around 42 kDa. A) ANP32A (ab189110) and Beta-actin proteins detected in Human Embryonic Kidney (HEK) cells, spleen (sp), trachea (Tr) and NPTr. ANP32A was detected in the HEK, spleen, trachea, and WT NPTr samples but not in the samples where ANP32A had a disrupted open reading frame from CRISPR-editing. The presence of ANP32A could only be detected in the trachea sample with a longer exposure time. All samples had an observable band for Beta-actin. B) ANP32B (ab4224) and Beta-actin proteins detected in HEK and NPTr cell lines. In the HEK, NPTr and AKO samples there was a visible band for ANP32B at the expected size range. For the BKO and DKO samples there was no band for ANP32B observable. All samples had an observable band for Beta-actin.

To assess whether functional deletion of ANP32A, ANP32B or both genes concurrently affected cell proliferation, a CCK-8 assay was performed. This is an assay for determining cell proliferation through activity of the electron transport chain. It was performed to check that cellular metabolism was not significantly affected by the loss of the ANP32 family proteins. The DKO cell measured here is DKO1, which has frameshift mutations in all alleles of ANP32A and ANP32B and the lowest abundance of mRNAs compared to the YWHAZ control.

Proliferation was measured at 4 timepoints spanning 48 hours. Across the timepoints, there was no differences observed for any of the ANP32 disrupted cell lines compared to the control (Figure 4-18) (n=3, one way ANOVA and post-hoc Dunnett's test, (p=0.05)). These results show that the loss of ANP32A or ANP32B individually or concomitantly had no observable effect on cellular proliferation and meant that any effects in the viral assays were not the result of a drastically modified cellular metabolism.

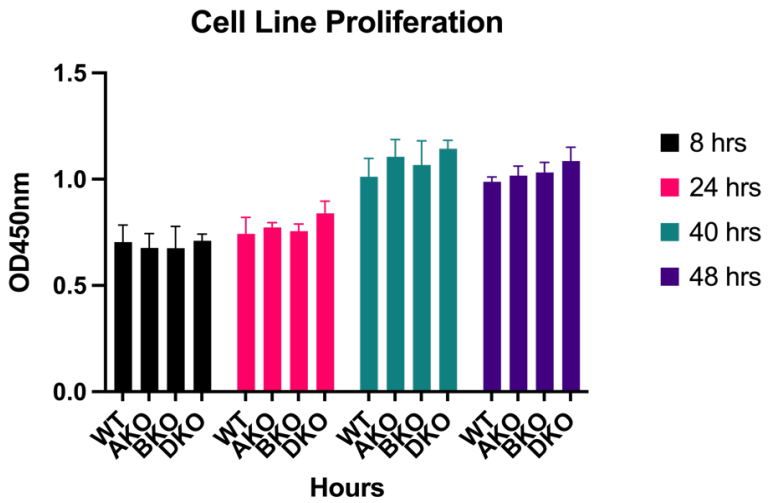


Figure 4-18: Determining the proliferation of WT NPTr and CRISPR-edited NPTr cell lines that lack ANP32A (AKO), ANP32B (BKO) and both ANP32A and ANP32B (DKO) by CCK-8 assay. OD_{450} measurements were taken from replicates (n = 3) at each timepoint. No statistical significance was observed in proliferative capacity of each cell line at each of the time points (two-way ANOVA and post-hoc Tukey's test (p > 0.05)).

IAV infection of NPTr

It had previously been demonstrated that NPTr is a viable cell line for infection with a range of human, swine and avian origin viruses (Ferrari et al., 2003; Moncorge et al., 2013; Zhang et al., 2017). Of the viruses we were specifically using, only PR8/H1N1 had been demonstrated to infect NPTr. To confirm that the swine origin H3N2 (Sw87/H3N2), UDL/H9N2 (5:3 PR8 reassortant) and H5N1 (5:3 PR8 reassortant) would successfully infect and replicate in NPTr we performed a single cycle infection at an MOI of 1 (Figure 4-19). All the virus strains successfully entered and replicated in NPTr and could therefore be used in future experiments investigating whether the loss of ANP32A and/or ANP32B affects viral propagation.

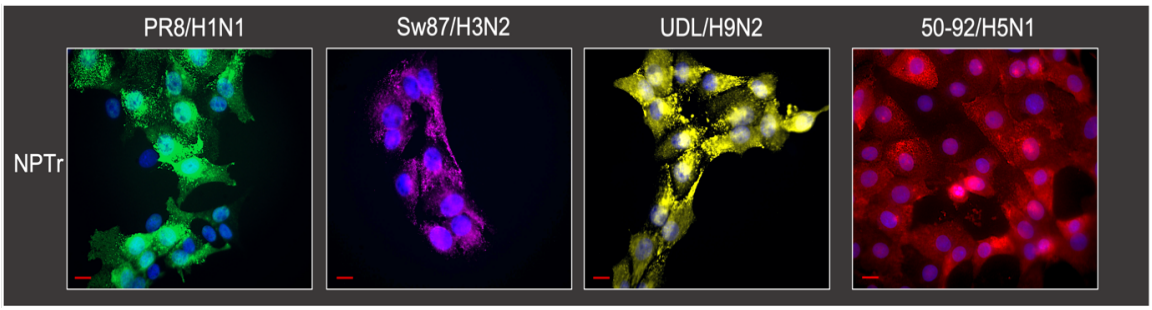


Figure 4-19: Confirming NPTr as a viable model for IAV infection assays from multiple species. Immunofluorescence of IAV infections in NPTr with IAV of human, swine and avian-origin by staining for IAV Nucleoprotein (NP) (ab20343). Cells were counter-stained with DAPI (blue). Scale bars are 20 µM.

4.3 Discussion

Following the discovery and refinement of CRISPR/Cas genome editors, the creation of knockout cell lines has become a fundamental experimental approach for molecular biological investigations. Knockout cell lines are valuable tools for studying the function of gene products. Disruption of a gene provides an understanding of the role a protein has in cellular function by seeing what changes occur when that protein is not present. However, the intended use of the cell lines we have created was not to determine how the protein affected cellular function, but to gain insight into how swine cells respond to IAV infection without the presence of ANP32 family proteins, and in turn, how replication of IAV originating from different species are affected by cells lacking these proteins. For this to be possible we first needed to create and validate our ANP32A and ANP32B knockout cell lines.

4.3.1 Identifying gene-editing targets

Genome-wide screens, whether through gene-knockouts, knockdown of gene expression, gene activation, protein interactomes or genetic variant association studies, have provided powerful tools for scrutinising molecular interactions between IAV and its hosts (Schaack and Mehle, 2020). There have been many host proteins that have been identified to be cofactors or inhibitory factors for IAV replication, such as cellular serine/threonine protein phosphatase 6 (*PP6*) (York et al., 2014), importin- α family proteins (Gabriel et al., 2011), SA synthesis protein *CMAS* (Zhao et al., 2021), SA transporter *SLC35A1* (Han et al., 2018), and *CMTR1* for viral cap-snatching from host mRNAs (B. Li et al., 2020). The approach of scanning the entire genome is powerful, but modest overlap between each screen indicates that the data must be carefully interpreted (Capitanio and Wozniak, 2012; Tripathi et al., 2015).

The use of GeCKO screens to identify human IAV host factors has been a powerful tool for identifying proviral factors and improving our understanding of the molecular progression during IAV infection. The application of chicken and swine GeCKO screens are in the pipeline at the Roslin Institute. The results will be informative as an experimental model for comparative biology research

to human IAV infection (Rajao and Vincent, 2015), provide insight into pigs' role as a mixing vessel and contribute to knowledge of understanding molecular pathogenesis of IAV infection specifically in pigs and chickens.

With consideration of our target genes from the ANP32 family in mind, their identification in only a single genome wide screen (Watanabe et al., 2014) demonstrates the influence that the genomic context of loss of function mutations can have on the results. Genomes have evolved through duplication events (Crow and Wagner, 2006), and the divergence of genes following duplication has resulted in families of proteins with high homology but distinct roles. Because genes often come in families, it is possible that proteins that have a functionally redundant role as IAV host factors will have been missed disproportionately when systematic disruption of single genes is the approach taken.

The discovery of the association of ANP32 proteins with IAV highlights the importance of performing research in zoonotic reservoirs (Long et al., 2016). The divergence of genes occurs at different rates between species due to varied selection pressures and evolutionary drift, and therefore the relationship between IAV and proteins will not be consistent across all hosts. The transposition of avian IAV knowledge to human and subsequently porcine IAV epidemiology was a significant step in developing the digenic target approach we have taken in this project.

A further issue in determining host factors across these screens that support viral replication is the esoteric nature of genetic nomenclature. With consistent titling of genes, it is plausible that overlap between screens could be higher. By example, a gene that is a host factor affected by variable name assignment is the nuclear export factor *CRM1*/*XPO1* (Chutiwitoonchai et al., 2017; Karlas et al., 2010). Because ANP32A and ANP32B are referred to with multiple different names, thorough literature research is more difficult. However, this discordance in naming in ANP32 proteins appears to be in decline with their increasing IAV-associated publication. Better nomenclature standardisation for all genes would mean that putative gene-editing targets could be better compared between organisms and prioritised.

4.3.2 The Selection of NPTr's as a Model System

The remarkable zoonotic dissemination of IAVs belies the fact that transmission between species requires significant barriers of infection to be overcome (Long et al., 2019b). Physiological context, such as pH and temperature at the site of infection in the host, and the biological environment, such as nucleic acids and proteins present, means that IAV must acquire specific adaptive features following the initial zoonotic transmission to successfully complete the viral replication cycle (Mair et al., 2014). The unique biological properties and signatures of immortalised cell lines are associated with their temporospatial origin. Because of IAVs specificity and differences between host environments and IAV strains, it is important to utilise the most relevant available *in vitro* model. Ideally, the *in vitro* model will be from cells that were originally isolated from tissue that is the site of viral infection and has maintained a similar gene expression profile and cellular metabolism.

The cell line we used, NPTr, was established by spontaneous transformation and validated as an IAV infection model in the original publication (Ferrari et al., 2003). This cell model was selected as of immortalised swine cell lines it most closely represents the native IAV infection site in swine. The use of Porcine Kidney-15s (PK-15s) in other research papers that are applying gene-editing for ANP32 and IAV investigations means that the results presented with NPTr should provide an improved point of reference for potential downstream field applications in animals (Zhang et al., 2020, 2019).

As an alternative to immortalised cells, primary cells isolated directly from pig respiratory tracts could be used for IAV infection studies (Sreenivasan et al., 2019). Primary cell cultures will more closely represent the tissue of origin as they will not have severe chromosomal aberrations and will maintain their biological identity. However, for repeated experiments their high heterogeneity and varied cellular metabolism introduces variation that is difficult to experimentally control for. This could be a large confounding factor when assessing viral infections if one host has alternative factors contributing to underlying genetic resistance. The genetic consistency of an *in vitro* system was

a key determinant in our decision in using immortal cell cultures for repeatability. Despite the fact that swine primary epithelial cells have been cultured and shown to be susceptible to IAV infection, clonal isolation of swine lung tissue by FACS has not previously been described and the ability to grow a cell population that had a homogenous gene-edited genotype would have presented a significant hurdle (Sreenivasan et al., 2019; Steimer et al., 2006).

4.3.3 Guide RNA Design

In designing gRNAs, only exons annotated in all isoforms in Ensembl for swine and human ANP32A and ANP32B genes were considered to increase the likelihood that any nonannotated porcine isoforms that are transcribed would be identified through conservation with the better annotated human genome. They were also designed to target exons without alternative start codons present after the gRNA target locus to reduce the possibility that a smaller transcribed protein would remain in-frame to maximise the likelihood that all translated proteins would be disrupted (Wang et al., 2014).

The technique that is used to deliver the Cas9 nuclease and the gRNA with *in vitro* models affects the outcome variably by cell type and gene-editing intention (Glass et al., 2018). In this project we selected to use a dsDNA based plasmid vector, as was outlined in an original methodology paper, except with modifications to the Cas9 amino acid sequence for reduced off-target activity (Kleinstiver et al., 2016; Ran et al., 2013b). The benefits of using a plasmid based delivery approach lies in its customisable design, co-expression of eGFP as a selection marker, the relatively low-cost of acquisition of the required reagents and the stability of expression. The stability of plasmid DNA is beneficial in cell culture models, but creates an issue when applied to animal models due to the potential for integration of the dsDNA into the host genome (Norris et al., 2020; Owen et al., 2021) and the more persistent expression and associated off-target activity due to the stability of dsDNA (Sung et al., 2014).

An alternative dsDNA strategy for CRISPR/Cas9 expression is from lentiviral transduction of an sgRNA expression cassette into cells stably expressing Cas9 (Doench et al., 2016). This has been successfully performed

with NPTr (Zhao et al., 2021). Although lentiviral transduction can be a more efficient method than plasmid transfection for creating knockout cell lines, the use of dsDNA constructs that randomly integrate into the host genome means that the methodology is less translatable as a method applicable to animal models.

To avoid the step of introducing nucleic acid which needs to be nuclear imported for transcription that is part of dsDNA based methods, Cas9 mRNA and pre-transcribed sgRNAs could have been used. sgRNAs can either be generated by *in vitro* transcription (IVT) or synthesised by a commercial supplier. The use of IVT transcription still requires plasmid cloning and purification steps, as well as IVT expression and purification of the expressed RNA. On top of this, the fact that Cas9 mRNA does not contain a selectable marker such as eGFP means that specific isolation of cells that have CRISPR reagents delivered is less exact than with a co-expressed plasmid marker. More recently, the use of CRISPR as precomplexed RNP has become increasingly popular. This can abrogate the need for any synthesis steps if commercial products are used. Direct delivery of precomplexed RNPs allows immediate nuclear localisation and CRISPR activity and reduces cell toxicity as no foreign mRNA or dsDNA is introduced (Givens et al., 2018), although introducing a 160 kDa protein requires alternative transfection optimisation and may not be suitable in all cell types. There are now commercially available eGFP-tagged Cas9 proteins that support observation of transfection and sorting of positively transfected Cas9 RNP cells, a system that was briefly tested and shown to work in a pilot experiment during this project (data not shown). The use of either mRNA and RNP CRISPR delivery systems are particularly relevant for dosage control and would provide useful insights towards application of this project for potential translation into an animal model.

Other alternatives for guide RNA design could have been formulated through changing of the Cas9 protein or the sgRNA distributions. One such alternative Cas9 is a Cas9 nickase, a modified Cas9 that only cuts a single DNA strand (Ran et al., 2013b, 2013a). With two gRNAs targeting either side of the loci for editing, Cas9 nickases require dual activity in *trans* and therefore the off-

target effects are reduced because if a single nickase interacts with an off-target region, by itself it has low potential for stimulating NHEJ repair. The use of multiple gRNAs with original Cas9 to remove an entire exon as a means of disrupting the coding frame could have been performed and would have allowed for rapid screening by PCR for shortened fragments. Multiple guides targeting within the same exon could also have increased the likelihood of indels being introduced, however creating multiple DSBs in proximity means that genomic architecture could be remodelled in the target region, a factor that could have unintended consequences (Canver et al., 2014). The plasmid-based system was effective for our desired outcomes, however if the project was begun again, an approach that would allow translation into an animal model with less contention than a dsDNA system would have been prioritised.

4.3.4 Determination of CRISPR Editing

When designing gRNAs, it should be acknowledged that the predicted cutting efficiency is likely to vary between different cell contexts. Chromatin packaging and DNA-bound proteins affect accessibility of Cas9 to the target locus and so targeting of DNA does not remain static in varying biological scenarios (Haeussler et al., 2016). The use of the T7 mismatch detection digest as a preliminary detection method is sufficient for preliminary assessment of CRISPR activity at the target site in cell culture, however with the ease of Sanger sequencing and the improved data received that allows discerning of the indels introduced, a Sanger sequencing-based approach should have been implemented immediately in. Beyond Sanger sequencing, screening for CRISPR gRNA efficiency can be done with Next-Generation Sequencing platforms to provide greater detail about the range of indels introduced. However, the time and cost-effective nature of the T7 assay meant that despite its inaccuracies (Sentmanat et al., 2018), it was a method which fit the purpose at this preliminary stage of gathering data.

In the original paper that established NPTr as an immortalised cell line (Ferrari et al., 2003), karyotyping of the cells observed the chromosome number to range from 45 to 65, with a mode of 61. Under normal ploidy, porcine cells

carry 38 chromosomes. This suggests polyploidy of most chromosomes. However, there is no indication as to the size of the chromosomes present within the original paper, and as we did not ourselves assess the ploidy of chromosome 1, where both ANP32A and ANP32B are present, we cannot ascertain from these data whether ANP32A or ANP32B are more than diploid. Karyotyping is an imperfect method of determining the number of specific loci in immortalised cells due to structural rearrangements of chromosomes and genomic alterations that make visual interpretation difficult. New methods that have been developed such as digital droplet PCR, which can determine the copy number of genetic loci with a high accuracy could have been used to resolve the issue of ploidy with accuracy.

To determine the specific nucleotide changes introduced at the target locus DNA sequencing is required (Sentmanat et al., 2018). A T7 assay would miss edits in a clonal population that has symmetrically edited homozygous alleles. There are several tools available that can be used to quantitatively assess the distribution of edited alleles. We have used ICE and Geneious' *Analyze CRISPR Edits* tool. For cross-checking the alleles to confirm that the output from ICE for each clonal cell population was correct we analysed the Sanger sequences using Tracking of Insertions and Deletions (TIDE)(Brinkman et al., 2014; Sentmanat et al., 2018). Both ICE and TIDE can only determine the amount but not the identity of the inserted or deleted bp, and they are restricted in size detection to ±50 bp for TIDE and -30/+14 bp for ICE (Bloh et al., 2021). These tools can also be used to infer gene copy number, albeit in a less reliable manner than ddPCR. For some clonal populations there were more than two alleles present in the ICE/TIDE analysis. This could be a result of two cells being seeded in a well instead of a single cell being isolated, a miscalling by the TIDE or ICE algorithms due to the presence of large aberrations in the edited DNA sequence at the target site (Sentmanat et al., 2018), from inherent base calling noise associated with Sanger sequencing or from ploidy related variation in NPTr.

The approach taken to edit to target ANP32 genes in previous human and swine studies was through sequential targeting (Staller et al., 2019; Zhang

et al., 2019). Targeting of ANP32A and ANP32B concurrently was a novel approach that meant only a single clonal isolation was required. This was intended to reduce the selection pressure towards cells that are particularly viable for culturing after being under mechanical stress and pressure from FACS. Because ANP32A and ANP32B are ~74 kb apart on chromosome 1 in the *Sscrofa11.1* annotation, it is possible that introducing dual DSBs could have stimulated an inversion or large chromosomal rearrangements. Because the PCRs used a primer that was externally flanking the gRNA target sites, the PCR for Sanger sequencing would only be able to amplify the entire 74 kb DNA region between ANP32A and ANP32B. Unless an inversion occurred which included the primer upstream from the DSB no PCR products would have been observed.

Of all the cell lines isolated for downstream analysis, only DKO3 has a symmetrical genotype for ANP32B. All alleles show a disrupted ORF, and this asymmetry between the genotypes has low potential to affect protein expression. It is possible that a genetic element affecting chromatin modelling, or an unannotated splice site remains viable in some genotypes only, however if this was the scenario it should be identified through the downstream analyses for mRNA or peptide expression. For future work that would translate into an animal model, the ideal scenario is to have two identical alleles to reduce confounding impacts of having two novel alleles present.

The DKO3 cell line was specifically selected for further investigation because of the in-frame deletion of 12 bp on one allele of ANP32A. The premise for this was that the protein could still be translated, but it would be lacking four amino acids (residues 25-28) that have been identified through cryo-electron microscopy to interact with PB2 of the FluPol complex (Figure 4-20) (Carrique et al., 2020). It may be possible that the remaining amino acids of ANP32A could remain interacting with FluPol, but the modified molecular interactions could affect the conformation of the entire complex that results in alterations to the efficiency of the IAV genome replication.

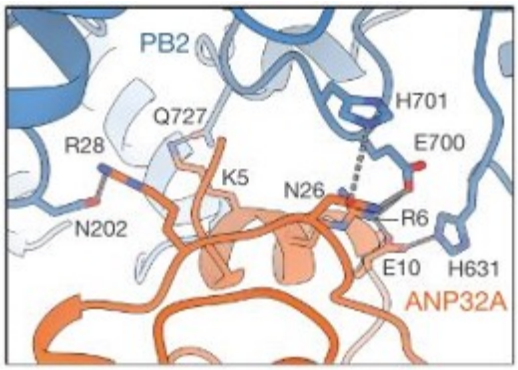


Figure 4-20: Schematic of a region where human ANP32A and PB2 interact directly. Determined through cryoelectron microscopy. Image adapted from Carrique *et al.*, 2020 with permission from Nature.

