THE ANTIVIRAL MECHANISM AND VIRAL ANTAGONISM OF HOST HISTONE DEACETYLASE 4 IN INFLUENZA A VIRUS INFECTION

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to my family and friends, for their unwavering support and encouragement
Abstract

Influenza virus continues to be an important global pathogen which poses serious health and economic challenges. Influenza virus causes regular seasonal epidemics, intermittent and unpredictable pandemics and deadly zoonotic outbreaks. The currently available antiviral strategies used against influenza are limited in their effectiveness. The rapid evolution of influenza viruses has precluded the development of a universal vaccine and has meant that the annually administered vaccine is only variably effective. Furthermore, the two classes of antiviral drugs used against influenza have only limited effectiveness due to rise in drug resistance. Therefore, there is a pressing need to develop a greater understanding of the virus-host interactions that are critical for influenza virus multiplication and pathogenesis. Such understanding would aid in the development of more effective and long lasting anti-influenza virus strategies.

This PhD investigates the role of host histone deacetylase HDAC4, in influenza A virus (IAV) infection, the most significant genus of influenza. Histone deacetylases (HDACs) are a family of enzymes that catalyse the deacetylation of acetylated histone and non-histone proteins. Previously, it has been shown that HDAC1, HDAC2, HDAC6 and HDAC11 all possess anti-influenza virus properties. HDAC1, HDAC2 and HDAC11 are involved in promoting the innate immune response during influenza infection. HDAC6 restricts influenza virus release by deacetylating α-tubulin and decreases viral replication by degrading the viral polymerase and enhancing the RIG-I innate immune response. Currently, no studies have investigated the role of HDAC4 in IAV infection. Given the similarity between different HDAC members, we hypothesised that HDAC4 also plays a role in inhibiting IAV replication. Hence, we investigated the effects of HDAC4 depletion and overexpression on IAV replication using primarily human lung epithelial cells and IAV PR/8/34(H1N1) strain as a model.

The outcomes of this project reveal that depletion of HDAC4 enhances the replication of influenza virus by up to 4-fold. Conversely, influenza virus replication was inhibited by up to 49% in cells overexpressing HDAC4, indicating an antiviral role for HDAC4. Mechanistically, HDAC4 was found to be involved in the influenza virus-induced host innate antiviral response. Specifically, we observed decreased phosphorylation of STAT1 in HDAC4 depleted cells, which corresponded to decreased levels of the interferon effector genes (IFITM3, ISG15 and viperin). Conversely, HDAC4 overexpressing cells enhanced STAT1 phosphorylation, resulting in greater expression of the downstream
interferon effector genes. We also found that influenza virus actively targets HDAC4 to downregulate its expression both at mRNA and polypeptide levels, likely as a means to inhibit its antiviral function. The HDAC4 mRNA is degraded by the IAV endonuclease PA-X while HDAC4 polypeptide is cleaved by IAV-induced host caspase 3.

In summary, the outcomes of this PhD implicate HDAC4 as an anti-influenza host factor that is a component of host innate antiviral response. In addition, it is revealed that influenza virus strongly antagonises HDAC4 to subvert its antiviral activity. Thus, the data presented here contributes to our molecular understanding of host HDACs-influenza virus interplay and strengthening the notion that HDACs are a family of anti-influenza host factors.
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CHAPTER I:
INTRODUCTION
Section A – Influenza and influenza A virus

1.1 The global burden of influenza

Influenza is an acute febrile disease, commonly referred to as “flu”. In humans it is caused by influenza A virus (IAV) and influenza B virus (IBV). The disease is typically associated with a somewhat mild upper respiratory tract infection, consisting of sore throat, cough, fever, muscle aches and headache [1]. However, in severe cases the disease can develop into pneumonia when the virus infects the lower respiratory tract and destroys the airway cilia allowing secondary bacterial infections to occur. In susceptible individuals such as young children (6-60 months), the elderly and immunocompromised, influenza infection can also lead to a variety of other health complications affecting the heart, brain and other organ tissues [2]. According to the World Health Organisation estimates, there are approximately 1 billion cases of flu every year. About 3 – 5 million of these are categorised as a severe illness, resulting in 300,000 – 500,000 deaths worldwide. In addition to the health related issues, an often underappreciated impact is the socio-economic burden: increase in hospitalizations and work absenteeism leading to loss of productivity and trade associated with influenza [3].

The spread of influenza is characterised by annual seasonal epidemics and unpredictable pandemics. Due to the introduction of IAV strains from avian or swine populations, there have been 4 resulting pandemics since 1918. The incidence of influenza infections increases during a pandemic due to a lack of pre-existing immunity to the new virus, however severity depends entirely on the pandemic virus itself. The 1918-1919 “Spanish flu” was the most severe pandemic in the last 100 years, which first arose in the United States and spread across the world. It is estimated that 500 million people were infected which resulted in 40 million deaths [4]. The second recorded pandemic was the 1957-1958 “Asian flu” which began in China and caused approximately 2 million deaths worldwide [5]. The third pandemic occurred from 1968-1969, first appearing in Hong Kong and was so dubbed “Hong Kong flu”. This pandemic caused between 1-4 million deaths worldwide, with a secondary outbreak occurring from 1970-1972 [6]. The most recent pandemic, dubbed “Swine flu”, originated in Mexico in 2009-2010 and resulted in about 18,000 deaths globally [4, 7].

In addition to the seasonal and pandemic outbreaks of influenza, there is growing concern regarding zoonotic outbreaks, in particular the emergence of highly pathogenic avian
influenza (HPAI). The first reported outbreak of HPAI occurred in Hong Kong in 1997 where an H5N1 strain infected 18 people, of which 6 died. The WHO has reported that between 2003 and June 2019, there have been 861 confirmed cases of H5N1 which caused 455 deaths [8]. In 2013 another HPAI strain, H7N9, emerged in China and between 2013 and 2018 1567 human cases were reported, resulting in 615 deaths [9]. In addition, outbreaks of HPAI is a major disease burden in animals. In the first 10 years since HPAI emerged, it has resulted in the deaths of more than 400 million birds with economic losses totalling US$10billion [10].

1.2 The structure of influenza A virus

Influenza virus belongs to the Orthomyxoviridae family, which encompasses seven genera of RNA viruses including the 4 influenza genera: influenza A, B, C and D [11]. All influenza viruses are enveloped with a negative sense, single stranded, RNA genome. IAV and IBV viruses contain 8 RNA gene segments, whereas influenza C and D viruses only contain 7. The latter do not cause substantial disease in humans [12-15]. IAV and IBV are of much greater concern in terms of global disease burden, with IAV being the most significant because of its unique pandemic potential. Unique among the influenza genera, IAV circulates not only in humans, but also in pigs, horses and poultry as well as migratory birds. The wild aquatic birds are considered the natural reservoirs of IAV [16].

IAV virions vary in size from 80-120nm and predominantly exist in a spherical form (Figure 1) [17]. The 8 gene segments that make up the genome of IAV encode up to 17 proteins [18]. The most characterised proteins are the RNA polymerase subunits, polymerase basic 1 and 2 (PB1, PB2) and polymerase acidic (PA) and together they carry out gene transcription and genome replication. Segments 4 and 6 encode the viral glycoproteins hemagglutinin (HA), which mediates viral entry and neuraminidase (NA) which is required for releasing newly formed virus particles from the plasma membrane, facilitating viral spread. Viral nucleoprotein (NP) is encoded on segment 5 and plays important roles in genome encapsulation [516], nuclear trafficking [517] and vRNA transcription and translation [518]. Segments 7 and 8 encode multiple proteins, most importantly matrix protein (M1), membrane ion-channel protein (M2), non-structural protein 1 (NS1) and nuclear export protein (NEP, also known as NS2). M1 provides a scaffold that helps to form the structure of the virus particle and together with NEP, regulates trafficking of the viral RNA segments inside the cell. M2 is transmembrane
protein that acts as a proton ion channel required for viral entry and exit. The protein is characterised by 4 transmembrane helices which are packed tightly together to keep the pore closed. Lowering the pH weakens the helix-helix interactions and opens the channel, allowing protons to flow through. NS1 is an important virulence factor that carries out a range of functions to promote viral replication and inhibit the antiviral immune response. More recently, additional IAV proteins have been identified including PA-X (a frameshift product of PA), M42 (a splice variant of M), PA-N155 (N-terminal truncation of PA), PA-N182 (N-terminal truncation of PA) and NS3 (point mutation in NS1) [19, 20]. The functional role of these proteins is not completely understood, however, PA-X has been recognised as an important virulence factor, owing to its ability to selectively degrade host mRNAs [21].

The envelope of IAV is composed of a lipid bilayer derived from the host cell. It contains the 2 viral surface proteins HA and NA which are the most antigenically variable and are used to classify antigenically diverse subtypes. Currently, a total of 16 HA and 9 NA subtypes have been identified (18 HA and 11 NA when including the phylogenetically similar bat IAV-like viruses, however these are unable to reassort with other IAVs) [22-25]. The diverse range of animal reservoirs is an important source of antigenically varied HA and NA genes that are able to be exchanged between different IAV strains during coinfection of the same host cell. This allows for a greater viral diversity and in some cases leads to the emergence of human pandemic strains with HA and/or NA derived from animal strains.
Figure 1. Structure of influenza A virus. This figure represents an IAV particle. IAV is an enveloped negative-sense single stranded RNA virus with 8 individual RNA segments contained within the virus particle. Adapted and modified from [26]
1.3 Pathophysiology of influenza A virus

In humans, IAV is transmitted via the respiratory route while in birds it is transmitted through the faecal-oral or faecal-respiratory route [519]. Depending on the route of transmission, the virus usually infects the epithelial cells of the upper respiratory tract or the epithelial cells of the intestinal tract [520]. Upon binding to the surface of a target cell, the cellular life cycle of IAV begins where it exploits many cellular components, in a complex, multistep process (Figure 2).

1.3.1 Entry

The binding of IAV to the host cell surface is mediated by the viral HA which bind to the sialic acid receptors present in the oligosaccharides and glycoproteins at the cell surface [27]. Human IAV HA preferentially binds sialic acids linked by an α-2,6 linkage to the rest of the oligosaccharide, whereas avian IAV HA favours α-2,3 linked sialic acids [28]. This is because these bonds are the predominant sialic acid linkages in the upper human airway epithelium and avian intestinal tract, respectively. It was thought that sialic acid receptors were a requirement for the successful absorption of IAV into the host cell, however, a recent study has shown that IAV can infect cells without sialic acid [29], indicating that perhaps IAV has evolved several strategies to enter a host cell. After binding, the viral particle is internalized in an endosome via receptor-mediated endocytosis. Endocytosis occurs in a clathrin-dependent manner, involving dynamin and the adapter protein Epsin-1 [30, 31], or by micropinocytosis [32].

1.3.2 Envelope fusion and nuclear import

Once internalized into an endosome, host proteases cleave the viral HA (or HA0) into HA1 and HA2 [33]. Acidification of the endosome induces a conformational change in HA that exposes the HA2 fusion peptide. The HA2 fusion peptide then inserts into the endosomal membrane which induces fusion of the viral envelope with that of the endosome [33]. Because the pH of endosomes varies between host species, the pH stability of HA is an important determinant of viral tropism [34]. After fusion of the 2 membranes, protons from the endosome enter the virion through the viral M2 ion channel which acidifies the virus particle leading to dissociation of M1 from the vRNP complex [35, 36]. Host vacuolar ATPase is important for both the acidification and fusion of the viral and endosomal membranes, allowing the genetic material of the virus bound in the form of 8 vRNPs to be released into the cytoplasm [37]. The vRNPs are then imported into the
nucleus through the nuclear pore complex. This process is aided by importin α and through the action of the nuclear pore complex proteins Nup98 and Nup153 [38-40].

1.3.3 Replication and transcription

Transcription and replication of the viral RNA occurs within the nucleus and is catalysed through the action of the viral polymerase complex (PB1, PB2 and PA) which is attached to the vRNPs [41]. Replication of the viral RNA occurs through positive-sense intermediate known as the complementary ribonucleoprotein (cRNP) complex [38]. Many copies of viral RNA are transcribed from the cRNP which are themselves transcribed into viral mRNAs [41]. Cap snatching of host mRNA is an important step in the transcription of the viral mRNA. The PB2 protein binds to the 5’-cap of the host pre-mRNAs which allows viral PA to cleave the 10-13nt long 5’ cap. This capped oligonucleotide primer is then used to initiate transcription of the viral RNA. Finally, the viral mRNA is matured by the addition of a poly(A) tail through the catalytic activity of PB1 [42]. Additionally, M mRNA is spliced into M1 and M2 mRNAs as are NS1 and NEP from NS mRNA [43]. This splicing occurs through the activity of host mRNA splicing factors Tat-SF1 and BAT1 [44]. The positive-strand mRNAs which are capped and polyadenylated are then exported into the cytoplasm for translation into viral proteins.