4.3.5 Staining of ANP32A and ANP32B in NPTr and Relevant Swine Tissue

It has previously been established that human, chicken, swine and murine ANP32A and ANP32B have a nuclear localisation in cell culture (Baker et al., 2018; Domingues and Hale, 2017; Long et al., 2019a; Staller et al., 2021). We have contributed to ANP32 localisation knowledge with the observation of their nuclear localisation in NPTr (Appendix 2) as well as in piglet trachea. This biological recapitulation of protein localisation in the porcine model is important in validating the use of our *in vitro* model for this research.

The localisation of ANP32 to nuclei was not restrictive. In actively dividing NPTr, as determined by a condensed chromatin state, ANP32A or ANP32B were only detectable diffuse in the cytoplasm. Both ANP32A and ANP32B are HuR ligands and are critical in the process of nucleocytoplasmic shuttling of Adenosine-Rich-Element mRNAs (Brennan et al., 2000; Fries et al., 2007). This HuR shuttling is a CRM1 related transport across the nucleus, the same nuclear transport protein that has been identified in many genome wide screens as IAV host co-factor related to its role in exporting vRNPs (Chutiwitoonchai et al., 2017; Watanabe et al., 2001). Although speculative, it is conceivable therefore that ANP32 proteins have a role in the nucleocytoplasmic shuttling of vRNPs due to their known interactions with FluPol and HuR as well

as in increasing the efficiency of FluPol activity. Because of the better-established role that ANP32 proteins have in affecting IAV polymerase activity, this was not an avenue investigated during this project.

4.3.6 Confirming the Biochemical Consequences of Gene Disruption

To determine whether disrupting the ORF of ANP32A and ANP32B affected had molecular consequences we assayed each step along the central dogma of biology for function. Genotyping the DNA, quantitatively measuring RNA, and antibody probing for translation of proteins. For the measurement of mRNA, qPCR measured against the relative expression of a house keeping gene. Because relative expression is normalised against a constitutively expressed gene to obtain a fold-change in expression, variation in RNA extraction protocols affects comparison between samples less.

The reduction in mRNA in the knockout cell lines may be because of NMD, where transcripts not recognised by host RNA processing machinery are degraded. NMD is a eukaryotic cellular surveillance mechanism for RNA quality control and for regulation of gene expression (Fatscher et al., 2015; Nickless et al., 2017). RNA transcripts that contain premature stop codons can be recognised and degraded to prevent aberrant proteins being produced that will affect cellular metabolism and physiology. The relative abundance of ANP32A and ANP32B mRNA transcripts in our knockout cell lines were appropriately reduced. For ANP32B disruption, the detection of ANP32B transcripts at such a low level could be transcripts that are partially degraded, or noise generated by use of random primers in cDNA generation or qPCR. For ANP32A mRNA measurement, the reduction in ANP32A transcripts in the AKO and DKO cell lines is indicative of a premature stop codon being detected by RNA processing machinery despite their relative expression still being at around 30% of YWHAZ compared to the WT control. The reduction of the ANP32A transcript observed in the BKO cell line was an unexpected result, but downstream analysis of translation by Western blot was performed to determine that ANP32A was translated despite the reduction in mRNA observed.

Because NMD is a translation independent process, the presence of mRNA transcripts for the disrupted genotypes does not indicate that translation of the wildtype mRNA is occurring. The cDNA primers were designed to bind post-gRNA target site, and therefore either contain the premature stop codon, as with ANP32B alleles, or be after the premature stop codon as with ANP32A. An alternative design considered was to use primers homologous to the target site that would have reduced homologous base pairing if indels were present. This was not pursued as the results would be affected by edited alleles having less homology than wild type alleles, and therefore genotype changes and not just alterations in mRNA transcripts would have been detected. The design of primers also considered the annotated transcripts for human ANP32A and ANP32B as available from Ensembl due to the more modest accuracy in the annotation of the swine genome (*Sscrofa11.1*). It was noted when assessing the Ensembl annotations that ANP32B in *Sscrofa11.1* is mistitled as N-acetylneuraminate synthase (NANS), an indication of the comparatively poor attention the porcine genome is exposed to.

Changes in gene expression do not always confer into changes in protein translation (Koussounadis et al., 2015; Moritz et al., 2019). The industry standard for confirmation of effective gene knockout is through Western blotting. The antibodies were raised against human peptides for both ab189110 and ab4224 for ANP32A and ANP32B, respectively. The human peptide used to raise antibodies to ANP32A had 95% identity to the porcine sequence, whilst the ANP32B antibody had 100% homology between humans and pigs. The antibody for ANP32B had previously been shown to work successfully against swine spleen lysate, however under the experimental conditions tested in our lab we were not able to get it working as effectively, despite optimisation trials. Together, the results of the gene expression and protein translation experiments gave us confidence that interruption of the ORF in ANP32A and ANP32B had biological consequences.

4.3.7 CCK-8 Assay

Removal of proteins that play critical roles and have multifaceted functions can affect cellular metabolism. Through the CCK-8 assay we aimed to gain insight into the growth and molecular metabolism of our cell lines that were lacking ANP32 genes. The results of the CCK-8 assay shows the level of proliferation in the WT and CRISPR-edited NPTr's remains consistent. The reduction in CCK-8 activity measured after 48 hours compared to after 40 hours could be due to the culture becoming confluent and the cells reducing in proliferative activity. This was not observed in the original NPTr paper, an effect which could be due to the different number of cells seeded relative to the flask size (Ferrari et al., 2003). Our CCK-8 assay was taken across three separately seeded samples and compared against WT proliferation in relative terms. It could have been performed to gain more insightful data by seeding a known number of cells to create an absolute cell number for each OD450nm reading. An alternative for providing insight into changes in cell activity would have been through transcriptomics, however the time and cost of transcriptome analysis was prohibitive to it being undertaken in this project, but it would be a critical step before translation into a swine animal model.

4.3.8 Conclusions

The data presented here has validated a model for investigating the role of ANP32A and ANP32B during IAV infection in swine. We have confirmed that the model system recapitulates the localisation of proteins observed in the native site of swIAV infection, that our genes of interest have been functionally ablated, and that the loss of these proteins has not significantly affected cellular proliferation. The progression of this work leaves these cells as a resource for future investigations into ANP32 proteins in swine, not only for IAV infections but potentially for other viral processes or for investigating their role in cellular function. The subsequent work in this project is interested in exploring the relationship between ANP32A and ANP32B with IAV during infection in swine.

5 Investigating the Role of ANP32A and ANP32B in IAV Infection in Swine

5.1 Introduction

The potential for gene-editing to be a successful antiviral strategy in domesticated pigs has already been demonstrated in a research environment (Burkard et al., 2017; Whitworth et al., 2019). These examples have bestowed viral resistance to the host through the removal of the host extracellular receptor that instigates viral internalisation. Cell entry is a key gate-keeping step for viral propagation. As obligate intracellular parasites, if a virus cannot enter a cell, there is no opportunity for it's replication. The SA moiety that is the extracellular receptor for IAV is a transmembrane glycoprotein. Removal of functional proteins involved in SA synthesis and transfer have been shown *in vitro* to affect IAV propagation (Zhao et al., 2021), however SA moieties on glycoproteins have an integral role in embryonic development, immune function and cellular reprogramming (Li and Ding, 2019). Because of their important role in maintaining physiological equilibrium, gene-editing of SA-related gene pathways does not represent a viable, or ethical, target for conferring IAV resistance in a mammalian or avian host.

Aside from proteins involved in SA synthesis or transfer, *TMPRSS2* is a gene that emerged as a putative target. A publication describes the creation of an *in vivo* swine model (Whitworth et al., 2017), but follow-up articles with IAV infection data are conspicuously absent. Beyond *TMPRSS2*, there is limited data on gene-editing for IAV resistance in swine. Given the impact of IAV in pigs and their role as a 'mixing vessel', investigating novel gene-editing targets is of benefit to the Influenza research community, the swine industry and all species that could be infected by zoonotic IAV transmission, including humans. Following on from ANP32 proteins being successfully demonstrated *in vitro* to be effective targets for IAV resistance in chickens and humans (Long et al., 2019a; Staller et al., 2019), the data presented in this chapter will allow us to further understand how the ANP32 family of proteins affect FluPol specifically in

swine and provide further information on whether they could be effective gene-editing targets for IAV resistance.

5.1.1 Host Specificity for FluPol

There are multiple factors that influence whether a virus can invade a cell and be successful in commandeering it as a factory for its replication. For IAV, environmental factors such as temperature and pH must fall within a certain range (Poulson et al., 2016), and the biological context must provide factors that can support its replication cycle as well as not create an environment that is hostile to the virus or the activity of its proteins or nucleic acids. Between the evasion of host restriction factors, the exploitation of host proteins to perform critical replicative actions and the biological background that allows catalysis of the necessary molecular reactions, there are significant barriers to infection for a zoonotic transmission of IAV to occur.

When an IAV originating from a particular species is capable of replicating in different host systems beyond its originally infected species, differences in pathogenicity and propagation are often observed. The variable cleavage capacity of HA was the first factor that was discovered as a determinant of the species that IAV can infect (Klenk et al., 1982, 1975). Expanding on host specificity, through analysing the capacity of two distinct avian FluPol's to form plaques on cells of chicken and mammalian-origins, it was identified that PB2 is a determinant in affecting the ability of IAVs to differentially infect particular species (Almond, 1977). The fact that many avian-origin FluPol's do not function efficiently in a mammalian context is now well documented. From data in mammalian *in vitro* systems, PB2, PB1, PA and NP were initially believed to be the minimal requirements for transcription and replication of the viral genome (Huang et al., 1990). It was later discovered that in fact there are host cell factors that the vRNP recruits to support efficient IAV polymerase activity (Sugiyama et al., 2015). Given that the IAV replication cycle occurs within a host cell, it is unsurprising that interactions of the vRNP proteins with other proteins present in the host environment affect its function. Because IAV has the capability of cross-species transmission, some mutations in the

polymerase proteins are related to its adaptation in a new host. Some of these mutations in FluPol proteins that are associated with host adaptation are discussed below.

A proteomic analysis that uncovered host proteins interacting with each of the FluPol subunits identified more than 300 human proteins that bound to PA alone (Bradel-Tretheway et al., 2011). The substitution of an avian for human-origin PA in the human FluPol complex can overcome the restriction of some avian-origin FluPol's in mammalian cells (Mehle et al., 2012), suggesting that alteration of the PA peptide sequence affects IAV suitability to specific host environments. Several residues in pH1N1 PA have been shown to be involved in host adaptation, including T85I, G186S and L336M (Bussey et al., 2011). In serial passaging of IAV in a murine model, these PA mutations were found to have a considerable effect on the level of polymerase activity, yet only minimal effects regarding viral pathogenicity were observed (Bussey et al., 2011). Thus, pathogenicity and host adaptation are not strictly entangled, as an increase in polymerase activity does not necessarily alter pathogenicity. PA N321K was identified during the third wave of the 2009 pandemic to have arisen in a selective sweep in a variant originating in the UK, and substitution of this residue results in improved polymerase activity in human cells (Elderfield et al., 2014). Further research has shown that PA N321K enhances IAV polymerase activity to a level 3.5 times greater in human cells than in swine, providing molecular context for why this substitution was under selective pressure after swine to human transmission (Peacock et al., 2020b).

The human IAV pandemics of 1918, 1957 and 1968 were caused by viruses harbouring PB1 gene segments from different origins (Kawaoka et al., 1989). Across all of these viruses, the avian signature asparagine (D) was substituted for a serine (S) at position 375 (D375S) (Naffakh et al., 2008; Taubenberger et al., 2005). As with many of the other adaptive mutations cited here, a serine at PB1 375 is not ubiquitous amongst human adapted IAVs, suggesting other mutations, and possibly a constellation of, are able to compensate for D375S to create a biochemically similar change that supports adaptation to mammals. Aside from residue 375, there is a relative paucity of

PB1 adaptive mutations that have been identified. As the estimated nucleotide substitution rate of PB1 is lower compared to PB2, PA and NP (Chen and Holmes, 2006), there may be evolutionary constraints that restrict the amount PB1 can diverge whilst retaining its multiple functions.

In contrast to PB1, the role that PB2 plays in species adaptation is well documented (Shirleen Soh et al., 2019). In 1993 it was established that a single amino acid change in PB2, at site 627, affected the phenotypic restriction of avian-origin viruses in mammalian cells (Subbarao et al., 1993). Amino acid substitutions of E627K from avian-origin IAVs have been observed in a single passage in porcine cell culture, and the significant improvement in replicative capacity suggests an important role in swine host adaptation (Mänz et al., 2012). For mammalian adaptation, conversion from the negatively charged, acidic, glutamic acid (E) residue, to a positively charged, basic amino acid, lysine (K) is a major determinant (E627K). For avian-origin IAVs, alteration of the glutamic acid 627 residue to the mammalian adapted lysine restores viral polymerase activity and infectious virion propagation in human cells (Long et al., 2019a, 2016).

Identified by crystal structure analysis, the 627-containing domain of PB2 (residues 535–684) is within an α-helix encircled by a loop, which is located next to a highly basic groove. Position 627 is located within the loop structure (Kuzuhara et al., 2009). The presence of glutamic acid forms a region of negative charge, whilst lysine is not charged. It is not solely PB2 627 that is responsible for this avian-mammalian adaptation, and infections in mammals with IAVs carrying PB2 627E does occur. With the 2009 pandemic strain (pH1N1), the presence of G590S and Q591R in PB2 were compensatory substitutions for the presence of 627E (Long et al., 2013; Mehle and Doudna, 2009). These substitutions mask the negatively charged glutamic acid in the α-helix encircled loop. The mutant PB2 D701N is also associated as a mammalian adaptation substitution which has been shown to compensate for the absence of 627K in mammals to support more efficient replication of avian-origin viruses (Gabriel et al., 2005; Li et al., 2005; Steel et al., 2009). Given the diversity of IAV and the species it infects, it is perhaps unsurprising that polymerase adaptation is

regulated by a more complex suite of interactions than that of a single amino acid substitution.

5.1.2 The Relationship of ANP32 Proteins and FluPol

Through crystallography and cryo-electron microscopy, the structure of the heterotrimeric FluPol complex for IAV and the conformational changes that occur when transitioning between cRNA, vRNA and mRNA transcription have been elucidated (Fan et al., 2019). Following on from this work, the same techniques were applied to discover the molecular interactions of ANP32A and the FluPol complex in a human *in vitro* model, albeit using Influenza C Virus as it is more amenable than IAV for these techniques (Carrique et al., 2020) (Figure 5-1A). Avian influenza polymerases are restricted in their ability to catalyse the synthesis of vRNA from the intermediary cRNA template in human cells (Sugiyama et al., 2015). The avian-origin influenza polymerases carrying the PB2 627E signature can still perform transcription of the vRNA to mRNA efficiently, but replication of vRNA from cRNA is impeded in the mammalian cellular context (Bi et al., 2019). It was found that in the IAV replicative cycle, ANP32A is a mediator in affecting the heterotrimeric structure of FluPol, leading FluPol that are bound with an ANP32 protein to bias viral transcription in favour of vRNA production as opposed to cRNA or mRNA (Bi et al., 2019; Sugiyama et al., 2015; Wei et al., 2019) (Figure 5-1B).

FluPol undergoes conformational changes to its structure between cRNA and vRNA transcription (Biquand and Demeret, 2016), and it is postulated that the association of ANP32A with FluPol affects the regulation of the vRNA/cRNA synthesis process (Long et al., 2019b). This theory is further supported from data in ANP32 double knockout swine cells, with cRNA being the viral RNA reduced in abundance the least (Zhang et al., 2019). With cRNA to vRNA transcription occurring at a low rate there is a lower accumulation of vRNA's that can serve as a template for mRNA transcription, hence the lower abundance of viral mRNA as well.

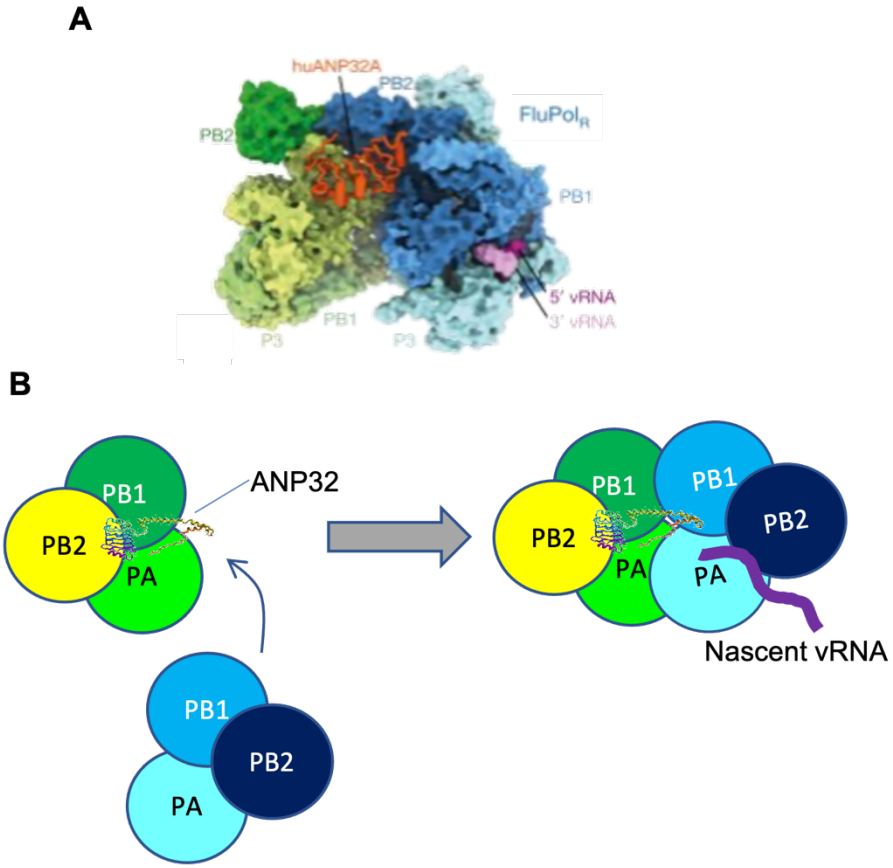


Figure 5-1: ANP32 proteins complex with the heterotrimeric FluPol dimer. A) Adapted from Carrique *et al.*, 2020. Crystallography has determined that ANP32A is embedded within the FluPol of ICV to form a complex. B) A schematic indicating the functional role of ANP32 proteins during IAV infection. When ANP32A is part of the heterotrimeric FluPol complex it supports transcription of negative-sense single-stranded vRNA from a cRNA template.

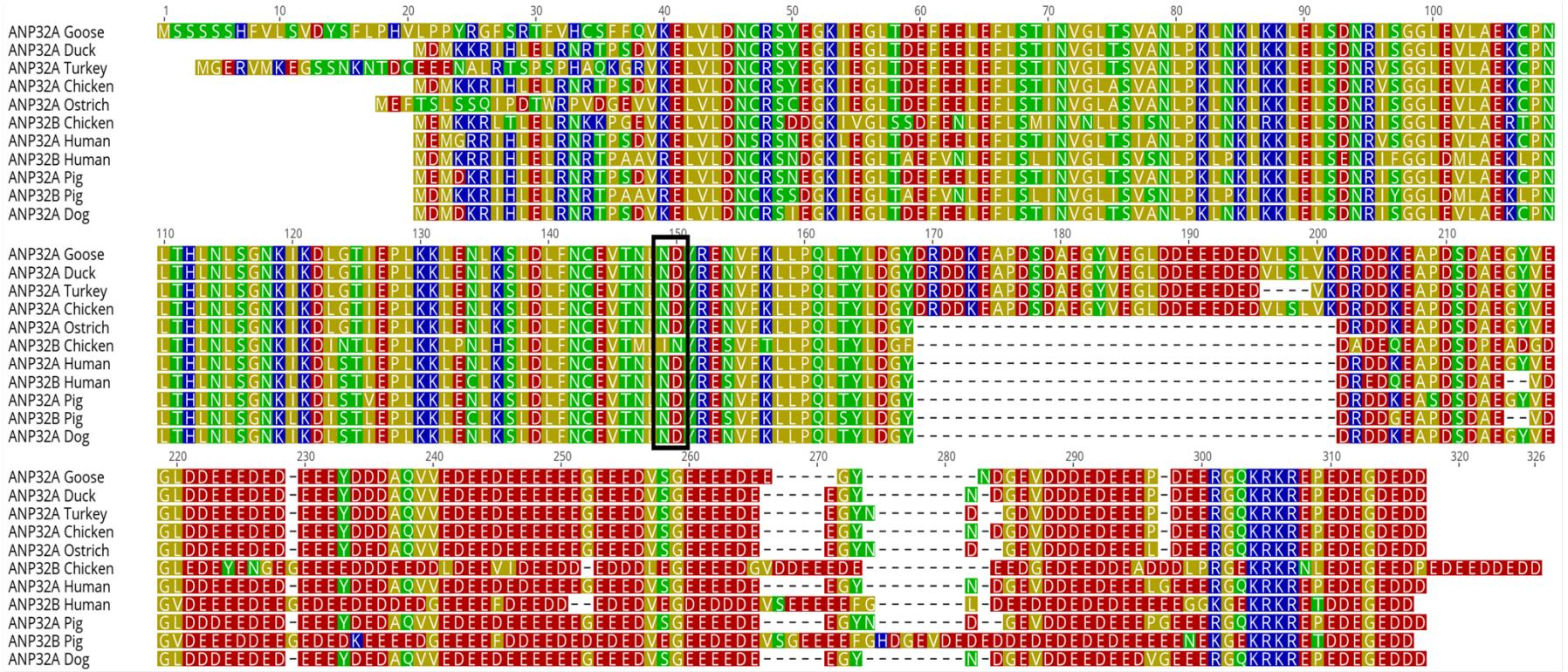


Figure 5-2: Alignment of the peptide sequence for ANP32 family proteins for selected IAV susceptible species. A 33 amino acid insert is present in avian ANP32A, apart from ratites such as ostriches, is not present in mammalian species. Amino acids 129/130, identified in the black box, are critical for ANP32 proteins being effectively recruited by IAV to promote more efficient IAV polymerase activity. When IAV transmits from the wild avian species, antigenic mutation in FluPol, such as in PB2 residue 627, promotes adaptation for recruiting mammalian ANP32 proteins

Differences between mammalian and avian ANP32 phylogeny are a critical factor in restricting the host range of IAV (Baker et al., 2018; Long et al., 2019a) (Figure 5-2). Unique to avian species is a 33 amino acid insert (residues 176-204). This stretch of acidic residues has arisen through an exon duplication encoding for 27 amino acids. As well as the exon duplication, in avian species there are an additional 6 amino acids that encodes for a SUMO Interaction Motif (SIM) (Domingues and Hale, 2017). This ANP32A insertion in bird species has been strongly implicated in affecting the level of FluPol activity for strains carrying PB2 627E, and insertion of the avian duplication into human ANP32A is enough to restore avian FluPol activity in human cells (Long et al., 2016).