1.3.4 Translation

Translation of the IAV mRNA is entirely dependent on the host cell translation machinery. The viral mRNAs are exported from the nucleus into the cytoplasm, where translation is divided between cytosolic ribosomes (for PB1, PB2, PA, NP, NS1, NS2 and M1) and endoplasmic reticulum-associated ribosomes for the membrane proteins HA, NA and M2. Newly synthesised viral polymerases (PB1, PB2 and PA) and viral NP, NS1, NEP and M1 are imported into the nucleus to further increase the rate of viral RNA synthesis while the membrane proteins (HA, NA and M2) are trafficked and inserted into the plasma membrane [45, 46]. To further aid in viral replication, many of the viral non-structural proteins actively inhibit host protein synthesis or promote viral mRNA synthesis to favour translation of viral mRNAs. For example, NS1 contributes to viral mRNA export by linking the viral transcripts to the cellular nuclear export components and the nucleoporin NUP98 [39]. Additionally, PB1, PB2 and PA interact with host DNA-dependent RNA polymerase II, which is believed to contribute to shutdown of host mRNA synthesis [47]. The viral protein PA-X targets host mRNAs transcribed by host RNA polymerase II (Pol II), while leaving viral mRNAs transcribed by viral RdRp intact. Initially, PA-X cleaves the
target which allows complete degradation of the mRNA to be carried out through the activity of the host 5’ -> 3’ exonuclease Xrn1 [48]. Interestingly, even non-coding mRNAs transcribed by Pol II are targeted by PA-X, likely as a means to free up ribosomes to translate more viral mRNAs (reduce ribosomal load), allowing for increased viral protein synthesis [48].

1.3.5 Post-translational modifications

Newly translated viral proteins are subject to a diverse range of post-translational modifications (PTMs) in order to diversify their function. IAV itself encodes no known protein-modifying enzyme and so all of these PTMs are carried out by host machinery. Recently, it has been discovered that viral NP is acetylated which is important for viral growth and replication. Lack of acetylation at lysine residue 229 results in decreased viral replication as a result of impaired particle release. Furthermore, hyper-acetylation at lysine residues 77 and 229 severely diminish viral polymerase activity [49]. Viral NP is both phosphorylated and SUMOylated, both of which affect the trafficking of NP to and from the nucleus. During the early stage of infection NP localises to the nucleus with the vRNP while in the late stage of infection, NP is important for exporting the vRNP complexes [6, 7]. Phosphorylation of NP at serine 3 residue, located at the N-terminal nuclear localization signal (NLS), inhibits interaction with nuclear import factors and prevents its nuclear localization [50]. Meanwhile, phosphorylation at the serine 296 residue prevents NP interacting with nuclear export factors [51]. SUMOylation plays an important role in the retention of NP within the nucleus as mutant NPs lacking SUMOylation sites are prematurely exported to the cytoplasm [52]. Ubiquitination of all of the RNP components serve as a means to promote the function of the RNP but in some circumstances also can lead to its degradation through the addition of polyubiquitin chains [53, 54]. Phosphorylation of NS1 disrupts its protein-RNA interactions as well as its protein-protein interactions [55-58]. In this way, the host cell is able to interfere with the antagonistic properties of NS1. Additionally, HA is subject to glycosylation which helps in immune evasion as it inhibits antibody recognition and neutralization [59]. Furthermore, glycosylation of HA has also been linked with an increase in virulence and fitness after immune escape [60, 61].

1.3.6 vRNP nuclear export

In the nucleus, vRNA is matured into vRNP complexes through assembly with NP, PA, PB1 and PB2. These vRNP complexes are then exported out of the nucleus through two
different pathways mediated by nuclear RNA export factor 1 (NXF-1) and chromosome region maintenance-1 (CRM-1) [62, 63]. NS1 is believed to aid in exporting viral components through the NXF-1 nuclear export pathway [64, 65]. Additionally, M1 acts as an adaptor protein and links NEP to vRNPs whereby NEP interacts with CRM-1 to guide the vRNPs to the CRM-1 nuclear export pathway to be transported to the cytoplasm [66-68].

1.3.7 Maturation of IAV membrane proteins

The viral membrane proteins, HA, NA and M2 are synthesised by ribosomes associated with the ER membrane [69-72]. Following synthesis, HA, NA and M2 oligomerize and are trafficked through the Golgi to the plasma membrane. During this trafficking process from the ER to the Golgi, HA is trafficked as a fusion incompetent precursor termed HA0. In order to gain its fusion function, HA must be cleaved into subunits HA1 and HA2 [73-75]. Different proteases are known to cleave HA, depending upon the particular strain of IAV, therefore the cleavage site of HA is important for the tissue tropism of the virus. All influenza viruses have a cleavage site that is recognised by extracellular proteases present in respiratory and epithelial cells except for HPAI viruses, which contain a multibasic cleavage site in HA that is recognised by furin, a ubiquitously expressed protease [76, 77].

1.3.8 Viral budding and release

During the late stages of infection, viral M1 and NEP localize to the nucleus where they bind with vRNPs to mediate their export to the cytoplasm. Then, through interactions with the recycling endosome and microtubules, they migrate to the plasma membrane and are packaged into the 8 vRNPs that makeup the viral genome [67, 78, 79]. Budding of new virions takes place through the clustering of HA and NA in lipid raft domains. M1 binds to the cytoplasmic tails of HA and NA and serve as docking sites for the vRNPs. Polymerization of M1 proteins cause the bud to elongate and then the M2 protein forms a positive curvature at the periphery of the bud. Membrane scission occurs via the insertion of the M2 amphipathic helix at the budding neck of the lipid phase boundary, resulting in the release of the budding virion [80, 81]. Once the virion is released it may still be bound to the cell membrane via interactions between host silica acid and virion-associated HA. NA then plays the final role in release as it is able cleave HA bound to sialic acid receptors through its sialidase activity [81]. Viral replication results in cell death, inducing pro-inflammatory responses that promote recruitment of innate and adaptive immune cells.
which clear the infection, but in overabundance can cause immunopathology and pneumonia.
Figure 2. Influenza A virus life cycle. IAV enters the cell once the viral surface protein HA binds to sialic acid receptors on the surface of the host cell. The virus is then internalized into an endosome where the low pH induces fusion of the viral and endosomal membranes. Following membrane fusion, the vRNP is released into the cytoplasm and is then imported into the nucleus where the replication cycle begins. The negative sense strand is converted into positive sense or complementary RNA (cRNA) which is used as the template for viral RNA synthesis. Viral mRNAs are exported from the nucleus and translated into viral proteins in the cytoplasm, and these are assembled into new virions together with newly synthesised vRNPs. Newly formed virus particles bud from the surface of plasma membrane and NA cleaves the sialic acid receptors to release the newly formed virions from the host cell surface. (adapted and modified from [26])
1.4 Evolution of IAV