However, even if birds carry this uniquely avian ANP32A gene, not all avian species are equally infected or affected by IAV. This is at least partly because the introduced 33 amino acids introduces novel splice sites for two alternative putative isoforms. Taking a view across all bird species, most species have retained the potential to express ANP32A WT, ANP32A$_{+29}$ and ANP32A$_{+33}$. Avian susceptibility to IAV is associated with the ratio of the ANP32A isoforms expressed, and the balance in gene expression of ANP32A$_{+29}$ and ANP32A$_{+33}$ is species dependent (Baker et al., 2018; Domingues et al., 2019) ANP32A$_{+33}$ is the most prevalent variant in waterfowl, which are the predominant natural reservoir of IAVs, and it is in the presence of this ANP32A isoform that avian-origin FluPol's have the most potent activity (Baker et al., 2018). ANP32A$_{+33}$ is also the predominantly expressed isoform in farmed avian species, such as chickens and turkeys, which are highly susceptible to high and low pathogenic IAVs. The avian ANP32A$_{+29}$ variant has a reduced capacity to restore FluPol activity in chicken ANP32A knockout cells, whilst the mammalian-like ANP32A WT was not observed to restore polymerase activity for avian-origin viruses in an avian context at all. A *cis*-element splicing site has been identified as being responsible for regulating the splicing at ANP32A exon 4 via an interaction with Serine and Arginine Rich Splicing Factor (SRSF) 10 (Fang et al., 2020). It was found that SRSF10 had no effects on the replication of mammalian-origin viruses, which lack any these ANP32A splice sites, but it acts as a negative regulator for avian virus polymerase activity and replication by biasing isoform

expression towards ANP32A$_{+29}$ instead of ANP32A$_{+33}$ when overexpressed. The mechanism by which SRSF10 expression alters ANP32A$_{+29}$ and ANP32A$_{+33}$ remains unclear, however it has been observed in chicken DF-1 cells that temperature affects ANP32A splicing, and thus temperature is a modifier of avian susceptibility and FluPol evolution.

Most avian species, with the exception of the ratites, have lost a protein orthologous to eutherian ANP32B, and have thus adapted to specific recruitment of the predominantly expressed ANP32A$_{+33}$ isoform (Long et al., 2019a). However, not all bird species have the same ANP32A genetic structure. Ratites (ostriches, emus etc) do not harbour the same genetic insertion in ANP32A (Figure 5-2). In avian ANP32A null cells, ostrich ANP32A is not sufficient to rescue activity of FluPol carrying PB2 627E (Long et al., 2016). The ratite lack of an exon duplication may go some way towards explaining why the acquisition of mammalian adaptive signatures in PB2 has been observed following ostrich IAV infections (Shinya et al., 2009; Yamada et al., 2010). The same mammalian adaptive signatures are identified in magpie IAVs, and magpies have been modelled to express each of ANP32A$_{+33}$ and ANP32A$_{+29}$ equally (Domingues et al., 2019). This bias in the presence of ANP32A variants in birds could drive IAVs towards mammalian adaptation, and close monitoring of IAV in birds that express the ANP32A isoform lacking the 33 amino acid insert could be used as a surveillance tool for detecting variants with potential for mammalian zoonosis.

As a result of the redundant role ANP32A and ANP32B have in supporting human adapted viral polymerase activity, ablating the function of either gene individually in humans has no effect (Zhang et al., 2019). In both avian and mammalian species, domain swapping analysis with cDNA constructs in ANP32A$^{-/-}$ ANP32B$^{-/-}$ cells has identified the 5th LRR and cap domain as being the most important region in enhancing FluPol activity (Long et al., 2019a; Zhang et al., 2019). Amino acids at residues 129 and 130 in the 5th LRR, identified from variation between avian ANP32A and ANP32B, have been shown to be critical in conferring optimal efficiency for IAV polymerase activity in both humans and swine (Long et al., 2019a; Zhang et al., 2019). This data was supported further by the cryo-electron microscopy observations that found

ANP32A N129 and D130 directly interact with the FluPol complex of ICV (Carrique et al., 2020). Human and swine ANP32E do not share the amino acid sequence in this 5th LRR domain, potentially explaining the lack of association with viral polymerase activity.

The SIM affects the interaction of avian ANP32A with 627E FluPol (Bi et al., 2019; Domingues and Hale, 2017), and in the ANP32A$_{+29}$ isoform the SIM is disrupted, explaining the reduced efficacy of avian-origin polymerases with ANP32A$_{+29}$ (Carrique et al., 2020; Domingues et al., 2015). ANP32A$_{+33}$, containing the entire SIM-like motif, induces a conformational change in the heterotrimeric FluPol complex with 627E that induces specific binding to cRNA promoters, which in turn initiates vRNA synthesis (Bi et al., 2019). In mammalian ANP32A and ANP32B proteins that have been associated with FluPol activity, the correspondingly aligned region to the SUMO motif region is entirely comprised of acidic amino acids, whereas due to the 33 amino acid insertion, the chicken peptide sequence contains acidic and basic residues. This has been suggested as the biochemical reason why PB2 has selective pressure to substitute an acidic residue at site 627 in mammals, as two acidic residues will not support a stable electrostatic interaction. Substitution of the avian basic and acidic region into human ANP32A conferred it with the ability to support avian flu isolates with PB2 627E (Carrique et al., 2020). The 2009 pH1N1 retained the avian signature 627E in PB2, but the PB2 Q591K adaptation compensates for this and has been mapped to also interact with the SIM region in avian ANP32A There is also an interaction between the highly acidic LCAR region of ANP32 proteins and the FluPol complex through amino acids 221-235. This region affects FluPol function through improving the binding affinity with PB2 (Domingues and Hale, 2017).

Swine have been identified to have ANP32A amino acid signatures unique to mammals at sites 106 and 156. These residues in swine ANP32A have been found to enhance FluPol activity, compared to the conserved residues in other mammalian species, in IAVs with the avian signature PB2 627E (Zhang et al., 2020). Although substitution of V106/S156 does not ablate the activity of any viral polymerases in an *in vitro* swine model system, their modification

significantly reduced the potency of swine ANP32A in enhancing avian-origin polymerase activity, and it is suggested that because they improve the efficiency of FluPol containing avian signatures and do not affect mammalian signature FluPol activity, they are another reason that swine can function as effective mixing vessels for IAV from multiple origins.

5.1.3 Chapter Aims

With research on the mechanistic molecular interactions of ANP32 proteins with IAV FluPol improving our understanding of how polymerase adaptation of IAVs affects zoonotic events, the role of swine in the IAV ecosystem is becoming better understood. The concomitant effect of ANP32A support and the presence of SA moieties that support avian IAV HA endocytosis puts swine in a unique position where they are more readily infected with avian-origin IAVs and can therefore act as a mixing vessel. If modification of swine ANP32A and ANP32B genes confers resistance to IAV, pigs that are resistant to avian IAVs, human and swine-origin IAVs, and also IAVs that are endemic in other species such as dogs, horse or bats could be a critical node of IAV zoonotic ecology removed. Resistance to avian-origin IAVs is particularly important when considering prevention of potential pandemic strains emerging via reassortment.

The aim of this chapter was to investigate the role that swine ANP32A and ANP32B have in NPTr cells during IAV infection through the previously created CRISPR-edited knockout cells. The data related to swine described in this introduction have almost exclusively been performed in cells of kidney origin (PK-15) and has used viruses of different origins to those that we will present. Therefore, these data contributes to understanding whether ANP32A and ANP32B could be viable gene-editing targets for IAV resistance in a more relevant context. The results highlight the concerns of conferring variable resistance to different viral strains and the potential that partial resistance could lead to viral circumvention of the resistance mechanism. Understanding the role of ANP32 proteins in swine also contributes to our understanding of IAV

evolution from a cross-species perspective, and provides knowledge when considering IAV ecology and control from a One Health perspective.

With an *in vitro* model validated in Chapter 4 as being ANP32A and ANP32B null, in tandem and individually, this chapter set out to investigate whether the loss of these functional proteins affected swine, avian and human-origin IAV propagation. The subsequent experiments were designed to gain insight into the stage of the viral replication cycle that was affected and to begin disentangling how ANP32 proteins support IAV infection in swine, as well as determining whether expression of ANP32A or ANP32B could restore activity of the stage in the viral replicative cycle that is affected by the absence of ANP32 proteins.

5.2 Results

5.2.1 Preliminary infections of NPTr cell lines

To gain a preliminary insight into whether the loss of ANP32 family proteins would affect IAVs ability to replicate in our *in vitro* model we infected the WT NPTr, each of the single knockouts, and the double knockout NPTr cell lines at an MOI of 2 with the human-origin lab-adapted PR8 strain. An uninfected control sample was maintained to observe cell growth in the absence of IAV (Figure 5-3). The images shown are selected as being representative of the infected cell cultures. In the wildtype, ANP32A (AKO) and ANP32B (BKO) knockout cells there was notable cytopathic effects (CPE), apoptosis and cell debris after 24 hours, with few cells remaining attached to the culture flask. After 48 hours, there were no morphologically normal cells in the image field for the WT or monogenic knockout cells. In the DKO1 cell line, a moderated cytopathic effect and less cell debris was visible when compared to the WT cells after 24 hours. After 48 hours there were few cells remaining attached, and the cells that were adherent had a distorted morphology. The DKO3 cell line displayed minimal CPE or cell debris visible after 24 hours. With 48 hours of infection there was an increase in the number of cells that had detached, but unlike all other cell lines, some cells remained adherent and retained a normal NPTr morphology. These pilot infections suggested that the roles of ANP32A and ANP32B are functionally redundant in the context of PR8 infection. Only the DKO cell lines disrupted viral replication dynamics, and the DKO3 cell line had an exacerbated effect of being less conducive to viral replication than all the cell lines tested.

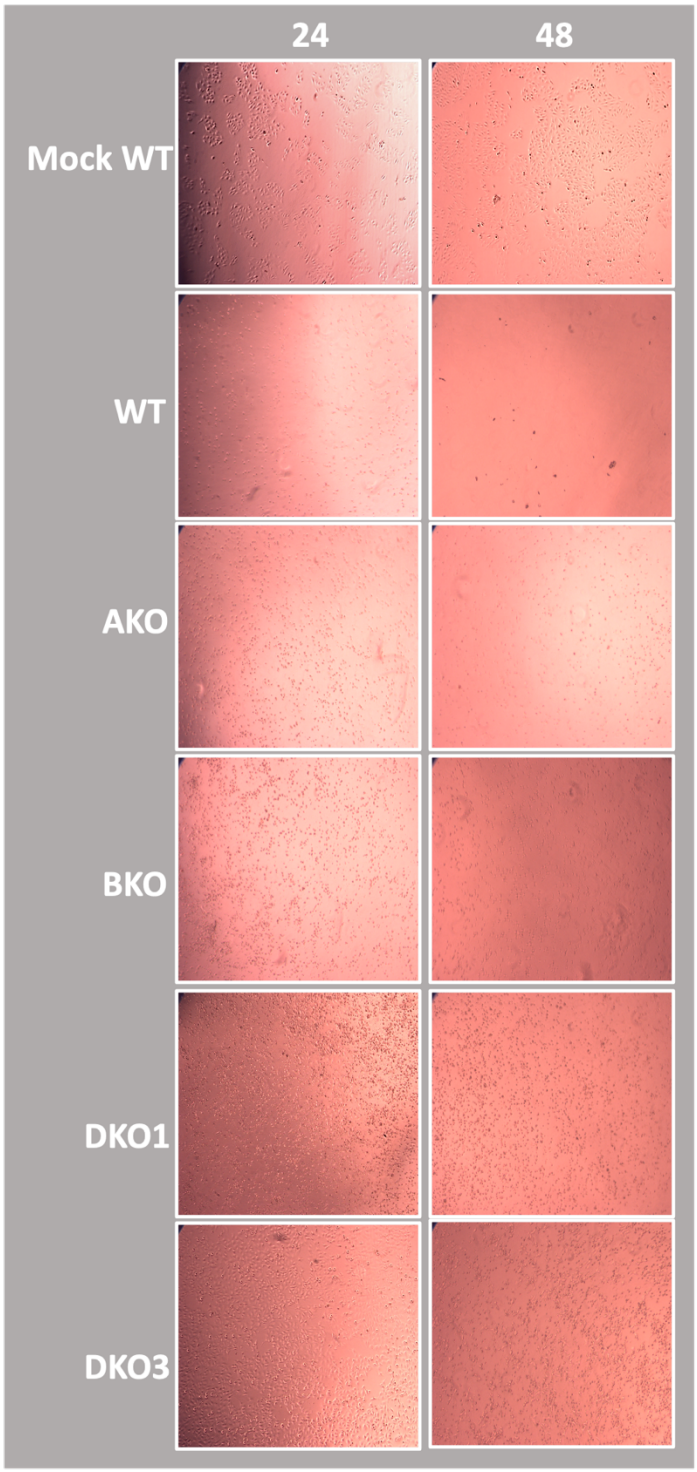


Figure 5-3: Pilot IAV infections of WT and CRISPR-edited NPTr's at an MOI of 2 to qualitatively assess the impact of functionally knocking out ANP32A and ANP32B. Brightfield images were selected as representative of the surrounding cultures infected with the PR8 strain of IAV after 24 and 48 hours.

5.2.2 Plaque Assays

Plaque assays were performed to measure the number of viable infectious virions that were produced from each of the ANP32 family protein knockout cell lines following infection with IAVs of human, swine, and avian origin. All viruses used in this chapter are described in Table 5-1. We hypothesised that the number of infectious virions produced would only be reduced in the double knockout cell lines because swine ANP32A and ANP32B are functionally redundant in their recruitment by FluPol. The NPTr cultures were infected with IAV in the presence of TPCK to allow for multiple IAV cycles to occur. The virus-containing supernatant was collected at the specified time points and titres were assessed by enumeration of plaques formed on MDCKs. Each time point in the figures represents data from three biological replicates that were plaqued once each (n=3 independent experiments, one-way ANOVAs compared to WT, post-hoc Dunnett's test; * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001).

Strain Name	Abbrev.	HxNx	Isolation Species	Description	590	591	627	701
A/Victoria/1975	Victoria	H3N2	Human	Human origin H3N2	G	Q	K	D
A/England/195/2009	Eng/195	H1N1	Human	'09 pandemic-like from UK	S	R	E	D
A/Hubei/73/2009	Hubei	H1N1	Human	'09 pandemic-like from China	S	R	E	D
A/swine/England/453/2006	EA453	H1N1	Swine	Pre '09 pandemic swine	G	Q	E	N
A/canine/Illinois/41915/2015	Illiniois	H3N2	Canine		G	Q	E	D
A/chicken/Pakistan/UDL-01/2008(H9N2)	UDL	H9N2	Chicken	Low pathogenic avian influenza	G	Q	E	D
A/turkey/England/50-92/1991	50-92	H5N1	Turkey	Highly pathogenic Galliformes restricted virus	G	Q	E	D
A/turkey/Turkey/5/2005	Ty/05	H5N1	Turkey		G	Q	E	D
A/swine/England/163266/1987(H3N2)	Sw87	H3N2	Swine	Swine origin H3N2	G	Q	K	D
A/PR8/1934	PR8	H1N1	Human	Mouse-adapted H1N1	G	Q	K	N

Table 5-1: The IAV strains used across all experiments in this thesis. The indicated amino acid residues in IAV PB2 are known to be key determinants of zoonotic potential.

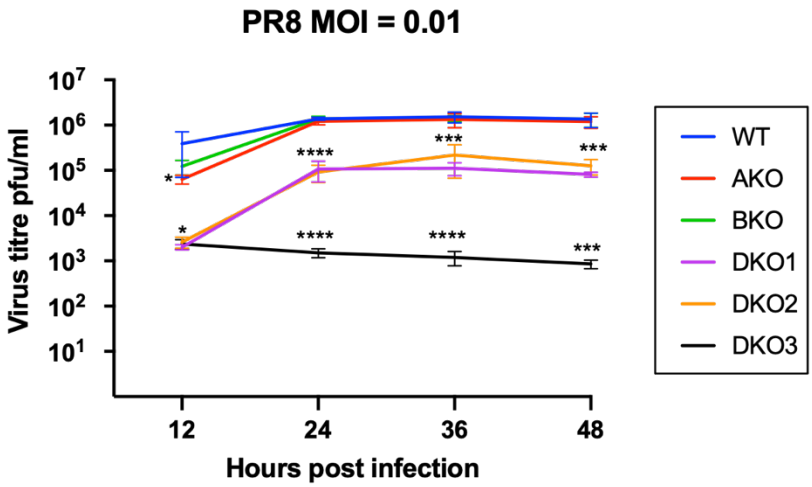


Figure 5-4: Swine ANP32A or ANP32B promotes the efficiency of PR8 replication. Comparative growth kinetics of IAV PR8 in WT and CRISPR-edited NPTr by titration of collected supernatants from cell cultures infected at an MOI of 0.01. Time points were taken in triplicate and mean viral titres were determined by plaque assays on MDCK cells. Cell lines were infected with TPCK to allow multiple infection cycles and incubated at 37 °C. WT (blue), AKO (red), BKO (blue), DKO1 (purple), DKO2 (orange) and DKO3 (black) Standard deviation is shown and statistical significance was determined by one-way ANOVA with a post-hoc Dunnett's test.* = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$, **** = $p<0.0001$

An initial infection was performed with PR8 at a MOI of 0.01 (Figure 5-4). IAV titre in AKO was reduced compared to wildtype NPTr's after 12 hours only ($p < 0.05$). Thereafter, viral replication was not impeded by the loss of either ANP32A or ANP32B individually. There was over a ten-fold reduction in viable PR8 virions collected from the supernatant from all DKO cell lines at each time point, aside from the DKO2 cell line at 36 hours, and these differences were statistically significant when compared to WT NPTr at the same time points.

We then performed infections in each of the NPTr cell lines using Sw87, an H3N2 swine-origin virus, and two 5:3 reassorted avian viruses (with HA, NA and M from PR8 to attenuate virulence), being 50-92, an H5N1 virus and UDL, a low pathogenic H9N2 virus. To investigate the impact of a higher initial infectious dose, we also performed the infection at a higher MOI of 0.1 (Figure 5-5). DKO2 was not included in the 0.01 MOI infections as its genotype caused the same peptide sequence changes as DKO1, and from the prior 0.1 MOI infections and the pilot PR8 infections, DKO1 and DKO2 were observed to

recapitulate each other's infection dynamics. For Sw87 at an MOI of 0.01, there was a reduction in virions collected from the supernatant of greater than 10-fold when compared to the WT cells for DKO1 at 12 and 36 hours, and a smaller reduction at 24 and 48 hours (Figure 5-5A). IAV titres from the DKO3 cell line were below the limit of detection (LOD) for all time points after 12 hours. The BKO cell line had a statistically significant difference at the 36-hour time point compared to the WT sample, and the AKO cell line was reduced after 48 hours. At an MOI of 0.1 for Sw87, the DKO3 cell line had significant reductions in viral titre compared to the WT cell line from 24 hours onwards (Figure 5-5B).

Regarding the reassorted avian virus infections, for 50-92 at an MOI of 0.01, the DKO cell lines had a reduced number of infectious virions collected from the supernatant at all time points (Figure 5-5C). DKO1 maintained over a 10-fold difference until 48 hours, whilst DKO3 was between 100 and 1000 times lower at each time point. The BKO cell line had a moderate but significant reduction observed at 24 hours and at each time point after, and the AKO cell line also had a modest reduction at the 48-hour time point only. At an MOI of 0.1 for 50-92, all DKO cell lines had a significant reduction in infectious virions collected at each time point, with DKO3 showing an exacerbated effect (Figure 5-5D). The BKO cell line was reduced after 24 and 36 hours only.

For the UDL strain, there was a significant reduction in the number of virions collected from the supernatant from the DKO infected cell cultures compared to WT cells after 12 hours, and subsequently only DKO3 at 24 hours was lower with statistical significance (Figure 5-5E). With the 0.1 MOI infections, the DKO cell lines were all lower with significance at 12 hours, and then only at 36 hours were the DKO and AKO cell lines lower with statistical confidence when compared to WT NPTr (Figure 5-5F).

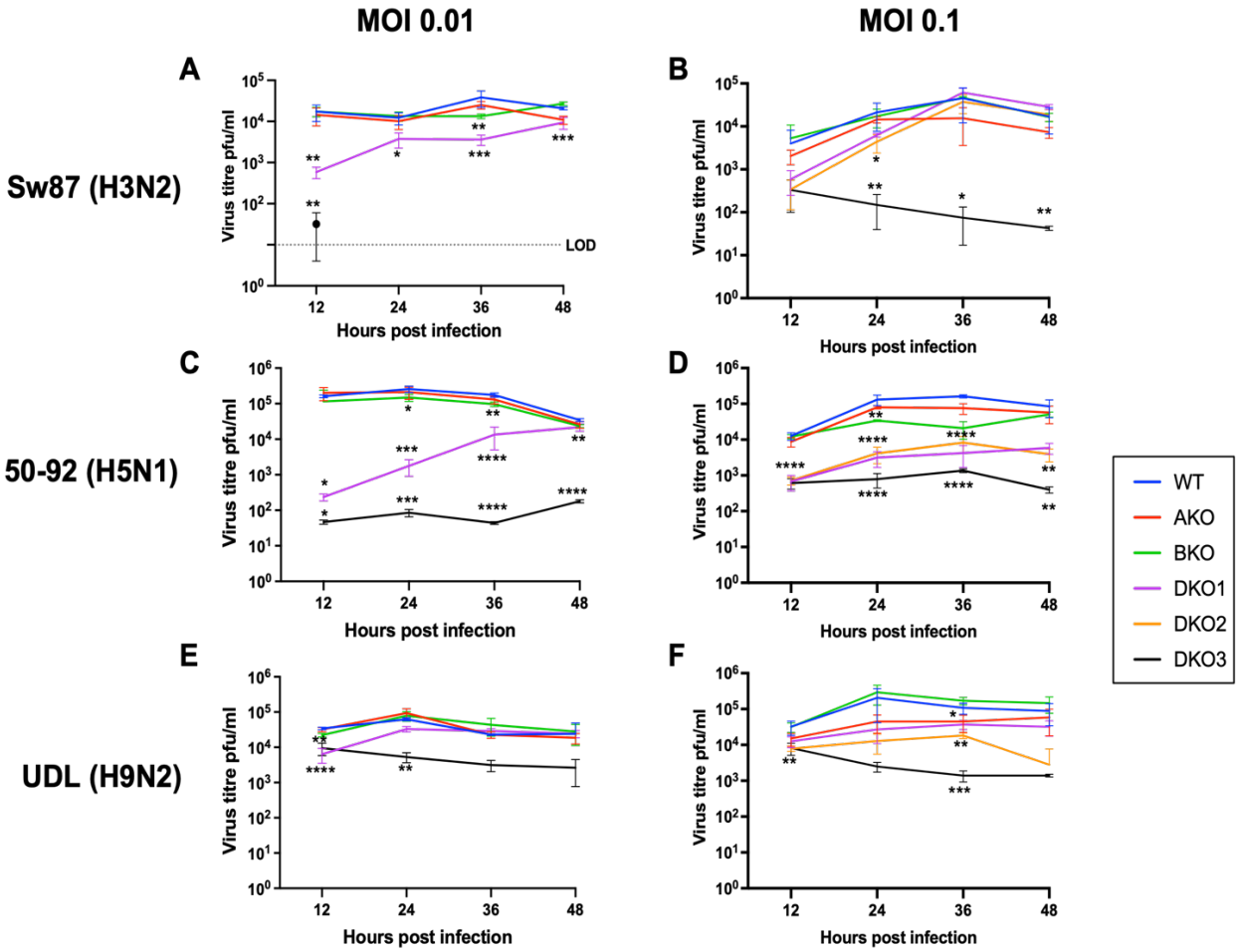


Figure 5-5: Swine ANP32A or ANP32B can improve the efficiency of replication for IAV originating from a swine or avian host. Comparative growth kinetics of Sw87, 50-92 and UDL in WT and CRISPR-edited NPTr by titration of collected supernatants from cell cultures infected at an MOI of 0.01 or 0.1. Time points were taken in triplicate and mean viral titres were determined by plaque assays on MDCK cells. Cell lines were infected with TPCK to allow multiple infection cycles and incubated at 37 °C. WT (blue), AKO (red), BKO (blue), DKO1 (purple), DKO2 (orange) and DKO3 (black) Standard deviation is shown and statistical significance was determined by one-way ANOVA with a post-hoc Dunnett's test.* = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001

5.2.3 Nucleoprotein Staining

To gain insight into the replication of IAV in our knockout cell lines, we performed immunofluorescence staining of IAV NP. The presence and distribution of NP functions as a molecular clock for IAV infection (Figure 5-6). Through qualitative analysis of NP, insights into the point at which infection is perturbed can be gained. By example, if no NP is present in the nucleus but there is a small cytoplasmic amount, there is an impediment to IAV replication between cell entry and nuclear transport of vRNPs. Using the same viruses as for the previous assessment of infectious virion propagation, we infected each ANP32 knockout cell line at an MOI of 1 for 10 hours without the presence of TPCK to limit IAV replication to a single infectious cycle. The images presented were selected on the basis that they are representative of the surrounding cell culture. The mock infected samples shown were exposed to culture medium that was collected alongside the viral propagation methods.

In the PR8 infected NPTr's there was no NP observed in the mock infected negative control, and NP was present throughout the cytoplasm and nucleus of WT cells (Figure 5-7). The intensity of fluorescence in the AKO and BKO cell lines is qualitatively reduced, but NP is still distributed throughout the nucleus and cytoplasm, as was observed in the WT cells. The majority of the DKO1 cells have their highest fluorescence signal intensity from NP detected in the nucleus, with sporadic cells also having NP puncta visible throughout the cytoplasm. For the DKO3 cell line the NP signal is tightly restricted to nuclei, with a lower intensity than in each of the other clonal cell lines observed.

For the Sw87 virus, no NP was detected in the mock infected cell line. In WT NPTr's, the distribution of NP was observed throughout the nucleus and cytoplasm (Figure 5-8). As with the PR8 infections, the AKO and BKO cell lines may have had a marginally lower signal intensity, but they retained the same distribution pattern as WT cells, with NP in the cytoplasm and nuclei of infected cells. Both the DKO1 and DKO3 cell lines had NP at a detectable level that was restricted to the nuclei. DKO3 had a lower fluorescence intensity and there were distinct puncta observable in the nuclei.

In the 50-92 IAV infections, NP was observable in the nucleus and cytoplasm for the WT, AKO and BKO cell lines, whilst in the mock infected cells there was no NP detected (Figure 5-9). The DKO1infected cells had a low level of nuclear NP visible and peripheral cytoplasmic NP distribution was detectable, whilst the NP in DKO3 cells was restricted to the nuclei at a level that was difficult to discern.

In the infections with the UDL strain, in WT infected cells there was NP distributed throughout the entire cell and no NP detectable in the mock infected control (Figure 5-10). In AKO and BKO cell lines, the presence of NP was distributed in a manner indistinguishable from the WT infected cells, with all cells showing a nuclear and cytoplasmic NP distribution. The DKO1 cell line had fluorescence signal restricted to most nuclei in the image field, and for DKO3 the detection of NP was limited, and its presence was only observed in a subset of nuclei.

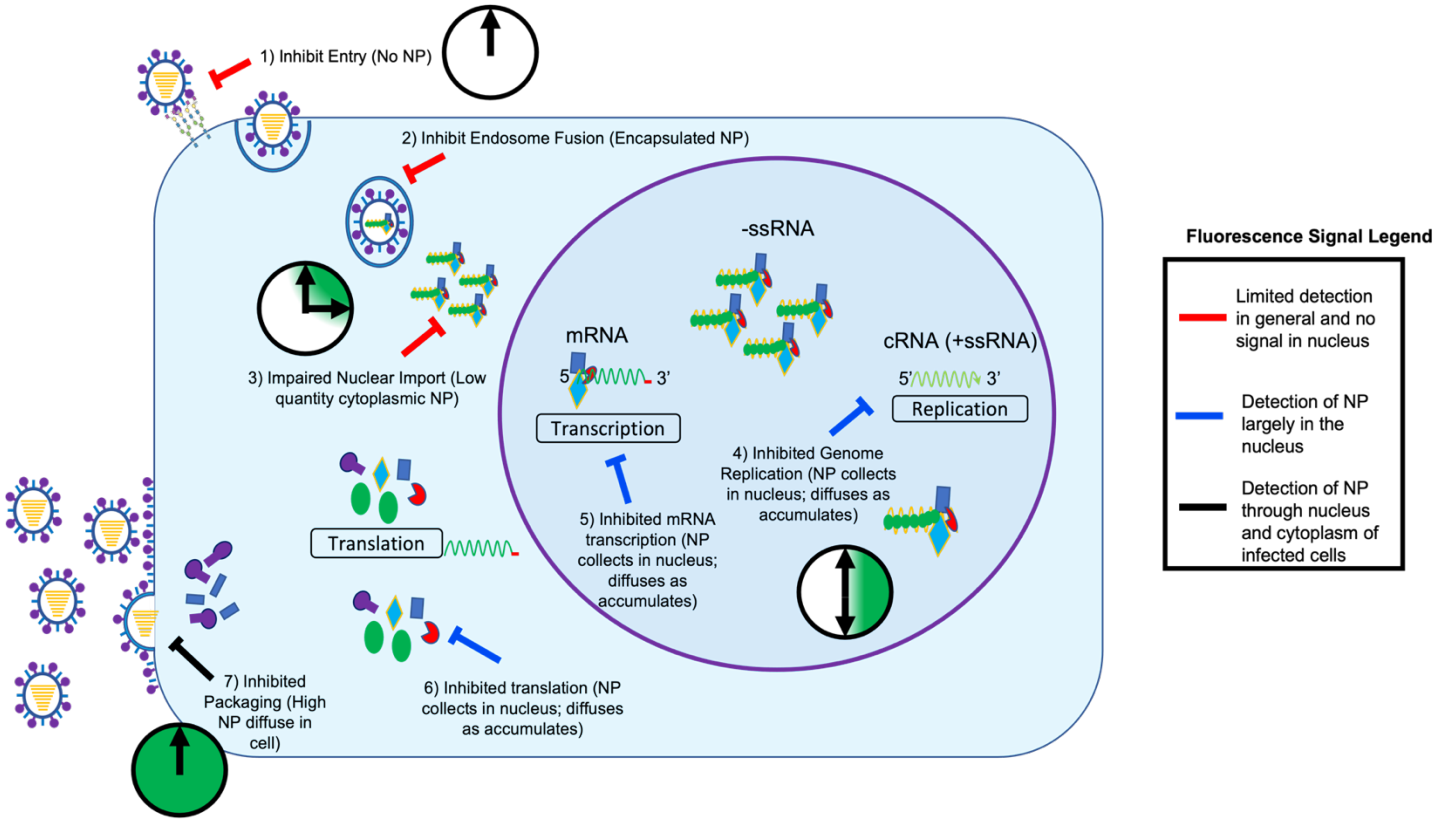


Figure 5-6: A schematic representation of the nucleoprotein (NP) clock. The distribution and quantity of NP present in an IAV infected cell can be used to infer the stage of the replicative cycle that has been reached. Through immunofluorescence detection tracking of NP is informative of the progression of IAV infection in a single cell context.

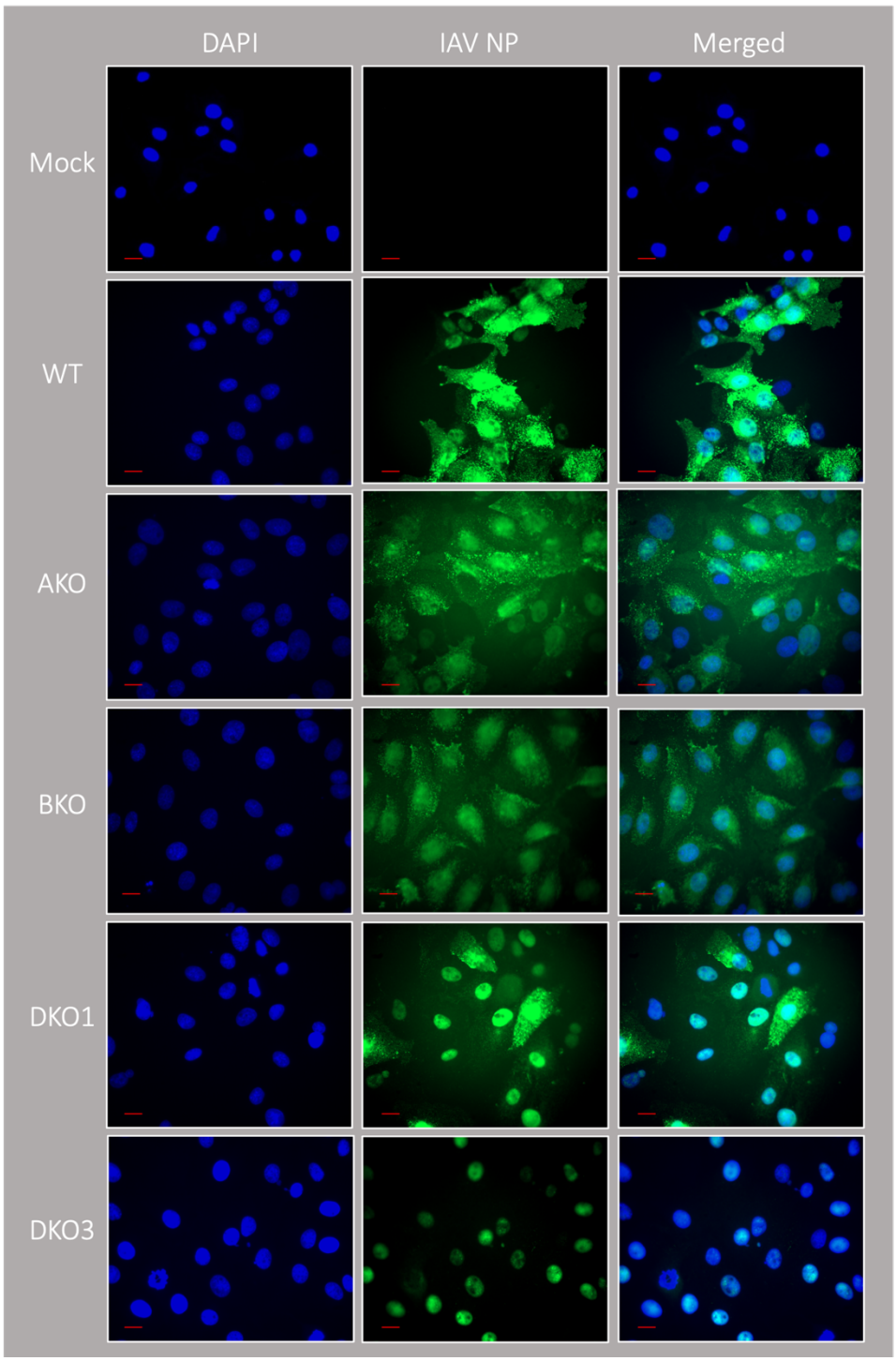


Figure 5-7: Immunofluorescence analysis of PR8 infections in NPTr WT and the CRISPR-edited cell lines shows that DKO NPTr are less permissive to PR8 replication. NP (green) was detected with ab20343 and the AlexaFluor-488 conjugated anti-mouse secondary (ab150113). Cells were counter-stained with DAPI (blue) to visualise nuclei. Scale bars represent 20 µm.

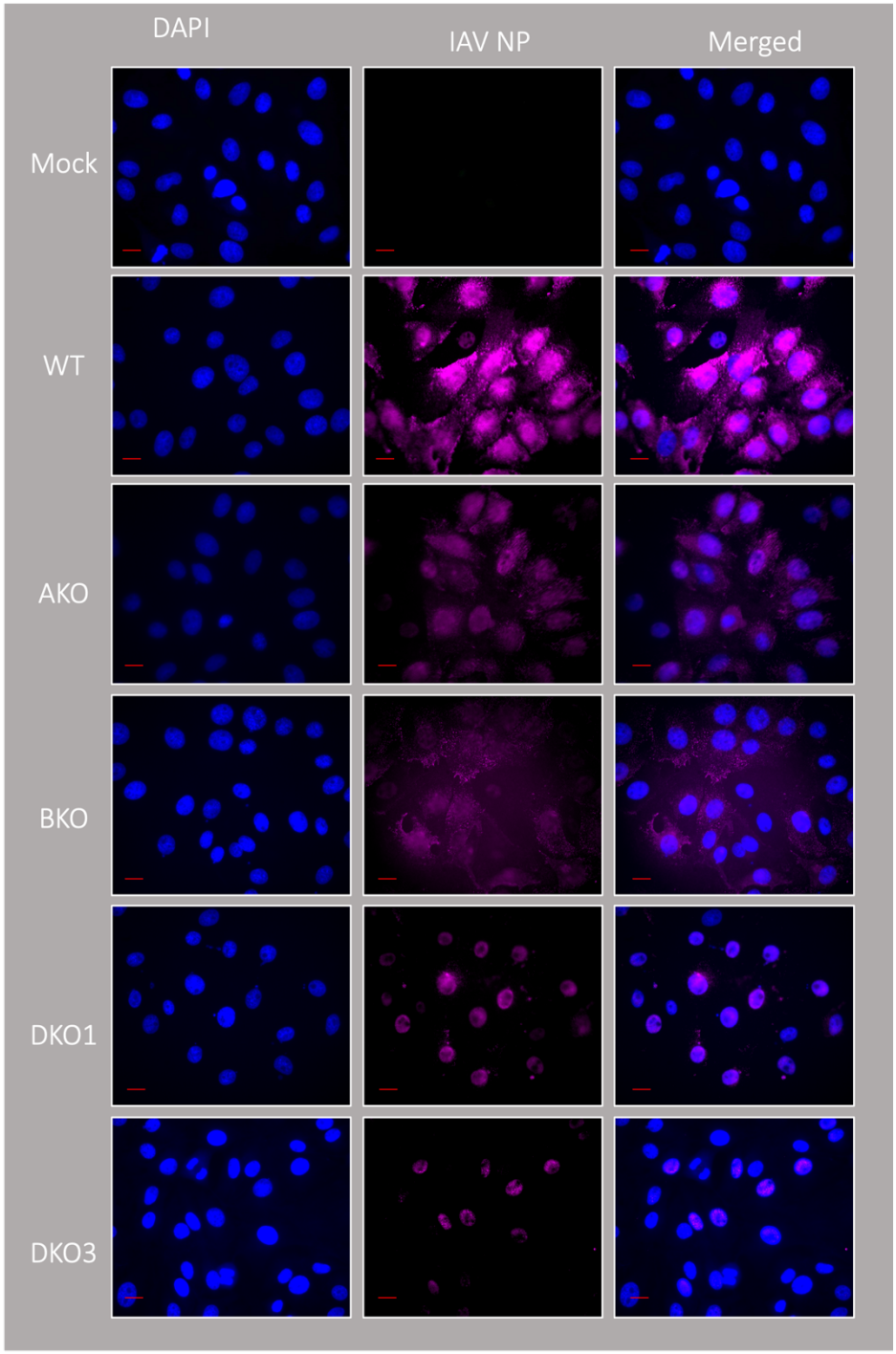


Figure 5-8: Immunofluorescence analysis of Sw87 infections in NPTr WT and the CRISPR-edited cell lines shows that DKO NPTr are less permissive to Sw87 replication. NP (magenta) was detected with ab20343 and the AlexaFluor-488 conjugated anti-mouse secondary (ab150113 Cells were counter-stained with DAPI (blue) to visualise nuclei. Scale bars represent 20 µm.