The most important driver of IAV evolution is the viral RNA-polymerase which lacks any proofreading function. Therefore, during viral replication, the integration of incorrect nucleotides occurs at rate of $10^{-3}$ to $10^{-4}$ which allows for a rapid mutation rate of the viral genome [82, 83]. The effect of this rapid mutation rate is most profound in the context of the two IAV surface proteins, especially HA. Due to its role in receptor recognition and attachment, HA is the principal determinant in IAV host range. The specificity of avian HA is for α2,3 sialic acid receptors found in the intestinal tract of birds, while human influenza HA preferably binds α2,6 sialic acid receptors, which are predominantly found in the respiratory tract of humans. However, sialic acids with α2,3-linkages are found in the lower respiratory tract in humans, allowing avian IAV subtypes to infect humans occasionally, usually at a much greater mortality rate than human IAV. Importantly, pigs and some avian species (pheasants, turkeys, quails) possess both α2,3 and α2,6 linkages particularly in alveoli epithelial cells, allowing them to be infected by both human and avian IAV strains. Thus, these animals can act as mixing vessels capable of generating re-assortment viruses during co-infection with different IAV strains. This is the mechanism by which the 2009 “swine flu” pandemic virus emerged [84-86]. In addition to determining host range specificity, the rapid mutation rate of IAV surface proteins also contributes to the viruses’ ability to effectively evade the host immune response [87]. There are three main mechanisms that drive evolutionary changes in the virus which include antigenic drift, antigenic shift and occasionally, recombination.

1.4.1 Antigenic drift

Antigenic drift is a mechanism of IAV evolution that is characterised by the gradual accumulation of mutations in key antigenic sites. This results in antigen migration to produce new influenza subtypes which escape the immune pressure of the population [88]. Immune escape occurs when the surface proteins HA and NA accumulate minimal structural changes that are enough to circumvent immune protection (acquired through previous infection or vaccination), but do not significantly alter the function of these proteins. Antigenic drift is important in altering host range as well as immune evasion, but
also plays an important role in the emergence of drug resistance. Some single point mutations in the drug targets of influenza have been identified as enough to change the structure of these targets so that drug binding becomes ineffective, allowing for the evolution of drug resistant strains. Antigenic drift is the main driver of new IAV variants with the ability to cause annual influenza epidemics [89]. While these changes are not sufficient to lead to pandemics, over long periods of time, antigenic drift can cause strains to become considerably different from the original pandemic virus.

1.4.2 Antigenic shift

One of the most distinctive advantages of the segmented genome of IAV is that it enables the exchange of RNA segments between genotypically different influenza viruses. This re-assortment of the viral genome allows for the production of entirely new IAV strains and subtypes that have the potential to become pandemic strains [90]. These novel strains are generated as a result of antigenic shift and contain elements from the parental strains but are significantly different antigenically. The 1957 and 1968 pandemics of last century as well as the 2009 pandemic of this century arose as a result of re-assortment between human IAV and IAV from other host species [91]. It is believed that the HA, NA and PB1 genes from the H2N2 1957 pandemic strain as well as the HA and PB1 segments from the 1968 H3N2 pandemic strain are of avian origin, while the remaining fragments may come from the 1918 “Spanish flu” strain [92]. The H1N1 2009 “Swine flu” pandemic strain is a re-assortment from multiple mixed recombination’s between European H1N1 swine IAV, North American H1N2 swine influenza, North American avian influenza, and human H3N2 influenza [93]. The re-assortment resulted in a novel H1N1 strain containing PB2 and PA segments from the North American swine influenza, PB1 from human H3N2 influenza, HA, NP and NS from a classic swine influenza virus and, NA and M from a European H1N1 swine influenza virus [92].

1.4.3 Recombination

Recombination is another means which can shape the evolution of IAVs and can occur through two mechanisms. The first is by non-homologous recombination which occurs between two different RNA segments [94, 95], while the other is homologous recombination which is thought to participate in template switching.
while the RNA is being copied by the polymerase. However, during IAV replication, the RNA genome is rapidly packed with ribonucleoprotein which prevents the occurrence of template switching, making homologous recombination in influenza viruses a rare event [96]. Nevertheless, several studies have provided evidence for homologous recombination in IAV, for example, between PB2 and PA (between H1N1 strains) as well as HA and NP (between human H1N1 and H3N2 strains) [97].

1.4.4 Adaptations to new hosts

Aquatic waterfowl and shorebirds are the main natural host species of IAV and are often referred to as the ‘reservoir hosts’ [98]. Some IAV strains (presumably from waterfowl), have adapted to be able to successfully infect other birds (including domestic poultry and ducks) and mammals (including humans, swine, equine, dogs, mink and marine mammals) [99]. The virus is often propagated through animal faeces and contaminated water which enables contact with a broad host range, significantly increasing the chances of genetic mutation and re-assortment. This also allows for greatly increased intra-host genetic diversity (referred to as quasi-species) resulting in a pool of viral mutants that facilitate the adaptation to new hosts and selection pressures [100]. Adaptation to transmission in new hosts requires mutations in the viral HA and NA to optimize receptor binding [101] and sialic acid cleaving activities [102], respectively, and to enable HA activation at different pH levels [103]. Additionally, changes in the viral RNA polymerase subunits can affect activity in different hosts as well as its temperature sensitivity which varies depending on the internal temperature of the host organism [104, 105]. Furthermore, changes in NP can affect susceptibility to the host interferon-stimulated gene (ISG), Mx1 [106], whereas changes in M1 and M2 affect viral morphology which is an important facilitator in respiratory transmission in new hosts [107]. These changes can also be acquired through re-assortment, as occurred with the 1957, 1968 and 2009 pandemics [108].