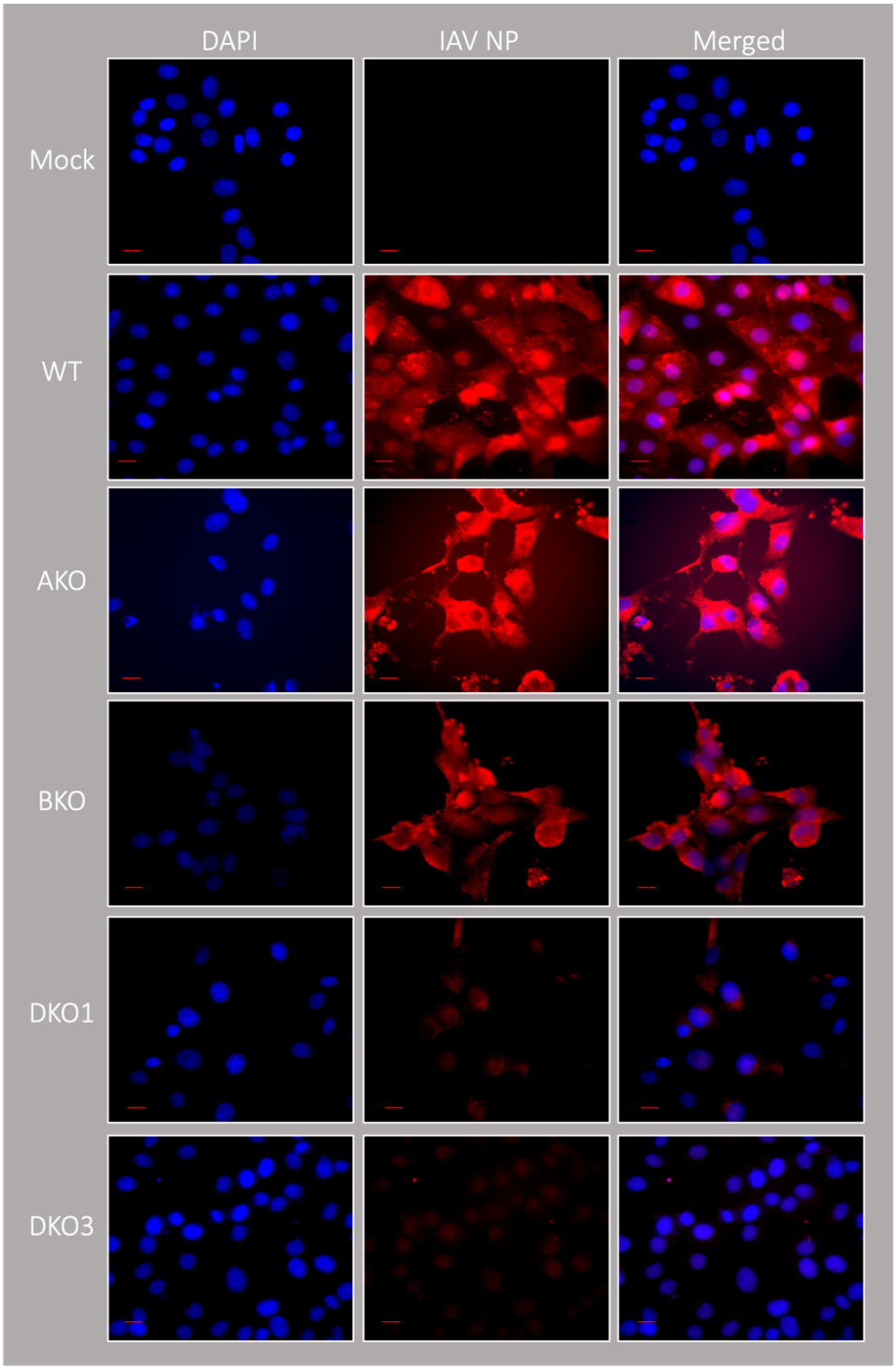


Figure 5-9: Immunofluorescence analysis of 50-92 infections in NPTr WT and the CRISPR-edited cell lines shows that DKO NPTr are less permissive to 50-92 replication. NP (red) was detected with ab20343 and the AlexaFluor-488 conjugated anti-mouse secondary (ab150113). Cells were counter-stained with DAPI (blue) to visualise nuclei. Scale bars represent 20 µm.

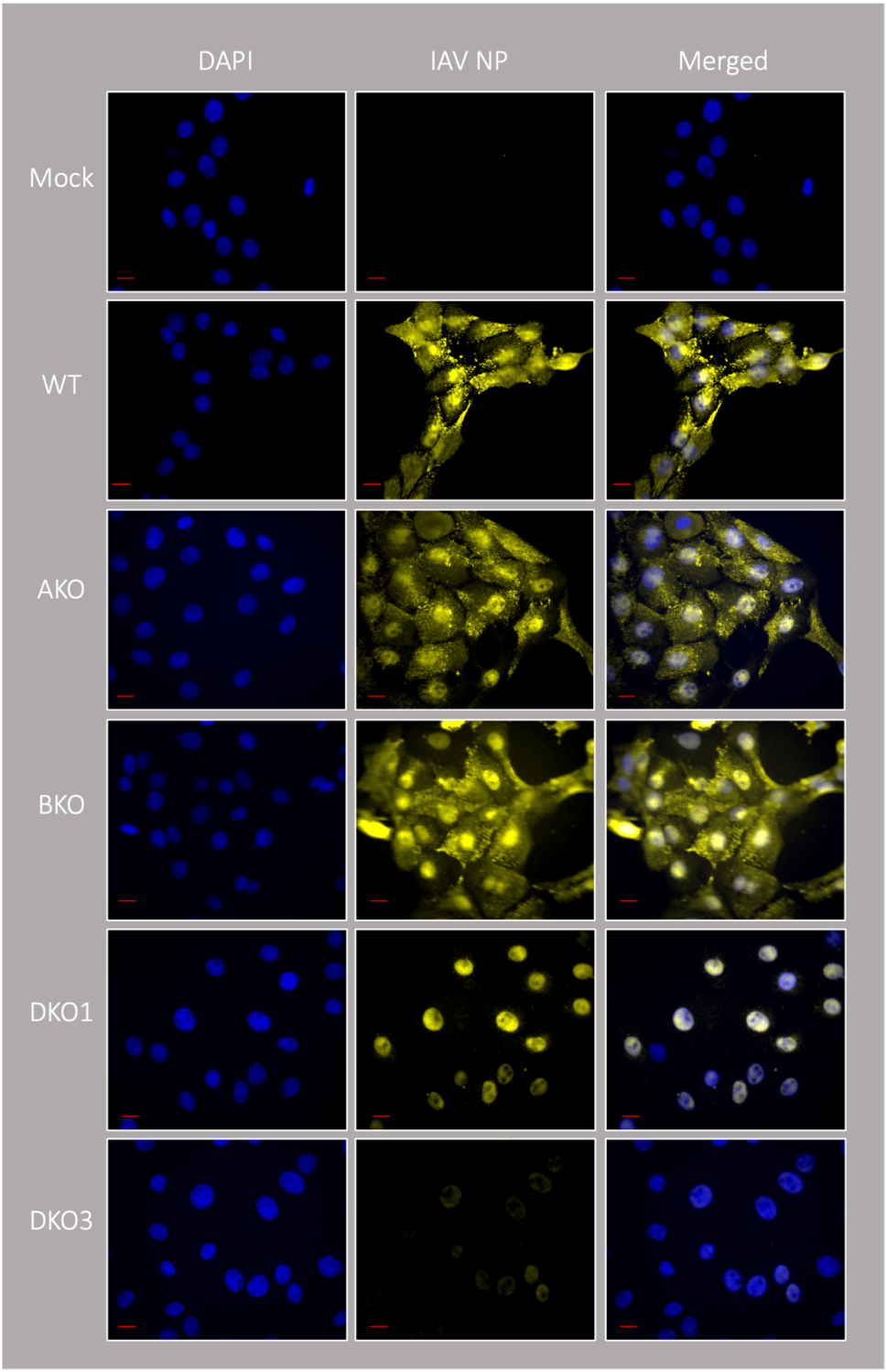


Figure 5-10: Immunofluorescence analysis of UDL infections in NPTr WT and the CRISPR-edited cell lines shows that DKO NPTr are less permissive to infection with UDL. NP (yellow) was detected with ab20343 and the AlexaFluor-488 conjugated anti-mouse secondary (ab150113). Cells were counter-stained with DAPI (blue) to visualise nuclei. Scale bars represent 20 µm.

5.2.4 Minigenome Assays

To gain further insight into the role that ANP32 proteins have during IAV infections in pigs, minigenome assays were performed. Minigenome assays report FluPol activity through the expression of a reporter gene that requires vRNA synthesis. Constructs expressing the minimally required components for cell based FluPol activity and reporter plasmids were transfected into the NPTr WT and CRISPR-edited cell lines. The activity of the strain specific FluPol's is reported as the detection of *Firefly* normalised to *Renilla*. Two biological replicates of the minigenome assays were performed (n = 2) and a representative plot using triplicate measurements from one is presented. No ANOVA was performed to determine statistical significance due to the small sample size. The negative controls were performed using the firefly to *Renilla* ratio in WT cells transfected without PB2. The negative control lacks the full enzymatic complement necessary for IAV polymerase activity.

For the mammalian-origin IAVs, the activity of the H3N2 Victoria strain's FluPol was reduced by over 10-fold in the DKO1 cell line compared to WT NPTr's (Figure 5-11A). DKO3 had a further reduction in FluPol activity observed. The human-origin 2009 pandemic strain tested was Eng195 (Figure 5-11B). With Eng195, the DKO strains had over 10-fold lower FluPol activity compared to the WT cells, and the AKO and BKO samples had similarly high levels of polymerase activity as the WT. For the swine-origin H1N1 pre-2009 pandemic IAV strain (EA453) FluPol's, there was a similar reduction in activity observed in both the AKO and DKO1 strains when compared to the WT cells. In DKO3 cells there was a further 10-fold reduction in polymerase activity (Figure 5-11C). The final mammalian strain tested was an H3N2 IAV strain of canine origin. The DKO cell lines both had close to a 100-fold reduction, whilst the monogenic knockout cell lines retained polymerase activity similar to the level observed in the WT NPTr's (Figure 5-11D). For all mammalian polymerases except H3N2 Victoria in DKO3, the double knockout cell lines had polymerase activity of at least 10 times more than the negative control samples, suggesting that despite the large reduction in

polymerase activity observed in DKO3 it remains at a higher level than when a FluPol component is absent.

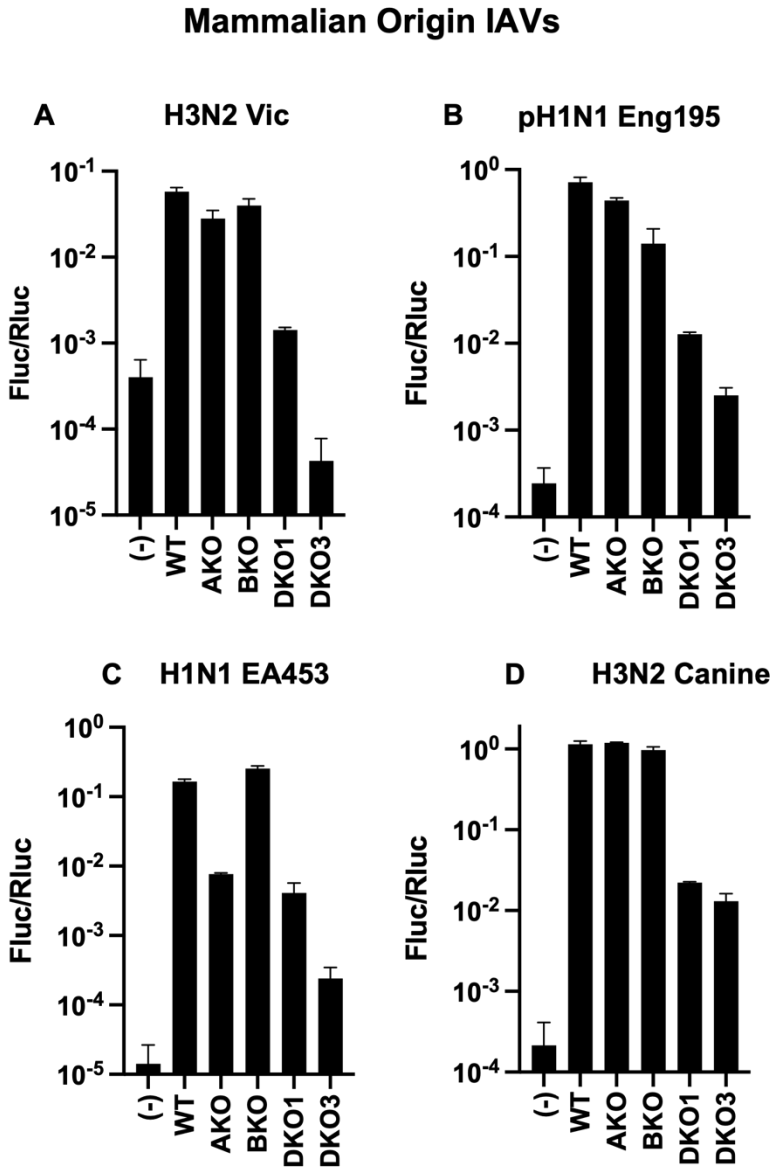


Figure 5-11: Minigenome assays performed in WT and CRISPR-edited NPTr's with mammalian-origin IAVs shows that in the absence of ANP32A and ANP32B there is a reduction in the level of polymerase activity**.** The loss of both ANP32A and ANP32B is necessary to reduce FluPol activity for mammalian-origin IAV. Firefly luciferase (Fluc) expression was normalised to *Renilla* (Rluc) A) Human-origin H3N2 Victoria. B) Human isolate FluPol sequences from pH1N1. C) Swine isolate FluPol sequences from pH1N1 D) Canine-origin H3N2.

The avian-origin IAV FluPol constructs used for investigating polymerase activity in the knockout cell lines were derived from the same IAV strains as the two used in the viral infection and NP immunofluorescence (50-92 and UDL) assays, alongside another H5N1 virus of turkey origin. Each FluPol set had a construct encoding the avian signature PB2 627E as well as a mammalian adapted signature PB2 627K variant construct. For the 50-92 carrying PB2 627E, the DKO cell lines had over a 10-fold reduction in polymerase activity, and the AKO cell line was reduced by less than 10 times compared to the WT and BKO cell lines (Figure 5-12A). With the PB2 627K substitution in 50-92, both monogenic cell lines had a similar detection of polymerase activity as the WT cell line, whilst the DKO cell lines had a reduction of 1.5-2 logs (10) (Figure 5-12B).

With the H9N2 UDL strain, for both the PB2 627E and 627K there was no change in polymerase activity between the monogenic cell lines (AKO/BKO) and WT NPTr. The DKO cell lines both had at least a 10-fold reduction for both PB2 isoforms (Figures 5-12C and D). The Ty/05 strain did not have transfections into the DKO3 cell line performed. With PB2 627E, there was a similar reduction in polymerase activity for the monogenic AKO cell line and DKO1. When PB2 627K was used in place of PB2 627E, the polymerase activity in the AKO cell lines was returned to a similar level as in WT cells, and only in the DKO cell line was a reduction of 100-fold observed (Figure 5-12 E and F). For all avian-origin viruses in each cell line, polymerase activity was higher with the mammalian adapted PB2 627K signature.

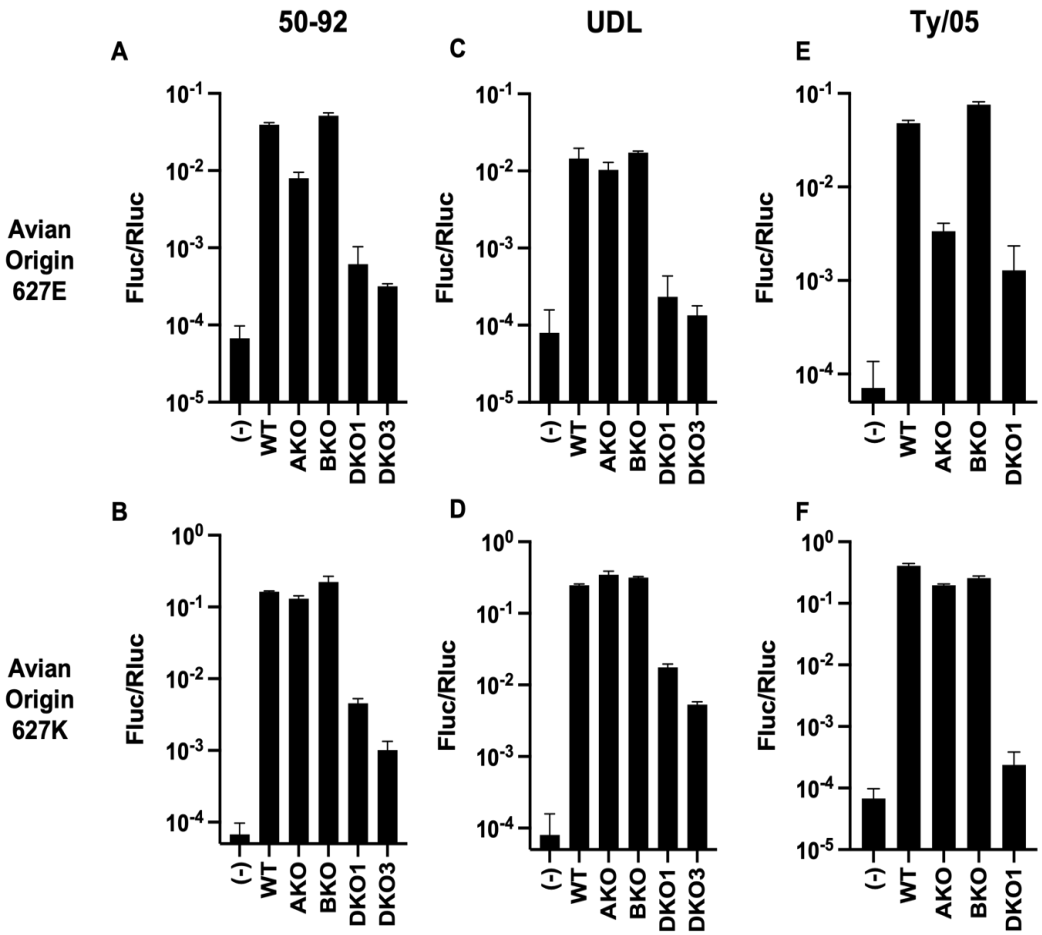


Figure 5-12: Minigenome assays performed in WT and CRISPR-edited NPTr's with avian-origin IAVs carrying either PB2 627K or PB2 627E. shows that in the absence of ANP32A and ANP32B there is a reduction in the level of polymerase activity**.** The loss of both ANP32A and ANP32B is necessary to reduce FluPol activity. PB2 627E is an amino acid that is widely associated as an avian adaptive signature, whilst PB2 627K is a mammalian adaptive signature, these assays show more potent activity for minigenomes with the 627K variant. Firefly luciferase (Fluc) expression was normalised to *Renilla* (Rluc). A) 50-92 with PB2 627E. B) 50-92 with PB2 627K. C) UDL with PB2 627E. D) UDL with PB2 627K. E) Ty/05 with PB2 627E F) Ty/05 with PB2 627K.

5.2.5 Restoring the function of ANP32 proteins in KO cells and investigating the impact of the -12bp in ANP32A

To further investigate whether the activity of the IAV FluPol was specifically affected by the presence and absence of ANP32A or ANP32B in swine, and to gain insight into how the ANP32A -12 bp allele could be exacerbating the phenotype of reduced FluPol activity, we restored expression in WT, AKO, BKO, DKO1 and DKO3 NPTr's with swine ANP32A and ANP32B cDNA constructs. With the human-origin H3N2 Victoria virus, vectors expressing ANP32A, ANP32B and ANP32A -12 bp supported FluPol activity to be returned to similar levels as were observed in the WT and monogenic knockout cell lines (Figure 5-13).

The same experiments restoring ANP32A or ANP32B in the CRISPR-edited NPTr's were performed with the UDL and 50-92 avian-origin viruses carrying either PB2 627E or the mammalian-signature PB2 627K amino acid residue (Figure 5-14). For the H9N2 UDL virus, both ANP32A and ANP32B cDNA constructs rescued polymerase activity to levels similar to the level observed in WT NPTr (Figure 5-14AB). Transfection with ANP32A -12 bp did not have a significantly reduced effect in restoring FluPol activity when compared to cells transfected with WT ANP32A or ANP32B in the DKO cell lines, and neither did it have an inhibitory effect for FluPol activity in the monogenic AKO or BKO cells. With the avian strain 50-92, all the ANP32 cDNA constructs restored FluPol activity by at least 10-fold (Figure 5-14CD). In 50-92 there also was no prohibitive effect observed with the ANP32A -12 bp construct. These results do not implicate the -12bp ANP32A allele as capable of impeding polymerase function or having role of steric hinderance in the WT or monogenic CRISPR-edited cells.