Genetic adaptations often occur between wild aquatic birds and domestic ducks or geese. It can also occur between aquatic and domestic poultry (which is how the avian IAV strains H5N1, H7N9 and H10N8 emerged) or within swine (as occurred
with the emergence of the 2009 pandemic). H3N8 was initially an endemic strain circulating in equine but has since been transmitted to dogs in the United States [109]. Recently, a species of bat from Guatemala and Peru have been found to carry a new influenza A-like virus with new subtypes of HA and NA (H17, H18 and N10, N11) [25, 110].
Figure 3. The ecological cycle of influenza A virus. Influenza A viruses have been found in a multitude of species all seemingly derived from viral ancestors in wild birds, with the exception of the bat influenza-like viruses which are still of uncertain origin. Viruses from wild birds can be transmitted to marine mammals and domestic free range ducks and poultry. Transmission from ducks occurs through ‘backyard’ farming where the animals are raised together or in live animal markets. Humans can be infected from poultry and swine viruses through aerosols, fomites or contaminated water. Other domestic animals known to be susceptible to IAV infections are dogs and cats. Dashed lines represent transmissions that bypass a domestic duck intermediate. Adapted and modified from [26].
1.5 Current anti-IAV strategies and their limitations

Currently, the annual influenza vaccination program alternating in the Northern and Southern Hemispheres is the main strategy that is used to prevent and control seasonal influenza epidemics. Because of the rapid mutation rate of IAV and IBV resulting in the emergence of novel strains, the vaccine needs to be reformulated, manufactured and distributed to the global human population each year, prior to the start of flu season [111]. The most important factor in determining the success or failure of the vaccine is the global influenza surveillance program known as the Global Influenza Surveillance and Response System (GISRS), spearheaded by the WHO. Laboratories and collaborating centres in 113 member states conduct surveillance on circulating influenza strains as well as provide recommendations on vaccine formulation and global influenza risk-assessment. Vaccine efficacy varies each influenza season and depends upon the strains included in the vaccine (recently, an IAV H1N1 strain, an IAV H3N2 strain and 2 IBV strains) and which strains end up being the dominant circulating strains [112]. In addition to the challenge of predicting the circulating strains, vaccine effectiveness is also dependent upon global vaccine uptake. New strategies are being utilised to improve vaccine efficacy through development of next-generation vaccines. These include synthesis of a universal vaccine which targets the extracellular domain of the M2 protein or the stalk domain of HA, both of which are highly conserved and less prone to mutation. Another approach includes vaccines which enhance the numbers of cytotoxic lymphocytes as these target more conserved IAV proteins. In addition, the use of DNA vaccines against other IAV antigens are being explored, as well as the use of different vectors such as baculovirus expression systems and influenza virus-like particles [113-115]. However, the development of a universal vaccine still remains an elusive goal and as such, if the vaccine becomes ineffective, we are reliant on the second line of anti-influenza strategies - the antiviral drugs.

The adamantanes: amantadine (Symmetrel), and rimantadine (Flumadine) were the first class of anti-IAV drugs approved, however, they are ineffective against IBV [116]. This class of drugs works by binding to the M2 ion channel and blocks the entry of protons into the virion, consequently inhibiting vRNP release and IAV replication [117, 118]. When amantadine and rimantadine were first introduced in 1966 and 1993 respectively, both drugs were highly successful in inhibiting IAV replication with an efficacy of up to 90% [119-121]. However, since 2005, resistance to adamantanes has risen exponentially to a point where they are no longer recommended by the CDC or WHO as an effective anti-IAV drug [122]. Widespread resistance arose mainly due to the emergence of a single serine
substitution at position 31 of the M2 protein for an asparagine (S31N) [123, 124]. The S31N mutation and others (L26F, L38F) effect the tetramer helix-helix packing, resulting in the destabilization of the helix-helix assembly which decreases the pore size [125-128]. Other mutations (V27A, A30T/V, G43E, L38F) occur within the M2 transmembrane domain and results in an increase in the pore size and hydrophilicity of the channel [129-131]. Neuraminidase inhibitors (NAIs), were the second class of approved anti-IAV drugs and are the only antiviral currently used against influenza. NAIs target the surface protein NA and function by acting as sialic acid or transition state analogous that compete with cell surface sialic acid-viral NA interactions and inhibit the enzymatic reaction and release of the newly formed IAV progeny [132-135]. This inhibits budding of the viral particle, preventing spread and further infection. However, similar to the M2 inhibitors, IAV has developed resistance to these inhibitors through mutations in several different amino acids within the NA protein, most notably, E119V, I222V, H274Y, R292K and N294S [136, 137]. These mutations occur in or around the active site of NA which subsequently alters the architecture of the active site and reduces the binding efficiency of NAIs by many fold [138-142]. Zanamivir (Relenza) and oseltamivir (Tamiflu) were the first approved NAIs [143-146], although due to the emergence of resistance to these drugs, two related NAIs, peramivir and laninamivir have since been developed [147-151]. However, mutations in the NA have also been detected in circulating strains that confer resistance to these new drugs [152]. While adamantane-resistant IAV strains have become the predominant circulating strains, the mutations that confer resistance to NAIs occur around the NA active site, which commonly decreases viral fitness [153, 154]. This means that NAI-resistant strains are often out-competed with NAI-sensitive strains in the absence of NAIs and so are not often maintained within the circulating population.

However, the lack of a universal vaccine and the emergence of drug resistant strains highlights vulnerabilities in our preparedness against IAV outbreaks. Therefore, there is a pressing need to further understand the interactions between virus and host that are important for the IAV replication and pathogenesis in order to aid in the development of alternative, effective and long lasting anti-IAV strategies. One such strategy is to target the naturally occurring antiviral defences already present in the cell as these are capable of targeting nearly every stage of the virus life cycle, and are potentially less likely to be rapidly overcome as a result of IAV evolution.
1.6 Host factors and their role in IAV infection

During the infection cycle, a virus interacts with a multitude of host factors in order to successfully replicate. These interactions can positively affect viral replication and are otherwise known as proviral host factors, while others will interfere with and inhibit viral replication and are known as antiviral host factors [155]. Recognizing the role that these host factors play in both promoting and inhibiting IAV replication would broaden our understanding of the virus-host interaction and could potentially uncover novel drug targets [156].