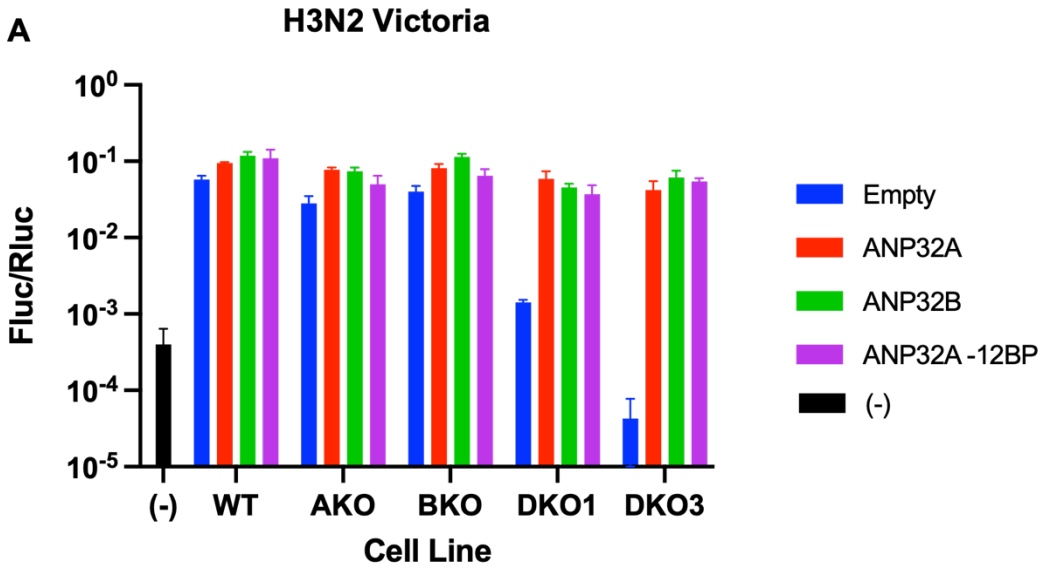


Figure 5-13: Minigenome assay in WT and ANP32 CRISPR-edited NPTr's using H3N2 Victoria FluPol minigenome constructs with restored expression of either ANP32A, ANP32B or ANP32A -12 bp**.** Cell lines were transfected with an empty pCAGGs vector (blue), pCAGGs expressing ANP32A (red), pCAGGs expressing ANP32B (green) or pCAGGs expressing the ANP32A -12 bp allele. Firefly luciferase (Fluc) expression was normalised to *Renilla* (Rluc). The (-) sample had no PB2 construct transfected to measure the minimal Fluc/Rluc ratio.

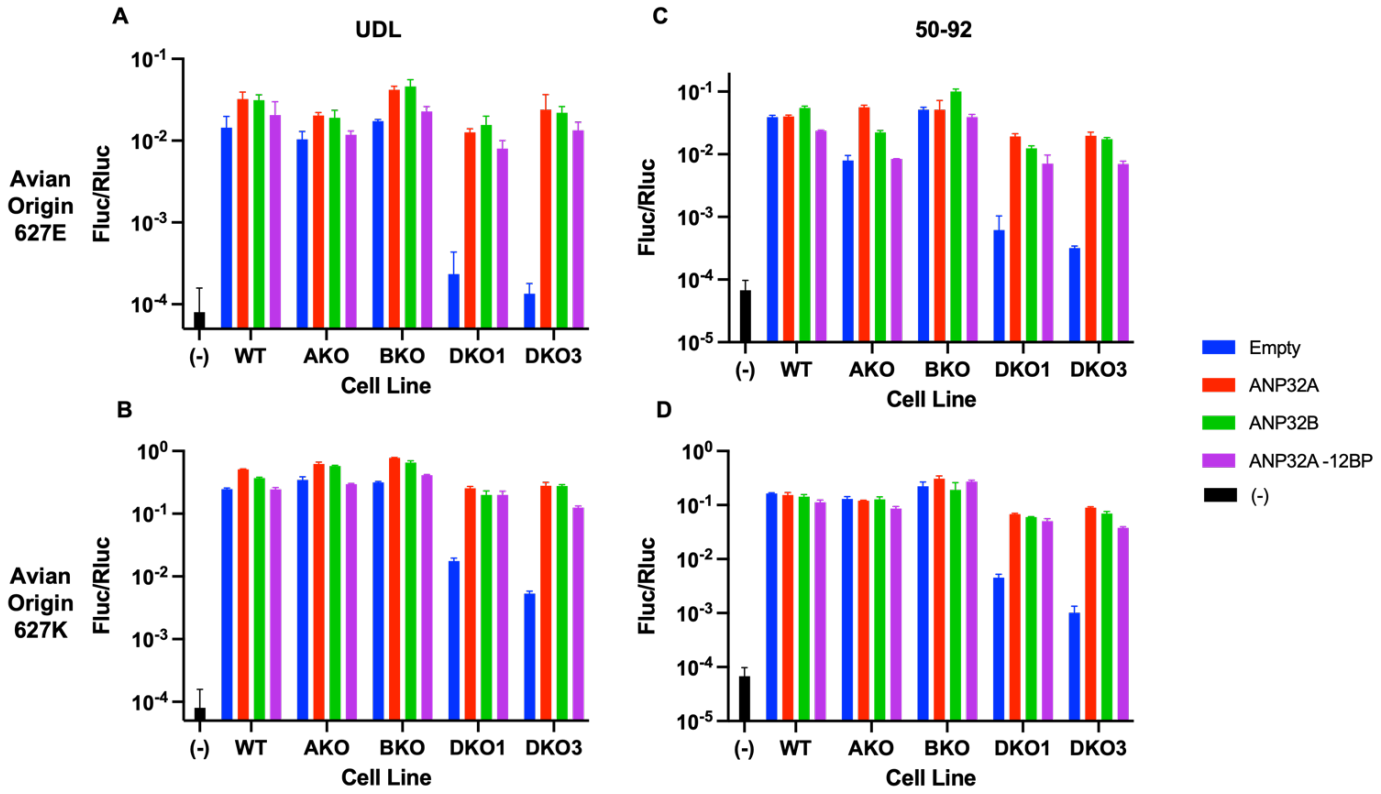


Figure 5-14: Minigenome assays performed in WT and ANP32 CRISPR-edited NPTr's using avian-origin IAVs carrying 627E or 627K in PB2 with restored expression of either ANP32A, ANP32B or ANP32A -12 bp. A-D) NPTr's were transfected with an empty pCAGGs vector (blue), pCAGGs expressing ANP32A (red), pCAGGs expressing ANP32B (green) or pCAGGs expressing the ANP32A -12 bp allele. Firefly luciferase (Fluc) expression was normalised to *Renilla* (Rluc) PB2 627E is an amino acid that is widely associated as an avian adaptive signature, whilst PB2 627K is a mammalian adaptive signature. A) FluPol activity for UDL with PB2 627E. B) FluPol activity for UDL with PB2 627K. C) FluPol activity for 50-92 with PB2 627E. D) FluPol activity for 50-92 with PB2 627K. The (-) sample had no PB2 construct transfected to measure the minimal Fluc/Rluc ratio.

5.3 Discussion

The experimental data that was collated in this chapter investigated if and how the removal of ANP32 proteins in NPTr's affects IAV propagation. The concomitant absence of functional ANP32A and ANP32B proteins reduced the ability of the selected human, swine and avian IAV strains to replicate in the *in vitro* swine tracheal model. The reduction in IAV's replication capacity was observed to occur after importation of the vRNPs into the host nuclei, and that the efficiency of FluPol activity was reduced in the absence of ANP32A and ANP32B together. These results agree with our original hypothesis based on available literature (Long et al., 2019a, 2016; Peacock et al., 2020b; Staller et al., 2019; Zhang et al., 2020, 2019) that IAV with avian, swine or human-origin could recruit swine ANP32A or ANP32B to improve the efficiency of FluPol, and thereby improve IAV replication dynamics.

5.3.1 Preliminary Infection Assays

The preliminary assessment of viral infections in the monogenic and digenic ANP32 knockout cell lines was performed by qualitatively assessing CPE following infection with the PR8 lab strain. In WT NPTr, infection with PR8 caused significant CPE. The CPE seen in IAV-infected cells is associated with the induction of programmed cell death (PCD) via the apoptotic pathway (Atkin-Smith et al., 2018). By visual assessment of the infected cultures, the phenotype of the CRISPR-edited NPTr cells was a recapitulation of the IAV titre data generated in the human *in* vitro models, in that monogenic knockouts had no resistance to viral propagation, but that digenic ANP32 knockouts conferred a level of resistance to infection, at least with infection of the H1N1 PR8 strain (Staller et al., 2019). The use of WT PR8 in the swine context for ANP32 gene-edited cells was a novel finding, and this was the first indication that functionally knocking out ANP32A and ANP32B in NPTr would align with the published human and swine data for IAV infections (Long et al., 2019a; Zhang et al., 2020, 2019).

An immediately distinct phenotype by qualitative comparison at the 24 hour time point that was not anticipated was the DKO3 cells displaying a lower

level of CPE compared to DKO1 cells, which had accumulated cell debris at a greater rate. It wasn't until the 48-hour time point that DKO3 had a similar amount of cell debris as DKO1 did at 24 hours. This delayed CPE observation flagged the ANP32A -12bp containing DKO3 cell line as being less supportive of IAV replication, and so at this stage it was decided to be continued as an *in vitro* model of interest for the further downstream analysis.

5.3.2 The IAV Strains Under Investigation

Because of the antigenic heterogeneity of IAV, its widespread ecology and the role of pigs as a mixing vessel, particularly in an agricultural scenario, it was important for this research to include IAVs originating from humans, pigs, and avian species (specifically from Galliformes (turkeys and chickens)). IAV subtypes are categorised according to their surface proteins, denoted by the HxNx nomenclature system. Beyond being used for naming, HA and NA have a significant effect on IAV affinity for different hosts (Cauldwell et al., 2014; Long et al., 2019b). HA are the critical interacting IAV protein with the host cell for entry, and as the determinants of cell entry, they are critical determinants of species specificity and therefore have a large bearing on viral evolution. The FluPol proteins do not define what cell types virions are capable of infecting, but they do evolve and become adapted to the molecular context into which they are introduced. Divergence in FluPol's arises through the natural drift that is introduced during the error-prone process of viral genome replication. Variability in the peptide sequence of FluPol constituents affects the biochemical interactions they have with other proteins, and one of these critical interactions is with ANP32's. We selected IAVs with different species origins to observe potential differences in their ability to recruit swine ANP32's to gain insight into the role that ANP32's may have in promoting swine as a species that support polymerase activity from multiple species-origins. The fact that IAVs from multiple species-origins were found to be capable of replicating in NPTr's highlights the role of swine as a mixing vessel where genome reassortment could occur.

PR8 is a well-established lab strain that has been used throughout human and avian IAV-ANP32 protein related research and was therefore a relevant benchmark for comparisons. The avian strains, UDL and 50-92, have also been widely used in IAV ANP32 related research, but these avian strains had not yet been investigated in a swine ANP32 DKO model (Zhang et al., 2020, 2019). The use of a swine H3N2 for viral infections and measurements was a novel inclusion for ANP32-related research, but given that H3N2 is a major global subtype in swine (Detmer et al., 2012; Simon et al., 2014), knowledge about how ANP32 proteins affect swine H3N2 viruses could be particularly relevant when considering any potential future role of gene-edited ANP32 pigs on-farm.

The avian strains used for infectious virus assays, UDL and 50-92 contain the HA, NA, and M segments from the lab adapted PR8 strain as a safety measure for attenuating virulence (Long et al., 2016; Staller et al., 2021). As we hypothesised that ANP32 proteins were specifically FluPol activity associated, it was not essential to use the avian viruses without reassorted PR8 segments that requires a higher Biological Safety Level. However, the use of the reassorted avian viruses carrying mammalian HA and NA will have altered replication capacity compared to the WT viruses. Interestingly, the replication of avian IAVs in human respiratory tract epithelial cells, which are in a 33°C environment, is compromised (Hatta et al., 2007; Scull et al., 2009). Avian IAVs replicate most efficiently at 37°C, and this temperature of incubation favours FluPol with PB2 627E. The fact that NPTr were cultured at 37°C may therefore be affecting FluPol activity of the mammalian strains whilst promoting avian FluPol activity more than in swine cells at 33°C. Incubation at lower temperatures would have possibly resulted in improved propagation of IAVs with PB2 627K and discriminated more the ability of mammalian-origin strains to replicate in a mammalian context. It is recommended that future studies consider this temperature effect when investigating polymerase activity and ANP32-IAV dynamics when using IAV that originate from both avian and mammalian species.

5.3.3 Plaque Assays

Plaque assays were adapted for use in animal virology in 1953, and have since provided a gold standard for assaying the presence of infectious virions (Dulbecco and Vogt, 1953). Our data shows that double ANP32 KO cell lines have incomplete resistance to IAV infection. For all cell lines infected, aside from DKO3, the infection curves mostly observed growth from 12-24 hours, followed by a plateau in the number of infectious virions aspirated. Titres were then generally observed to reduce after the 36-hour time point in a similar manner to that observed by Peacock et al., 2020. The DKO1 and DKO2 cell lines were generally observed to follow a similar growth trajectory, but either at lower titres or with a delayed growth curve. The DKO3 cell lines showed no increase in the virus number beyond the number initially inoculated in the culture flask, and at 48-hours, all apart from 50-92 at an MOI of 0.01 were reduced in titre when compared to the 12 hour time point.

At the 12 and 24 hour time points, the presence of infectious virions present in the supernatant of DKO NPTr's had greater differences to WT NPTr than after 36 or 48 hours. The closing of this gap at later time points could indicate IAV mutating to evade the resistance mechanism. To test if the ANP32 null phenotype was evaded, we used the supernatant of potentially mutagenic virions for subsequent infections in WT and DKO NPTr's, however these experiments unfortunately did not result in successful plaques.

Throughout the available published literature with viral titration data in ANP32 knockout cell lines, there are discrepancies between the experimental time points presented. Given that the data in this thesis shows that titred infectious virions from infected cultures become more similar between the WT and DKO1/DKO2 cell lines after 48 hours, the fact that some literature is concluding experiments after 24 or 36 hours affects interpretation between results (Long et al., 2019a, 2016; Park et al., 2021). Visually, after 48 hours most cells in the DKO1/DKO2 cultures were observed to have significant CPE, and it is recommended that future research conducts infection assays through to at least 48 hours to record whether the phenotype is delayed or the resistance is persistent. Because of variation in time points, and some data being presented

as a bar plot from a single time point of 24 hours (Park et al., 2021), care in comparing data between literature must be taken.

As well as time point being important for valid comparisons, the MOI appears to have a bearing on ANP32-associated resistance and should also be considered in experimental planning and when analysing data. The selection of different MOIs and allowing multiple infection cycles affects the nature of the IAV infection established, as cellular metabolism and the level of the innate immune response mounted is distorted in a manner proportionate to viral load and exposure (Ramos et al., 2019; Westenius et al., 2014). Data from avian primordial germ cell derived fibroblast cells with loss of function ANP32A edits by Long *et al.*, 2019 and the avian-origin IAV 50-92 found that for their infection at an MOI of 0.0001, no viral growth was detected in the AKO cell line. However at an MOI of 1, there was measurable infectious virions with a delayed titre compared to WT cells in an experiment that concluded at 24 hours (Long et al., 2019a). The use of an MOI 100 times smaller than in our assays is significant, and perhaps the IAV replication kinetics data in NPTr may have altered, and our results could have better recapitulated this greater resistance effect seen if we had used a similarly small MOI (Long et al., 2019a; Staller et al., 2021). Viral infections with 50-92 from Peacock *et al.*, 2020 and Staller *et al.*, 2019 also used lower MOIs than selected for the data presented here, yet they observed a similar growth trajectory of infectious virions, with differences between WT and the DKO NPTr's being the greatest at 24 hours and reducing in difference as they progress to 48 hours.

As well as variation in time points and MOI affecting interpretation between datasets, the experiment used to determine viral growth is not always plaque assay, but instead can be measured as Tissue Culture Infectious Dose for 50% of infected cells (TCID50) (Reed and Muench, 1938; Zhang et al., 2020, 2019). This means that although the results can be informative of each other, they are not directly comparable. These publications using TCID50 (Zhang et al., 2020, 2019) also used IAVs of different origins to this thesis, which also must be considered when comparing data. Despite the use of 0.01 as an MOI, which was also used in our plaque assay research, different viral subtypes have different

replication kinetics in a context dependent manner. Their use of PK-15s may also affect the ability to compare infectious virion data, as the metabolism and immune response in a cell line originating from kidney tissue and is likely to differ to that which is of tracheal origin (Freeman et al., 2012).

The differences observed in infection dynamics across all publications raises questions regarding how these results should be interpreted when considering translation towards an animal model. and how relevant some *in vitro* parameters are when considering the biology of complex organisms that are infected in wild or agricultural ecosystems. In an *in vivo* system, the challenges of infection are different to those present in cell culture, and care must be taken in interpreting the available data when considering prospects of translation into an animal model.

Because ANP32 proteins have been implicated in catalysing vRNA synthesis from the cRNA intermediate (Sugiyama et al., 2015; Wei et al., 2019), collecting total RNA from the supernatant of these infected cells is an experiment that would have furthered our understanding of the role ANP32 proteins have in IAV genome replication. Comparing the presence of vRNA, cRNA and viral mRNAs from the supernatants collected from infected cell cultures (Kawakami et al., 2011; Staller et al., 2019; Zhang et al., 2020) is indicative of the stage of infection and would have provided insight into the specific role of swine ANP32's with IAV in NPTr.

5.3.4 NP Localisation Investigations

The qualitative analysis of NP localisation in the WT and ANP32 KO cell lines provides an insight into how the loss of ANP32A and/or ANP32B affects the IAV replication cycle in NPTr. From previous data published, and the results from the plaque assays and immunofluorescence data, we hypothesised that a reduced presence of NP would be seen as a result of lower FluPol activity in the absence of ANP32A or ANP32B. Although the imaging data was not quantified, qualitative assessment was supportive of observations across all other IAV data gathered in this project. The NP staining data is novel, as previous research investigating swine ANP32 proteins and IAV has not published NP

immunofluorescence data, and only a single example from the avian and human *in vitro* research in a haploid cell line is found (Staller et al., 2019).

The images from Staller *et al.*, 2019, are from a time point of 5 hours, at an MOI of 0.2. After 5 hours of infection, they observed that NP localisation remained restricted to the nucleus, suggesting that the amount of vRNPs which had been exported for virion production was below the detectable limit. In the immunofluorescence data presented here, the MOI used for the nucleoprotein staining was 1, and the infected cells were fixed after 10 hours. The 10-hour time point was selected on the basis of the IAV replicative cycle taking at least 4 hours (Frensing et al., 2016), but to observe modulated replication kinetics in the DKO cells, a longer replication cycle time should be allowed to discern differences in the scale of resistance. We also hypothesised that the longer timeframe would also provide data on the stage of infection that was disrupted, given that the full IAV replicative cycle would occur even if entry was delayed. The observations of the immunofluorescence data corroborated the plaque assays data, in that DKO3 cells impeded the propagation of human, swine and avian-origin viruses more than DKO1, and that DKO1 cells had diminished IAV replication efficient the viral NP protein beyond what was observed in WT NPTr's.

The investigation of lower MOIs and completion of the experiments at the 5- and 10-hour time points would have been beneficial in dissecting the immunofluorescence data further and would have allowed a better comparison between data in human and swine cell lines. Transfection of the cDNA expression constructs to rescue ANP32 knockout cells from defective IAV propagation has previously been valuable as supporting evidence (Carette et al., 2009; Han et al., 2018; B. Li et al., 2020), and evaluation of NP after ANP32 restoration would have supplemented the observations made here. Having optimised the ANP32A and ANP32B antibodies in NPTr against the swine peptides, co-staining of ANP32A and/or ANP32B with NP in infected samples could have shown when and where ANP32 proteins are interacting with FluPol in swine. This could also have been reliably done using the FLAG tagged cDNA expression constructs, and therefore insight into whether the antibody bound to

the ANP32A -12 bp isoform and any differences in its distribution could have been acquired.

5.3.5 Minigenome Assays

In order to experimentally assess the efficacy of FluPol activity we used minigenome assays, which have become the most widely used method for assaying the activity of IAV polymerases (Schaack and Mehle, 2020; te Velthuis et al., 2018) (Section 2.4.5). In a minigenome assay, the level of FluPol activity is assayed through measurement of a luciferase reporter gene expressed by an IAV primer, and therefore it provides a proxy for the level of vRNA synthesis. All polymerase expression constructs that were used were acquired from the Barclay Group at Imperial College, London (Peacock et al., 2020b). Their sharing of these constructs was gratuitous and ensured that for these assays there was some experimental consistency between this research and other prominent ANP32 research being performed (Long et al., 2019a, 2016; Peacock et al., 2020b). In the minigenome data presented in this thesis there was a lower Firefly:*Renilla* expression ratio in the DKO cell lines than in WT NPTr, AKO or BKO. These results support the infectious assay and immunofluorescence data generated, in that IAV infection in NPTr is impeded in the DKO cell lines.