1.6.1 Proviral host factors

Significant progress has been made in identifying many proviral host factors that are important in promoting IAV replication, these are summarised in Table 1. An initial groundbreaking study using an RNA interference (RNAi) screen in *Drosophila* cells, was able to identify a plethora of host genes involved in influenza virus infection. The screen targeted 90% of the host genes and of these, 100 were found to either promote or inhibit influenza replication. Most notably, vacuolar ATPase subunit D (ATP6V0D1), cytochrome c oxidase subunit 6a 1 (COX6A1) and nuclear RNA export factor 1 (NXF1) were found to positively regulate influenza replication. Most significantly, the depletion of these genes resulted in a decrease in viral replication [157]. In a similar study using an RNAi screen, 295 host factors were identified to be involved in IAV replication of which, 219 were considered to be essential for virus replication. A subset of 23 proteins from this list were found to be crucial for virus entry including members of the vacuolar ATPase family, coatomer protein I (COPI) family, fibroblast growth receptor (FGFR) and glycogen synthase kinase 3 beta (GSK3B). Additionally, 25 proteins were found to be important post-entry, including nuclear import components, proteases, and calcium-calmodulin proteins [158]. Another RNAi screen identified 135 host proteins that were regulated during H1N1 infection while only 81 were regulated during H3N2 infection, highlighting the differences in the host response to infection with IAV different strains [159]. A further genome-wide RNAi screen identified another 287 host factors that were important for IAV replication. Upon depletion of these host factors, 119 were found to inhibit the replication of the influenza strain A/WSN/33 and 121 inhibited the replication of the pandemic strain A/Hamburg/04/2009. Comparison analysis revealed that 72 of these host factors were common between the 2 strains, indicating their strong inhibitory potential in IAV infection [160]. Another study conducted a genome-wide RNAi screen using shRNA
and was able to identify 110 host factors important for IAV replication. Most notably, the study revealed the role of Itch, an E3 ubiquitin ligase that plays an important role in the release and transport of vRNP from the endosome into the nucleus. The knockdown of Itch decreased endosomal trafficking of the viral RNA and subsequently viral titres were reduced by up to 10 fold [161]. Itch has also been shown to play a similar role in other viral infections such as T-cell leukemia virus 1 [162]. A more recent study employed affinity purification followed by mass spectrometry in order to identify cellular proteins that interact with the IAV RNA-dependent RNA polymerase (RdRp). Using this method, they were able to identify 171 host factors involved in IAV infection consisting of chaperones, cytoskeletal proteins, kinases, phosphatases and ubiquitin ligases. Most importantly, they were able to identify that serine/threonine protein phosphatase 6 (PP6) interacted directly with the PB1 and PB2 complex to promote viral RNA synthesis. Unsurprisingly, when the researchers knocked down PP6, viral replication was inhibited [163]. Another study using RNAi was able to identify the importance of TNSF13, TNSF12-TNSF13 and USP47 in promoting IAV replication. Depletion of USP47 inhibited IAV entry, whereas silencing of TNFSF13 and TNFSF12-13 inhibited the late stage of IAV replication [164]. A microarray study was able to determine that the host factor DR1 was involved in viral RNA and protein synthesis and also involved in inhibiting the host innate immune response. DR1 was found to bind directly with the viral RdRp while also inhibiting IFNβ synthesis [165]. Further analysis of the microarray data revealed that prodilase [166] and cathepsin W [167] were both important in the early stages of IAV infection by action of their dipeptidase and proteolytic activities, respectively. Additionally, the PI3K pathway has been shown to be beneficial in the early stages of IAV infection by promoting viral entry into the host cell, however, during the later stages of infection this pathway promotes the antiviral response through expression of interferon (IFN) and ISGs [168]. Another study showed that silencing of the squamous antigen recognized by T-cell 1 (SART1) decreased viral HA, NP and M2 expression as viral protein synthesis was impaired. Furthermore, they found that vesicular transport complex and COPI are important host factors in the secretion and trafficking of viral HA to the plasma membrane via the ER-Golgi transport network [169, 170]. Recently, the nuclear protein ANP32, was shown to be essential for viral RNA polymerase activity, where cells depleted of ANP32 that were infected with IAV resulted in an approximate 70% decrease in IAV replication [171].
<table>
<thead>
<tr>
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<th>Stage of IAV infection</th>
<th>Proposed mechanistic role</th>
<th>Reference</th>
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<tr>
<td>ATP6V0D1</td>
<td>Entry</td>
<td>Low pH dependent entry</td>
<td>[157]</td>
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<tr>
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<tr>
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<td>RNA replication</td>
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<td>[157]</td>
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<tr>
<td>COPI</td>
<td>Entry/late endosome</td>
<td>Endosomal trafficking</td>
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<tr>
<td>Proteases, CTSW</td>
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<tr>
<td>CAMK2B</td>
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<tr>
<td>TNSF12-13</td>
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<td>DR1</td>
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<tr>
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<td>Unknown</td>
<td>[169, 170]</td>
</tr>
<tr>
<td>ANP32</td>
<td>RNA replication</td>
<td>RdRp interaction</td>
<td>[171]</td>
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1.6.2 Antiviral host factors

Infection of host cells by viruses, including influenza viruses induces host cells to secrete type I IFN. An infecting virus is detected by pattern recognition receptors (PRRs) which recognise viral conserved components known as pathogen associated molecular patterns (PAMPs) [172, 173]. Depending upon the virus and the host cell that is infected, different pathways can be activated that lead to the secretion of type I IFN. In the case of RNA viruses such as influenza, there are two different classes of PRRs that detect the invading virus, these are Toll-like Receptors (TLRs) and retinoic acid inducible gene (RIG)-I-like receptors (RLR) [174, 175]. TLR3 and TLR7 are expressed in endosomal compartments and sense dsRNA and ssRNA respectively to promote IFN expression [176-178]. In the cytoplasm of cells, members of the RLR family including RIG-I and melanoma differentiation associated gene (MDA)-5 are capable of recognizing cytoplasmic viral RNA. RIG-I detects short RNA ligands with 5’-triphosphate caps which are generated during viral replication [172, 173] whereas MDA-5 recognizes the long genomic viral RNA segments and replication intermediates [179]. Generally, in airway epithelial cells (the primary target cell type of IAV), IFN induction in response to IAV infection is predominantly RIG-I dependent [179-181]. Upon intracellular sensing of viral PAMPs, type I IFNs are secreted from virus-infected cells which then bind to the interferon α/β receptor of neighbouring cells which activates the IFN signalling pathway. This signalling pathway results in the transcription of hundreds of ISGs and induces the cell into an ‘antiviral state’ which limits further spread of the virus [182]. Cells have evolved a plethora of proteins to inhibit viral replication, some of which are mentioned below and are summarized in Table 2.

1.6.2a Host factors that target viral entry

The interferon-inducible transmembrane (IFITM) family of proteins are some of the most widely studied host factors that restrict IAV replication. The IFITM family consists of IFITM1, IFITM2, IFITM3, IFITM5 and IFITM10 with IFITM1, IFITM2 and IFITM3 recognised as anti-influenza host factors [183, 184]. The anti-influenza activities of IFITM3 are the most well studied, whereby it prevents acidification of endosomes, trapping the virions inside and resulting in their degradation [183, 185]. IFITM3 knockout mice studies have shown that they are more susceptible to IAV infection with exacerbated disease and mortality [185, 186]. Additionally, in humans infected with 2009 pandemic IAV, correlations have
been made between the enrichment of IFITM3 expression and the severity of influenza [186]. The role of IFITM1 and IFITM2 in IAV infection is not well understood.

More recent studies have identified the antiviral role of zinc metallopeptidase STE24 (ZMPSTE24), specifically against viruses that require endosomal compartments in order to enter the host cell [187]. Interestingly, ZMPSTE24 has been detected in association with IFITM protein complexes and is known to inhibit a similar assortment of viruses to the IFITM family of proteins. Furthermore, it has been shown that ZMPSTE24 is required for the antiviral activity of IFITMs, however, the exact mechanism by which ZMPSTE24 acts is still undetermined.

Another study used CRISPR/Cas9 technology to perform a genome-wide overexpression study to identify anti-IAV host factors. Using this method, they were able to show that upon overexpression of the glycosyltransferase B4GALNT2, cell surface attachment by IAV was inhibited in strains with the HA preference for α2,3-linked sialic acids [188]. It is believed that this inhibition is due to the activity of B4GALNT2, which adds GalNAc residues to the sub-terminal galactose moiety of α2,3-linked sialic acids, which impairs HA binding.

1.6.2b Host factors that interfere with viral transcription and translation

The majority of the currently identified host factors that restrict IAV replication are known to target the genomic replication and translation steps of the virus lifecycle. Myxovirus resistance (Mx) proteins are expressed in almost all vertebrates and are well known for their antiviral activities. In humans, Mx1 proteins display potent antiviral effects against IAV by targeting the vRNPs in two ways. Firstly, vRNPs are inhibited from entering the nucleus and so are retained in the cytoplasm [189, 190]. Secondly, the amplification of vRNA from cRNA is blocked, possibly through sequestering of viral NP and PB2 [191, 192]. The biggest determinant to the viral sensitivity to Mx1 is the viral NP as this is the main structural component of the vRNP [191].

Protein kinase R (PKR) is an IFN-inducible protein kinase that is activated by the presence and binding of dsRNA and displays broad antiviral activity against a range of viruses, including IAV. Upon activation, PKR phosphorylates itself and downstream substrates; two of the most important being eukaryotic initiation factor 2α-subunit. (eIF-2α) and IκB. Once phosphorylated, eIF-2α drastically impairs viral protein synthesis and as a result, viral
replication is inhibited. PKR also phosphorylates IκB which then goes on to activate NF-κB to promote the expression of IFN genes [193].

The 2',5'-oligoadenylate (2-5A) synthetases (OAS) are a family of ISGs which synthesize 2-5A, which results in RNA degradation through the activation of RNaseL. Upon binding to dsRNA, OAS1, 2 and 3 become activated and begin to synthesise 2-5A from ATP. The OAS-synthesised 2-5A then binds to cytoplasmic RNaseL and trigger its dimerization and subsequent activation. After becoming activated, RNaseL is then able to degrade ssRNA (both viral and cellular) to limit viral replication and induce apoptosis of the infected cell [194]. In addition, it has been shown that these degraded products can then go on to activate RIG-I to further promote the IFN response [195].

There are 4 members of the IFN-induced protein with tetratricopeptide repeats (IFIT)-family of proteins in humans, IFIT1 (ISG56), IFIT2 (ISG54), IFIT3 (ISG60) and IFIT5 (ISG58) [196]. This family of proteins are known to directly bind with viral RNA which suppresses initiation of translation. Also, they bind to uncapped vRNA and can sequester viral proteins and RNA in the cytoplasm [197]. However, in the context of IAV infection, these functions remain to be determined, although it has been determined that IFIT bind directly with IAV RNA and that depletion of IFT1, IFIT2 and IFIT3 results in enhanced replication of IAV [198].

Other antiviral host factors function by binding directly with viral proteins to inhibit their function. Moloney leukemia virus 10 (MOV10) is an IFN-inducible host factor that binds directly with IAV NP to inhibit its interaction with importin-α. This results in NP being retained within the cytoplasm, preventing formation of the vRNP complex [199]. In addition, DDX21 RNA helicase functions to inhibit the assembly of the viral RNA complex through interacting with PB1, reducing viral RNA and protein synthesis. However, DDX21 itself is the target of NS1 to impair its antiviral function and even promotes its interaction with other viral proteins as a means of promoting viral replication [200]. Plakophilin 2 (PKP2) is another host factor that competes with PB2 for binding with PB1 in order to reduce the activity of the IAV RdRp [201]. Cyclophilin A (CypA) is a member of the immunophilin superfamily that has been shown to interact with the IAV M1 protein [202]. Recently, this has been to accelerate the degradation of M1 via the ubiquitin proteasome 2 pathway [203]. Another member of this family, CypE, also interacts with IAV NP which interferes with NP self-association, NP-PB1 and NP-PB2 interactions [204]. Zinc finger antiviral protein (ZAP) is another IFN-inducible host factor that has two
isoforms that are expressed as a result of alternative splicing, giving rise to long (ZAPL) and short (ZAPS) isoforms, differing at the C-termini [205]. ZAPL is able to bind with IAV PA and PB2 proteins to induce their proteasomal degradation [206]. Meanwhile, ZAPS is able to inhibit the translation of PA, PB2 and NA through downregulating the viral mRNA [207]. Another IFN-inducible gene, ISG15, is an ubiquitin-like protein that binds covalently to target proteins through the enzymatic activity of the E1 activating enzyme UbE1L, the E2 conjugating enzyme UbcH8, and the major E3 ligase Herc5 [208]. During IAV infection, the major target of ISG15 is the viral protein NS1, whereby conjugation of ISG15 with NS1 impairs its functional activity [55]. The tripartite motif (TRIM) proteins are a group of at least 80 members that function as antiviral host factors through their involvement in the innate and adaptive immune responses. Many members of the TRIM family regulate the PRR-mediated signalling pathways induced as a result of viral infection. Others act as direct antiviral restriction factors such as TRIM22 which induces E3 ligase-dependent polyubiquitination of the IAV NP in order to induce its degradation via the proteasome degradation pathway [209]. Additionally, TRIM32 is known to bind with and ubiquitinate viral PB1, again, tagging it for proteasomal degradation [210].

1.6.2c Host factors that block assembly and viral release

There are several cellular host factors which have been identified that target the later stages of the IAV replication cycle. For example, cyclin D3, an important regulator of cell cycle has also been shown to play an antiviral role during IAV infection. Overexpression studies on cyclin D3 have revealed that it directly binds with viral M2, which subsequently interferes with M1-M2 binding, which is an important requirement for the proper assembly of viral progeny [211].

Bone marrow stromal cell antigen (BST)-2 is an IFN-inducible protein that inhibits the release of IAV from the surface of infected cells [212]. BST-2 is a transmembrane protein that is able to tether enveloped viruses including IAV to the plasma membrane of the host cell, however, in the context of IAV, the inhibitory effects appear to be strain specific [213, 214].