The role of swine in the IAV ecosystem is critical due to their role as an intermediate species for zoonotic IAV transmissions and a mixing vessel for novel IAV generation (Nelson and Worobey, 2018; Yassine et al., 2011). Swine ANP32A has been implicated as a host factor that improves function of avian FluPol's more than ANP32A from other mammals due to the unique amino acid residues at site 106 and 156 (Zhang et al., 2020). For EA453,the swine-origin minigenome strain used here, the reduction in FluPol activity observed in AKO and DKO1 cell lines was similar, suggesting swine ANP32A is more potent in supporting EA453 polymerase activity than ANP32B. In both the human-origin strains used it was only in DKO cell lines that a lower level of FluPol activity occurred, implicating swine ANP32A and ANP32B as host factors exploited by these IAVs. The avian-origin strains carrying the avian signature PB2 627E had lower firefly:*Renilla* ratios, and thus FluPol activity, in comparison to those with

the PB2 627K residue. This highlights the role that PB2 627 can have as a mammalian adaptive signature. The pilot data of canine-origin minigenome constructs in a swine *in vitro* model suggests that this IAV strain can recruit either swine ANP32A or ANP32B to enhance FluPol activity. Given that this is an H3N2 canine-origin virus, even though not an entire virus, the functioning polymerase in NPTr and presence of surface proteins that are known to infect swine means that there may be limited species barriers in this strain transmitting from a dog into a pig. This canine-origin virus has been used to create the first published example of a live-attenuated IAV vaccine available for dogs (Rodriguez et al., 2017).

Although the pattern of results between the minigenome assays shows a consistent effect, gaps remain in the data presented. Each set of data plotted is from technical replicates from a single biological replicate. A second repeat was performed and the same pattern of luciferase expression was observed, however because of a large variance in the raw data from the same samples, data representative from a single experiment is presented. This is the same format of presenting data as in Staller *et al*, 2019 (Staller et al., 2019), however it is acknowledged that more biological repeats are required before a complete analysis of the data can be performed.

Further regarding the presentation of data, the FluPol activity plots presented in this thesis have a *y*-axis with a log scale (Zhang et al., 2020, 2019), whereas much of the published literature uses a linear *y*-axis for presenting minigenome assay data (Long et al., 2019a; Peacock et al., 2020b; Staller et al., 2021, 2019). The use of a log axis allows discrimination between the luciferase detection in the negative controls and in the DKO cell lines, which was difficult to observe using a linear *y*-axis. Being able to discern that the DKO cell lines had firefly luciferase activity beyond what was detected in the negative control, aside from with the H3N2 Victoria constructs, is an important observation. This presentation of the minigenome data illustrates a connection between the infection assays and immunofluorescence data, where there was still replication of influenza virions without the presence of swine ANP32A or ANP32B. It therefore appears that the role of ANP32 host factors have a significant effect

on the efficiency of polymerase activity, however they are not essential catalysts for FluPol activity, and without their presence there is still background activity (Domingues and Hale, 2017).

The rescue of function experiments with ANP32A and ANP32B cDNA constructs were performed to support the data by observing whether reintroduction of ANP32A or ANP32B directly affected FluPol activity. The reliability of the data showing FluPol activity was reconstituted in the DKO cell lines would have been improved with Western blots as a way of quantifying the presence of ANP32 and FluPol proteins to confirm that differences in transfection or translation efficiency between the samples was not the cause of FluPol activity variance. The issue of the potential variable expression of transfected plasmids is partially negated by the decision to transfect 100 ng of the cDNA constructs into each well, as the effect of ANP32 proteins for FluPol activity is dosage dependent and a saturation effect has been observed in human ANP32A-IAV experiments when over 10 ng is transfected (Peacock et al., 2020a). Accounting for the fact that these experiments by Peacock et al., 2020 were performed in 24-well and not the 12-well plates used in this study, we were still beyond the saturation threshold they observed. Considering this saturation effect, performing a dilution series and using lower concentrations of the ANP32A -12bp cDNA construct may have provided some insight into how it affects the potency of swine ANP32 proteins in the swine context. It also would have been beneficial to perform Western blots on samples transfected with the ANP32A -12 bp allele to test whether the ANP32A antibody used was able to detect this peptide lacking 4 amino acids.

The reliance on plasmid transfections for IAV polymerase activity creates a bias in that the data generated can only arise from cells that are amenable to transfection and those that do not have an excessive cytopathic response to exogenous dsDNA. This subset of cells could have specific metabolic characteristics that affect FluPol activity. Furthermore, the translation of the dsDNA vector introduces novel peptides, which are also IAV proteins that could trigger an innate immune response (Delgado-Ortega et al., 2014; Schmidt et al., 2019). During IAV infection, there is an induction of host cell shut-off (Levene

and Gaglia, 2018) and alterations to host transcription and translation are caused by molecular manipulation by IAV proteins (Rodriguez-Frandsen et al., 2015). Therefore, introducing constructs that rely on host transcription functioning canonically, whilst also introducing constructs that will express factors that are known to disrupt host transcription, the assay may be affected by the negative feedback from IAV proteins being present. However, with increases in polymerase activity observed between the heterotrimeric transfected cells and those lacking the PB2 construct (Figure 2-6), and consistency between our minigenome data and published literature, there is confidence that the assays were sensitive enough to detect true differences in polymerase activity and not just stochastic effects.

Variation in the data may have also been introduced due to the protocol in practice. The methodology used in the minigenome assays was performed by the addition of *Renilla* and firefly detection reagents manually with a multichannel pipette. The need to remove the plate from the plate reader to add the secondary detection buffer introduced the potential for human error to affect the results. The use of an automated system may have improved the accuracy and consistency of the data output. However, the results presented are comparable in raw terms to other minigenome data published using these particular viral constructs (Long et al., 2019a; Peacock et al., 2020b; Zhang et al., 2019).

5.3.6 The Curious Case of DKO3

Across all the experimental data generated, the DKO3 cell line had a higher level of phenotypic resistance to infection from all of the IAV strains tested. Understanding whether this is a direct result of the ANP32A -12 bp allele, and how this could confer phenotypic differences in resistance, or whether it is an artefact of underlying biology from that particular clonal cell line is an issue that remains unresolved.

In the process of clonally isolating cells, any genetic alteration that has spontaneously arisen in the isolated cell becomes fixed. It is therefore possible that an unidentified genetic alteration is conferring IAV resistance that is

additive to the ANP32-related resistance, but through an alternative mechanism. The experimental design of performing a multiplexed transfection as opposed to the more common CRISPR knockout pipelines of sequentially targeting genes was chosen to avoid two rounds of selection where *de novo* mutations could become fixed. Full transcriptomics of the DKO1 and DKO3 cell lines would have provided a better understanding of any critical differences between them that may have been present. Although performing genome-wide transcription analyses on the knockout cell lines would have also helped in understanding how the loss of ANP32 proteins affects gene expression networks, undertaking RNA-seq was beyond the scope of this project.

Although there was no protein detected by Western blotting in DKO3, and qPCR data shows ANP32A and ANP32B transcription in DKO3 is reduced similarly to the other DKO cell lines, the detection capacity of Western blotting by chemiluminescence could have meant that low-level translation may have been occurring at an unobservable but still biologically pertinent level. Whether the protein translated would remain functional is unknown, however the fact that the ANP32A LRR domain is highly structured means that small deletions could lead to global unfolding of this domain (Domingues & Hale 2017). This premise is supported by the output prediction of the Robetta software for the -12 bp ANP32A peptide sequence that shows a loss of structural integrity. In the instance of reduced and not ablated translation, a low abundance ANP32A protein lacking 4 amino acids could have retained the capacity to interact with FluPol through the LCAR amino acids (Domingues and Hale, 2017; Wei et al., 2019). If this were the case, its association with the FluPol complex could have resulted in a form of steric hinderance. Further experiments would be required to gain insight into whether with the ANP32A -12bp allele a stable interaction is maintained through the LCAR and the presence of a mutated LRR region affected the level of FluPol activity.

Amongst further recommended research in to the -12 bp ANP32A allele present in DKO3, and for that matter any modified ANP32 protein, it is important to investigate how the presence of the modified ANP32 protein affects its functions and interactions with host proteins in the absence of any

infection. Given that ANP32A is implicated in apoptotic regulation, gene expression and tumour suppression, dysregulation of its function could exacerbate or prevent its natural function, resulting in undesirable biological or metabolic outcomes.

5.3.7 Conclusions

The data collected in this chapter supports our hypothesis that both ANP32A and ANP32B can be recruited by FluPol to improve the efficiency of its activity in swine. When both genes are concurrently knocked out, the *in vitro* model had an increased tolerance of infection with IAV. However, the CRISPR-edited NPTr were not completely refractory to IAV replication and the level of resistance varied by IAV strain and infectious dose. In accordance with other published data, swine ANP32A and ANP32B are important factors in improving the efficiency of FluPol activity, but they are not essential for viral propagation or polymerase activity in swine. By selecting an *in vitro* model that is specific to the site of IAV infection in swine, these results also provide further insights into the role swine have as a mixing vessel for IAV through supporting replication of swine, avian and human adapted viruses.

6 Discussion

IAV is a disease of significant concern from a One Health perspective. It can be a highly pathogenic disease in humans, livestock and wild animals that negatively affects economic activity and impacts the well-being of humans and other susceptible species. Between humans and farmed animals there are close interactions. The regularity and closeness of the interactions has created an interface which represents a significant opportunity for bidirectional zoonotic transmission to occur. Through research we can improve the understanding of zoonotic transmissions and aim to create barriers to IAV host-jumping which could be critical in preventing novel IAVs becoming endemic or instigating a novel pandemic.

The original aims of this project were to make preliminary investigations into whether gene-editing could be a viable method of creating pigs with resistance to IAV. In Chapter 3, we created a simulation model that observed how alleles flow through a commercially styled pig breeding system. These modelling results provided conceptual data that gene-editing can be a viable method for creating IAV resistant swine herds (with alleles that confer full resistance and in the absence of escape mutations), whilst it also highlighted that without improving gene-editing parameters in zygotes, the implementation of gene-editing will require significant improvements in effectiveness to be efficient. Subsequently, in Chapter 4 we created a swine *in vitro* model to investigate IAV resistance. In a cell line originating from the *in vivo* infection site in swine, CRISPR gene-editing reagents were used to disrupt two genes that encode for host factors which were hypothesised to be recruited by IAV in swine. In Chapter 5, these gene-edited cell lines were assayed to determine whether resistance to IAV infection had been introduced. In the cell lines in which the two target genes, ANP32A and ANP32B, were disrupted together, IAV from swine, human and avian origins were impeded in their capacity to replicate. This corroborates data previously observed in swine and confirms our original hypothesis that swine ANP32A and ANP32B have a functionally redundant role in supporting IAV polymerase function for viral strains from multiple species.

Improving our knowledge surrounding the relationship between IAV and proteins from the ANP32 family in porcine cells presents multiple potential opportunities for improving IAV control strategies in swine. The first control strategy arises from direct resistance to IAV by modifying swine ANP32 genes so that the proteins cannot be recruited by FluPol, or that they will have a dominant-negative effect that prevents viral replication. Secondly, a deeper understanding of ANP32 proteins and their relationship with ANP32 proteins could lead to the development of antiviral drugs that target host proteins as opposed to traditional antivirals that target viral protein function. Thirdly, knowledge of how ANP32 proteins affect zoonotic transmission of specific IAV strains could help in understanding IAV evolution, and through insight into the evolution of IAV, we could create better opportunities to accurately control particular infections of concern. Outside of direct pathogen control strategies, understanding how ANP32 proteins interact with IAV is particularly interesting because their evolutionary conservation may provide insight into how IAV jumps species barriers and replicates its genome in different physiological contexts.

6.1 Resistance, Resilience or Tolerance?

When considering the challenge that pigs face from pathogens, there are important differences in how gene-editing could affect the relationship between the pathogen and host, and how this may affect clinical presentation, transmission, and the economic impacts associated with disease. The three ways in which gene-editing can affect the pathogen-host relationship to reduce the effects of disease can be categorised as tolerance, resilience or resistance (Bai and Plastow, 2022). Depending on the pathogen, the gene target and the molecular mechanism affected, each outcome could have its place in the arsenal for fighting against disease in livestock.

As described here, tolerance of a pathogen is the ability to maintain close to normal physiological function whilst hosting a pathogen (Knap and Doeschl-Wilson, 2020; Richardson, 2016). An example of disease tolerance relevant to gene-editing in swine is found in the status of warthogs that are infected with African swine fever virus (ASFV). Infections do not cause notable

disease in warthogs, however when ASFV spreads from warthogs to domesticated pigs, it causes a severe and often lethal disease (Anderson et al., 1998). Through gene-editing with ZFN's, Lillico et al., 2016 introduced the warthog haplotype of 3 amino acids substitutions in an immune modulatory gene (NF-kB) that had been associated with disease tolerance by Palgrave et al., 2011, into domesticated swine. The results were pioneering in that it was one of the first examples of genome-editing with endonucleases in zygotes, however, the gene-edited pigs did not show tolerance or resilience against ASFV infection, and therefore this haplotype is not a significant contributor to disease outcome in the genetic context of *Sus scrofa*. Because ASFV has not been identified to infect non-swine species and only causes disease in *Sus scrofa*, the presence of ASFV resistant pigs is relatively unlikely to drastically affect the ecology of ASFV, and tolerance against disease alongside an increase in the prevalence of the infectious agent would have limited impacts.

For IAV, a consequence of creating a gene-edited pigs that are tolerant to infection is that they could become an endemic reservoir for more IAV strains, in the same way as wild waterfowl currently are (Webster et al., 1992). If the productivity or fertility of pigs is not reduced by swIAV infection, the economic incentive for producers to vaccinate is lost. A higher prevalence of swIAV could arise, and this could potentially have knock on effects for the whole IAV ecosystem. If it became widespread that pigs could carry a higher than current viral load, the opportunity for antigenic drift and antigenic shift to occur is increased, and this could create a situation where IAV could be more likely to jump hosts. For ASFV, there are arguments to be made that conferring tolerance to domesticated pigs, as seen in warthogs, would be of significant value because there are a very limited number of other species that would be affected by the increase in virus prevalence. However, for IAV related gene-editing in swine, virus tolerance is likely to be a bad outcome for the IAV ecosystem.

In the context discussed here, resilience to disease refers to the ability of a host animal to limit pathogen load and disease, but not to entirely prevent infection (Doeschl-Wilson et al., 2021; Richardson, 2016). Within a herd or species, resilience to a pathogen is naturally variable, and in a herd of pigs with

endemic swIAV, individual pigs will display variable resilience (Wilkinson et al., 2015), in the same way that is observed across a human population (Clohisey and Baillie, 2019). Many individuals will be sub-clinically affected, reducing productivity but not displaying disease symptoms. These animals still retain their status as a disease carrier, and probably the ability to transmit the pathogen to a new host animal. Understanding or identifying the genetic variants that affect resilience means that it is a trait which could be selected for in animal breeding programs or introduced through gene-editing as a disease control method (Bai and Plastow, 2022).

The data presented in this thesis showing NPTr resilience to infection does not mean that ANP32A and ANP32B should be disregarded as putative gene targets for reducing IAV burden in swine. A mammalian candidate genetic polymorphism that confers resilience through ANP32 proteins has been identified in human ANP32B by Staller *et al.*, 2021, where a single nucleotide variant was shown to affect virus propagation. In the human population, it is postulated that this variant could improve resilience to IAV infections through a dominant-negative impact on FluPol activity. Because individuals can retain the ability to be a host, the issue that they could act as a reservoir that promotes viral evolution or escape from the molecular mechanism of resilience makes it an unattractive outcome if adaptation towards human or avian-origin IAVs could occur.

The third outcome possible from gene-editing is full resistance to viral infection, which was observed with the pigs gene-edited for the CD163 receptor which confers PRRSV resistance (Burkard et al., 2018, 2017). Full resistance is the most desirable result when applying gene-editing in livestock when the goal is to remove the burden and threat a pathogen represents. Pigs that cannot be infected with IAV would not have to face the burden that disease incurs, and will therefore have improved standards of welfare, better productivity, and they cannot not be a reservoir for onwards transmission to their peers, humans, or wildlife. Using gene-editing methods, genetic variants that have been identified through phylogenetic, evolutionary and functional research that could provide full resistance can be tested. However, from the

natural genetic composition of animals, it is uncommon to find variation that confers full resistance (Doeschl-Wilson et al., 2021). This means that for full swIAV resistance in pigs, an allele that is not yet identified in nature or by research is the most likely option for introducing complete IAV resistance. These considerations are broadly similar for all viral pathogens causing disease in livestock and posing a zoonotic threat.

6.2 The Knockout Effect

Functionally knocking out ANP32A and ANP32B affects the propagation of the selected swine, human and avian-origin IAVs tested, but the effect was not consistent between IAV strains, MOIs and timepoints. The reduced viral growth kinetics in the DKO1 and DKO2 cell lines was not sustained over 48 hours. For DKO3, there was no increase the number of infectious virions from what was initially inoculated. We did not identify whether the virions collected from the supernatant were the originally inoculated virions that did not enter cells successfully, or whether these were novel viruses generated. The question of what is causing the difference between the DKO3 and DKO1/2 cell lines ability to support IAV replication remains. The enhanced resistance phenotype observed in DKO3 could be because DKO3 is a genuine KO, whilst the other two cell lines are not. It could be due to the expression of ANP32A-12bp at levels below the detection threshold, or it could be due to an off-target effect that is as yet unidentified. Whatever the underlying reason, even DKO3 does not appear to completely ablate the viral replicative cycle and there is a low level of viral replication that persists. In the absence of ANP32A and ANP32B, mutations in FluPol could occur that allow it to recruit swine ANP32E to function in the same role for enhancing the efficiency of vRNA synthesis. Swine ANP32E is 70.5% and 67% homologous in peptide sequence to swine ANP32A and swine ANP32B, respectively (NCBI; BLAST). In swine proteins ANP32E and ANP32A, the amino acid residues at site 106, 130 and 156, which have been associated as critical in swine ANP32A recruitment by avian-origin FluPol's, the amino acids residues are conserved, and it is only at site 129 that ANP32E has a glutamic acid (E) instead of an asparagine (N). Therefore, adaptation by FluPol to support an

electrostatic interaction with ANP32E despite the acidic residue at site 129 could allow a functional interaction to occur. In the absence of mutations in constituents of FluPol, the electrostatic interactions between ANP32E and FluPol may result in a less effective but still possible catalysis of FluPol RNA synthesis, hence the lack of its recruitment in nature when selective pressure originates from recruiting avian ANP32A proteins.

Going forwards in this research, understanding the role of ANP32E in the absence of ANP32A and ANP32B will be important. Creation of a swine triple knockout (TKO) cell line that lacks ANP32A, ANP32B and ANP32E would provide insight into the role of ANP32E with FluPol activity. A TKO cell line has been created in 293T cells (Zhang et al., 2020b). Here, it was observed that the TKO had no reduction in polymerase activity beyond what was seen with the DKO cells, using constructs from an avian-origin IAV. When assaying an IBV, the TKO cell line did have less polymerase activity than the DKO cells, and the rescue of polymerase activity with human ANP32E was only partially restored as compared to using human ANP32A or ANP32B. The use of a swine ANP32E plasmid in rescue assays in this thesis may have provided insights into the role of swine ANP32E in the swine context with IAV from a range of hosts. With only one viral titration, which used IBV, in this publication (Zhang et al., 2020b), there is an over reliance upon minigenome assays to understand how viral propagation is affected by the absence of ANP32 proteins. Comparisons of the replication dynamics for different viral strains in the DKO versus TKO cell lines would help in understanding viral adaptation, and identifying mutations that are acquired within the IAV genome. In the journey towards considering the practical implementation of gene-editing for swIAV resistance, further investigations of how resistance could be evaded will be crucial.

6.3 Genome-editing in Pigs For IAV Resistance – How far to Reality?

The integration of biological technologies like artificial breeding with molecular techniques such as gene-editing and genomics could provide a platform for a revolution in animal breeding (McFarlane et al., 2019). Disease management in livestock should capitalise on the availability of novel

technology to improve animal welfare and for potential zoonotic diseases, to limit the threat posed to humans. There are already multiple instances of genome-editing of porcine zygotes being performed using CRISPR reagents that were initially tested *in vitro* (Burkard et al., 2018; Hai et al., 2014; Park et al., 2017; Tanihara et al., 2018, 2016; Whitworth et al., 2019, 2017). When this project began, it was within the realms of possibility that if the hypotheses were correct and specific amino acid substitutions in ANP32A and ANP32B were confirmed in the *in vitro* model to provide resistance to swIAV, attempts at *in vivo* application of the CRISPR reagents could have been made. Although this stage of the project was not achieved, the prospects of introducing IAV resistance into swine through genetics is not dead.

When looking for potential ANP32 alleles or variants that could be considered for integration into traditional breeding programs, in *Sscrofa11.1* there are no naturally occurring variants within the 5th LRR domain (the region containing amino acids 129 and 130 and other FluPol interacting residues). Identification of naturally occurring variants would mean that concerns from regulatory bodies of introducing novel variants with unknown consequences could be avoided. However, experimental clarification and a mechanistic understanding of any variants identified that affect FluPol activity, in a similar manner to the experiments performed by Staller et al., 2021, would be beneficial for insight into how IAV evolution may be affected in the presence of this allele. Selection of a naturally existing resistance allele could be performed by conventional breeding. Genome and transcriptome sequencing of more pigs may lead to the discovery of relevant genetic variation, however it is unlikely that animals carrying genetic variants conferring resilience would be from the elite brood stock, meaning their integration into the breeding herd would be likely to incur a significant regression of genetic gains in other traits in return for more marginal benefits to animal health from reduced IAV burden.

With ANP32 proteins established as gene targets that could influence susceptibility to IAV in chickens and swine (Long et al., 2019, 2016; Zhang et al., 2020a, 2019), it remains that the *in vitro* data needs to be translated into *in vivo* research before a true understanding of their potential as resistance alleles for

livestock can be reached. Given the perinatal lethal effect observed in digenic ANP32A and ANP32B mice (Reilly et al., 2011), the level of genetic conservation in ANP32A and ANP32B in mammals, and the diverse functions of ANP32 proteins, creation of pigs from the data presented here would be a brash step from an ethical perspective. To support any potential translation of ANP32 gene-editing into swine, it is essential that specific amino acid substitutions could be made that do not notably affect normal host molecular activity. Given the role of ANP32 proteins in regulating gene expression through histone acetylation, assessing transcription by RNA-seq *in vitro* before translation into an animal model would need to be undertaken.

There are several sites in swine ANP32A and ANP32B that have been identified to affect polymerase activity in *in vitro* swine models (ANP32A residues 106, 129, 130 and 156; ANP32B residues 129, 130) (Zhang et al., 2020; Zhang et al., 2019). Using traditional CRISPR-based HDR to introduce these changes would have led this project further towards translational data. Attempts at achieving HDR in NPTr were made using single stranded oligonucleotide templates, however no introduction of the DNA template into the NPTr genome was detected. Given that NPTr are conspicuously absent in HDR literature, and HDR rates are known to vary considerably between cell types (Liu et al., 2019; Ran et al., 2013), the decision to use NPTr's because of their IAV relevance as a cell line may have negatively affected our ability to generate site specific substitution mutants by HDR.

Because resilience to infection and not complete resistance was observed in the IAV infection data in this thesis, it is possible that for ANP32 proteins to be deployed as IAV resistance targets in swine, they should be investigated alongside other gene targets that confer resilience to infection, to observe whether cumulative effects of resilience can effectively confer total resistance in a host. Using a multiplexed gene-editing approach would reduce the likelihood of IAV evading the molecular mechanism of resistance or resilience. For a multiplexed approach to be possible, other targets must be identified. One potential target in mammals is importin-α7. Alongside a weight of evidence implicating PB2 627 in mammalian adaptation for enhanced

polymerase activity, there is also data that shows it has a role in affecting nuclear importation of vRNPs. Avian IAVs switch in bias from importin-α3 to importin-α7 during mammalian adaptation (Gabriel et al., 2011). In human *in vitro* models, silencing of importin-α7 reduced the polymerase activity of mammalian adapted polymerases (Gabriel et al., 2011; Hudjetz and Gabriel, 2012). Additionally, using mice as an animal model, knockout of functional importin-α7 resulted in less susceptibility to mammalian adapted (627K) but not avian (627E) influenza virus infection. Although our ability to concurrently gene-edit multiple targets in a single step *in vivo* remains limited, a multiplexed approach to genome-editing for improving traits of livestock seems an inevitability as CRISPR matures as a methodology. There are considerable improvements to be made in CRISPR efficiency for site specific substitution to be achieved before this will become a reality, but progress is being made (Anzalone et al., 2020).

6.4 Where Would This Project Continue

The facets of this project that were not achieved and would have been pursued as the next steps relate to the *in vitro* model used (NPTr's), and to the CRISPR reagents that were optimised for use. In the situation of continuing this research on, the intention would have been to use alternative CRISPR-based approaches to achieve HDR in NPTr, to test whether other genome-editing tools such as ZFNs or TALENs would have been a better alternative for ANP32 target sites for HDR, and to use different *in vitro* models that were more representative of pig respiratory tracts that would have potentially been more amenable to successful HDR.

To improve the prospects of creating an amino acid substitution model in NPTr's, modified Cas9 proteins were considered for use. A wide range of modified Cas9 proteins have been developed with the intention of increasing the efficiency of the desired highly specific DNA sequence edits (Liu et al., 2019). Cas9 proteins that support direct interaction with the DNA repair template to improve its proximity to the DSB where they have homology, such as PCV-Cas9 (Aird et al., 2018) or streptavidin-Cas9 (Pineault et al., 2019) could be tested in

future attempts to improve NPTr HDR rates. Base editors were also considered (Gaudelli et al., 2017), however the PAM sites in proximity to the target loci were not conducive to base editing of amino acids 129 or 130. An innovative method that arose in 2019 that purportedly improved the efficiency of making specific genome edits, called prime-editing, was developed (Anzalone et al., 2019). Prime-editing utilises a Cas9 nickase, which cuts a single strand of DNA, fused with a RT. The guide RNA has an extended tail that functions as a template and primer for the RT to create a DNA copy that can be integrated at the nicked DNA region by homologous base pairing. Reagents for prime editing were designed and preliminarily tested, however there were no edits in DNA sequence observed in NPTr when targeting either ANP32A or ANP32B.

Outside the modified Cas9 approaches, there are opportunities for optimisation to be considered through the format that CRISPR reagents are delivered to cells. Due to the ease of development and modification, plasmid based dsDNA was predominantly used in this thesis. A single plasmid vector can express both the Cas protein and guide RNA, and these can be readily modified with new gRNA sequences using basic molecular biology equipment and techniques. We could have delivered CRISPR reagents as RNA or protein instead of DNA. Using mRNA or Cas9 protein removes the transcription/translation steps and the need to introduce a plasmid vector into cells. For RNA delivery, the sgRNA and Cas9 mRNA can be transcribed using T7 *in vitro* transcription (IVT) methods or commercially ordered. Cas9 protein can also be commercially ordered and precomplexed with sgRNAs so that immediately upon entry into a cell or zygote the RNP complex can localise to the nucleus and commence endonuclease activity. Pilot experiments in NPTr with GFP-Cas9 showed GFP-positivity after transfection, but no further experiments were performed to compare Cas9 RNP editing efficiency to the plasmid based system that was initially successful.

Testing for viral replication dynamics in an *in vitro* system provides preliminary data to construct a hypothesis around how a gene-edited organism will respond to pathogen challenge, but it cannot provide the context of how an entire organism will respond to infection. It is possible that in an animal model,

if it were viable and ethical, the reduction in viral titre seen in the growth assays would translate to IAV infections having difficulty in becoming established in the face of an active innate immune system and systemic immune response. Using a different *in vitro* model that is not an immortalised cell line would have been of benefit. From porcine somatic cells, induced pluripotent stem cells (iPSCs) can be generated (Ezashi et al., 2009). Swine iPSCs have been shown to be amenable to CRISPR genome editing and can be subsequently differentiated into multiple cell types (although not yet demonstrably to lung lineages) (Meek et al., 2022). Furthermore, because pluripotent cells are in the S/G2 phases for longer than somatic cells (Zaveri and Dhawan, 2018), and these stages of the cell cycle are more conducive to HDR (Liu et al., 2019), the efficiency of CRISPR experiments with HDR intentions may benefit from using these resources. This offers promise for the development of an *in vitro* system that has dual benefits of better HDR rates and a retention of the metabolic characteristics to the native IAV infection site in swine.

Human iPSCs can be differentiated into lung epithelial cells (Jacob et al., 2017), and embryonic or iPSCs can be differentiated into lung organoids (Dye et al., 2015). Organoids are a 3D system that recapitulates the native tissue context *in vitro* with the presence of diverse cell types. The use of gene-edited progenitor cells to create lung organoids with genetic mutations could be a powerful tool in IAV research (Archer et al., 2021; Strikoudis et al., 2019; Zhou et al., 2018), and the modification of these protocols to swine, as performed by Nash et al., 2021 in generating avian organoid models without the CRISPR experiments, would provide an optimal intermediary step between *in vitro* and *in vivo* translation.

6.5 Alternative ANP32 Targeting

The diverse ecology of IAV in distinct species, alongside the highly conserved nature of ANP32 proteins means that in different species that are infected with IAV, ANP32 proteins could be a host factor which promotes viral propagation through its interaction with FluPol. The same premise pertains to other viruses from the Orthomyxoviridae family, including IBV that has been shown to

function in an improved manner with mammalian-origin ANP32A or ANP32B present (Zhang et al., 2020b). Briefly investigated by Carrique et al., 2020, polymerase activity in DKO 293T cells was ablated for ICV constructs also, and FluPol activity could be rescued with human ANP32A or ANP32B, as well as chicken ANP32A. Experimental analysis of swine ANP32 proteins with ICV and IDV, which are known to infect pigs (Chiapponi et al., 2016; Hause et al., 2014), could be beneficial in understanding why ICV and IDV are more species restricted than other Orthomyxoviridae genera.

Although not an influenza virus, Infectious Salmon Anaemia Virus (ISAV) is part of the Orthomyxoviridae family and causes a significant level of mortality and productivity loss in farmed Atlantic Salmon (Dean et al., 2022). The isavirus, ISAV, has a heterotrimeric FluPol complex similar to Influenza (Mérour et al., 2011), and the ANP32A peptide sequence of Atlantic Salmon, the most susceptible species to ISAV, is 94% homologous to swine ANP32A (BLAST; NCBI), including homology at residues 129 and 130. The testing of whether resistance to ISAV can be conferred through the disruption of ANP32A and ANP32B in salmon was being tested by Dr. Maeve Ballantyne at the time of writing this thesis.

The role of ANP32 proteins in IAV replication means there is potential that they could be viable as therapeutic targets through transient reduction of gene expression, protein translation or molecular inhibition. In an *in vitro* model siRNAs targeting ANP32 proteins can lower FluPol activity (Fang et al., 2020). The development of molecular inhibitors would need careful research to ensure no off-target effects, but as a strategy targeting host factors, it could offer an alternative approach to drugs targeting IAV itself, which to date has resulted in the emergence of IAV strains emerging that are drug-resistant (Hussain et al., 2017).

6.6 The Future of Gene-editing in Agriculture

Gene-editing with CRISPR has been an incredibly powerful tool for life sciences research. But moving from the laboratory bench into a commercial setting has proven difficult. With the difficulties of commercialisation

considered, it is remarkable that within 10 years (Jinek et al., 2012), CRISPR is beginning to reach consumers. It has been redeveloped as a diagnostic tool (Jolany Vangah et al., 2020), a medicinal therapy (Mullard, 2019), a crop improvement tool (Miao et al., 2018; Waltz, 2016) and a method for improving animal agriculture (market approval, yet to be sold) (Burkard et al., 2017; Proudfoot et al., 2020).

For animal products, movement from a research background into a consumer product is a long road. For instance, in the USA, gene-edited animal products must be assessed through the new animal drug regulatory evaluation if they contain DNA that has been intentionally altered (Van Eenennaam et al., 2019). Regulatory approval for gene-edited organisms that have naturally occurring variants introduced with molecular tools are scrutinised by their process of creation, and the connotations that genetic technologies have for some, as opposed to on their scientific merits and genuine safety profile.

Although Canada has no gene-edited food products currently available to consumers, their legislation regulates CRISPR as another tool for breeding and will assess products based on the outcome, and not the process of creation (Ellens et al., 2019). Brazilian regulations allow for gene-edited livestock products to be sold, and a survey suggests that for welfare related issues these would not be met with significant public resistance (Yunes et al., 2021), however there was a difference in attitude when considering 'natural' variants compared to genome editing that creates genetic changes not identified within the gene pool already. These concerns speak of an ethical hangover that arose with resistance to genetic modification technology, without reconsideration of the precision with the newly developed methods. Regulations in Japan (Otsuka, 2021), Australia, Chile, Colombia and Brazil (Ishii and Ishii, 2022) all explicitly state that if it can be shown that no foreign genetic material is present, gene-edited organisms can be treated equally to organisms created by conventional breeding. This regulatory approach essentially is limited to allowing for deletions to be introduced via NHEJ, and although it may not be entirely scientifically sound to allow for the ablation of a protein's function and not for the specific modification of proteins where negative effects on cellular

metabolism cannot be identified, it appears to be the best currently possible appeasement that allows for the benefits that CRISPR products can offer to reach consumers.

6.7 Final Remarks

When embarking on this project, the overall goal was to develop a model for IAV resistance in swine that, if successfully validated, could be translated into an animal model that would potentially have commercial utility for swine breeders. Project goals were modified to include the data generated in Chapter 3 when modelling the introgression of swIAV alleles into a commercial style pig breeding unit. These results complement the wet lab data, which show that in a swine *in vitro* model from cells relevant to the site of IAV infection, targeting ANP32A and ANP32B with multiplexed CRISPR gene-editing, a significant reduction in IAV replication can be achieved. From these data directly, the creation of knockout pigs would be unethical, and further studies that show site specific substitutions in an appropriate *in vitro* model will be required prior to translation into a live animal model.

This project is by no means completed and it may ultimately be a very small part that contributes to the larger story of the relationship between ANP32 proteins and IAV in swine, and an even smaller part of how ANP32 proteins affect zoonotic transmission of IAVs, with particular relevance to chickens and humans. If it is not ANP32 proteins, there will be a role for gene-editing to play in reducing the threat of IAV to swine welfare, and to human pandemic emergence. CRISPR has had a big decade, and with the benefits it can offer to humans, livestock, and helping to create a sustainable environment, there's every reason to believe that the next decade will be even bigger.

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Appendices

Appendix 1 – Influenza control in swine review

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Porcine Health Management

REVIEW Open Access

Current and prospective control strategies of influenza A virus in swine

Hamish A. Salvesen* and C. Bruce A. Whitelaw

Abstract

Background: Influenza A Viruses (IAV) are endemic pathogens of significant concern in humans and multiple keystone livestock species. Widespread morbidity in swine herds negatively impacts animal welfare standards and economic performance whilst human IAV pandemics have emerged from pigs on multiple occasions. To combat the rising prevalence of swine IAV there must be effective control strategies available.

Main body: The most basic form of IAV control on swine farms is through good animal husbandry practices and high animal welfare standards. To control inter-herd transmission, biosecurity considerations such as quarantining of pigs and implementing robust health and safety systems for workers help to reduce the likelihood of swine IAV becoming endemic. Closely complementing the physical on-farm practices are IAV surveillance programs. Epidemiological data is critical in understanding regional distribution and variation to assist in determining an appropriate response to outbreaks and understanding the nature of historical swine IAV epidemics and zoonoses. Medical intervention in pigs is restricted to vaccination, a measure fraught with the intrinsic difficulties of mounting an immune response against a highly mutable virus. It is the best available tool for controlling IAV in swine but is far from being a perfect solution due to its unreliable efficacy and association with an enhanced respiratory disease. Because IAV generally has low mortality rates there is a reticence in the uptake of vaccination.
Novel genetic technologies could be a complementary strategy for IAV control in pigs that confers broad-acting resistance. Transgenic pigs with IAV resistance are useful as models, however the complexity of these reaching the consumer market limits them to research models. More promising are gene-editing approaches to prevent viral exploitation of host proteins and modern vaccine technologies that surpass those currently available.

Conclusion: Using the suite of IAV control measures that are available for pigs effectively we can improve the economic productivity of pig farming whilst improving on-farm animal welfare standards and avoid facing the extensive social and financial costs of a pandemic. Fighting 'Flu in pigs will help mitigate the very real threat of a human pandemic emerging, increase security of the global food system and lead to healthier pigs.

Keywords: Swine, Influenza, Pandemic, Disease control

Background

Influenza viruses are significant pathogens of humans, livestock and a multitude of wild species. They have a diverse and complex ecology stemming from their ability to cross species barriers. Comprising four genera within the *Orthomyxoviridae* family, Influenza A Virus (IAV), Influenza B Virus (IBV), Influenza C Virus (ICV) and Influenza D Virus (IDV) are enveloped virions with segmented negative sense RNA genomes (Fig. 1a). Seasonal epidemics of IAV and IBV occur in humans whilst only IAV has been attributed to cause epidemics in swine [1]. IBV [2], an ICV-related pathogen [3, 4] and IDV [5] have been reported and associated with mild morbidity in domestic pigs [4, 6]. Herein, the focus will be on IAV due to its more significant historical impacts

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Appendix 2 – ANP32A in swine original article

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VIRUS-CELL INTERACTIONS

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Swine ANP32A Supports Avian Influenza Virus Polymerase

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ABSTRACT Avian influenza viruses occasionally infect and adapt to mammals, including humans. Swine are often described as "mixing vessels," being susceptible to both avian- and human-origin viruses, which allows the emergence of novel reassortants, such as the precursor to the 2009 H1N1 pandemic. ANP32 proteins are host factors that act as influenza virus polymerase cofactors. In this study, we describe how swine ANP32A, uniquely among the mammalian ANP32 proteins tested, supports the activity of avian-origin influenza virus polymerases and avian influenza virus replication. We further show that after the swine-origin influenza virus emerged in humans and caused the 2009 pandemic, it evolved polymerase gene mutations that enabled it to more efficiently use human ANP32 proteins. We map the enhanced proviral activity of swine ANP32A to a pair of amino acids, 106 and 156, in the leucine-rich repeat and central domains and show these mutations enhance binding to influenza virus trimeric polymerase. These findings help elucidate the molecular basis for the mixing vessel trait of swine and further our understanding of the evolution and ecology of viruses in this host.

IMPORTANCE Avian influenza viruses can jump from wild birds and poultry into mammalian species such as humans or swine, but they only continue to transmit if they accumulate mammalian adapting mutations. Pigs appear uniquely susceptible to both avian and human strains of influenza and are often described as virus "mixing vessels." In this study, we describe how a host factor responsible for regulating virus replication, ANP32A, is different between swine and humans. Swine ANP32A allows a greater range of influenza viruses, specifically those from birds, to replicate. It does this by binding the virus polymerase more tightly than the human version of the protein. This work helps to explain the unique properties of swine as mixing vessels.

KEYWORDS ANP32, ANP32A, ANP32B, host factors, influenza, pandemic, swine, swine influenza, zoonotic, replication

Influenza A viruses continuously circulate in their natural reservoir of wild aquatic and sea birds. Occasionally, avian influenza viruses infect mammalian hosts, but these zoonotic viruses have to adapt for efficient replication and further transmission. This limits the emergence of novel endemic strains. Avian-origin, mammalian-adapted influenza viruses have been isolated from a range of mammalian species, including humans, swine, horses, dogs, seals, and bats (1–6).

One mammalian influenza host of significance are swine, which have been described as susceptible to viruses of both human and avian origin (6). It has been hypothesized that swine act as "mixing vessels," allowing efficient gene transfer between avian- and mammalian-adapted viruses. This leads to reassortants, which are able to replicate in humans, but to which populations have no protective antibody responses, as best illustrated by the 2009 H1N1 pandemic (pH1N1) (7). The ability of

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Appendix 3 – On-farm livestock genome-editing perspective article

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PERSPECTIVE
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On-Farm Livestock Genome Editing Using Cutting Edge Reproductive Technologies

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The global demand for animal-based food products is anticipated to increase by 70% by 2050. Meeting this demand in a way that has minimal impact on the environment will require the implementation of advanced technologies. Genome editing of livestock is a tool that will allow breeders to improve animal welfare, performance and efficiency, paving the way to a more sustainable future for livestock agriculture. Currently, genome editing of livestock is limited to specialized laboratories due to the complexity of techniques available for the delivery of genome editing reagents into zygotes and reproductive cells. The emergence of three cutting-edge reproductive technologies—(i) zygote electroporation, (ii) zygote transduction of recombinant adeno-associated virus (rAAV), and (iii) surrogate sire technology—will provide livestock breeders with a new toolkit of delivery strategies for genome editing. The simplicity of these technologies will enable widespread on-farm application in major livestock species by seamlessly integrating into current breeding systems. We believe it is timely to highlight these three cutting-edge reproductive technologies for genome editing and have outlined pipelines for their implementation in on-farm settings. With a nuanced regulatory framework these technologies could fast-track livestock genetic gain and help secure a sustainable future for livestock.

Keywords: genome editing, CRISPR, genetic engineering, pigs, cattle, livestock, sustainability

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INTRODUCTION

Preparing to feed a balanced and nutritious diet to the projected 9.7 billion people on the globe by 2050 will be one of the greatest challenges humanity has ever faced. The FAO estimates demand for animal-based food products will increase by 70% in this time (Alexandratos and Bruinsma, 2012). Increasing reliance on plant-based diets and artificial meat production will contribute to improving food security and the sustainability of commercial agriculture, however outright omission of animal protein from human diets risks nutritional deficiencies and malnutrition, particularly in developing regions. Meeting the anticipated increase in demand for animal food products in a way that has minimal impact on the environment and ensures high animal welfare standards will likely require the implementation of advanced technologies, including genome editing and cutting-edge reproductive technologies. Considering the huge potential of these technologies, it would be negligent not to examine their inherent possibilities further.

Traditional livestock breeding is restricted by genetic linkage and the available genetic variation within a breed. Genome editing allows animal breeders to overcome these biological impediments