Viperin (virus inhibitory protein, endoplasmic reticulum-associated, interferon inducible) is another host factor which has been shown to inhibit virus replication by several mechanisms [215]. In the case of IAV, viperin acts on the later stage of the lifecycle by preventing virion release from the plasma membrane. Viperin reduces biosynthesis of
isoprenoids and thereby disrupts lipid raft formation which IAV uses to bud from the plasma membrane [216].

Plasminogen activator inhibitor 1 (PAI-1) is an ISG that inhibits IAV spread through its ability to inhibit airway proteases required to cleave IAV HA glycoprotein, which is an important step in that maturation of the protein. In humans, complete or partial depletion of PAI-1 due to genetic polymorphism in the SERPINE1 gene leads to increased susceptibility to IAV in vitro [217]. PAI-1 is the first ISG shown to inhibit IAV replication within the extracellular environment.

1.6.3 Histone deacetylases, a recently discovered family of antiviral host factors

Recently, there has been growing interest in a class of cellular enzymes known as histone deacetylases (HDACs) and their emerging role in a number of human diseases including cancer [218], neurodegenerative [219] and cardiovascular complications [220] and various other diseases [221]. Interestingly, there is growing evidence for the role of HDACs in viral infection with numerous studies identifying their dual role in both promoting and inhibiting viral infections [222, 223]. Recently, our lab has identified the role of several different HDACs and the inhibitory role they play against IAV infection. Currently, we have shown that HDACs 1, 2, 6 and 11 all play antiviral roles during IAV infection, and that they themselves are targeted by IAV to downregulate their expression, likely as means to reduce their antiviral function [79, 224-226].
<table>
<thead>
<tr>
<th>Host Factor</th>
<th>Stage of IAV infection</th>
<th>Proposed mechanistic role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFITM3</td>
<td>Entry</td>
<td>Prevents acidification of the endosome</td>
<td>[183, 187]</td>
</tr>
<tr>
<td>MX1</td>
<td>RNA replication</td>
<td>Interferes with viral RdRp</td>
<td>[189, 190, 191, 192]</td>
</tr>
<tr>
<td>eIF-2α</td>
<td>Viral protein synthesis</td>
<td>Inhibits translation</td>
<td>[193]</td>
</tr>
<tr>
<td>OAS</td>
<td>RNA replication</td>
<td>Degradation of viral RNA via RNaseL activity</td>
<td>[194]</td>
</tr>
<tr>
<td>IFIT1, 2, 3, 5</td>
<td>Viral protein synthesis</td>
<td>Bind to viral RNA and prevent translation</td>
<td>[196, 197]</td>
</tr>
<tr>
<td>MOV10</td>
<td>RNA replication</td>
<td>Prevents NP from entering nucleus, inhibiting vRNP complex formation</td>
<td>[199]</td>
</tr>
<tr>
<td>DDX21</td>
<td>RNA replication</td>
<td>Inhibits vRNP complex formation by binding PB1</td>
<td>[200]</td>
</tr>
<tr>
<td>PKP2</td>
<td>RNA replication</td>
<td>Competitively binds PB1, to prevent RdRp formation</td>
<td>[201]</td>
</tr>
<tr>
<td>CypA/E</td>
<td>RNA replication</td>
<td>Prevents NP binding with PB1 and PB2, preventing RdRp formation</td>
<td>[204]</td>
</tr>
<tr>
<td>ZAPS</td>
<td>RNA replication</td>
<td>Inhibits viral mRNA synthesis</td>
<td>[207]</td>
</tr>
<tr>
<td>ISG15</td>
<td>Viral replication</td>
<td>Cellular protein conjugation and antiviral defence</td>
<td>[208]</td>
</tr>
<tr>
<td>TRIM32</td>
<td>Viral replication</td>
<td>Induces proteasomal degradation of viral NP and PB1</td>
<td>[209, 210]</td>
</tr>
<tr>
<td>Cyclin D3</td>
<td>Virion assembly</td>
<td>Interferes with M1-M2 binding, inhibiting proper virion assembly</td>
<td>[211]</td>
</tr>
<tr>
<td>BST-2</td>
<td>Virion release</td>
<td>Tethers enveloped viruses to the plasma membrane</td>
<td>[213, 214]</td>
</tr>
<tr>
<td>Viperin</td>
<td>Virion release</td>
<td>Reduces synthesis of lipid rafts needed for budding</td>
<td>[216]</td>
</tr>
</tbody>
</table>
Section B – Histone Deacytelases (HDACs)

HDACs are a family of enzymes that were originally discovered to regulate gene expression by inducing conformational changes in the structure of chromatin. Chromatin is a highly condensed and packed structure comprised of a dynamic-protein DNA complex. Chromatin is comprised of nucleosomes which are made up of core histones (H2A, H2B, H3 and H4). The posttranslational modification of these histones can cause changes in gene expression and chromatin structure. One of the most common modifications of histones (and the first discovered) is acetylation, which occurs at the ε-amino group of lysines. The acetylation of these lysine residues is a reversible process under the influence of two families of enzymes. Acetylation of these histones is under the influence of the histone acetyl transferases (HATs), while removal of the acetyl groups is catalysed by the HDACs [227]. The acetylation reaction is processed by generating acetyl groups from Acetyl-CoA which is generated as a result of cellular metabolism. Acetyl groups are negatively charged and the addition of these groups generates a repulsive force between the histones and the DNA [228]. This opens the DNA to becoming more accessible to transcription factors, thereby promoting expression of genes [228, 229]. In contrast, deacetylation of histones induces the histone-DNA complex to become more condensed, thus inhibiting transcription factor binding and silencing gene expression [230]. However, recent findings have shown that the activity of HDACs can also be associated with active gene transcription [231-233]. In addition, HDACs have also now been shown to deacetylate non-histone proteins, indicating a highly diverse role for this family of enzymes [234].

1.7 Classification of HDACs

HDACs are evolutionarily conserved enzymes found in plants, animals, fungi and bacteria [235]. In humans, there are 18 different HDACs which are grouped into 4 separate categories referred to as classes based upon their structure, enzymatic activity, intracellular localisation and expression pattern (Figure 4) [236, 237]. The Class I, II and IV HDACs are considered ‘classical HDACs’ and their activity is zinc dependent whereas the Class III HDACs require NAD⁺ for their enzymatic activity [238-241]. The Class I HDACs (HDAC1, HDAC2, HDAC3, and HDAC8) have sequence similarity to the yeast equivalent, Rpd3 protein. They are generally located in the nucleus and are ubiquitously
expressed. However, recent studies have shown that HDAC3 expression is restricted to certain tissues and that these HDACs can be localized to the cytoplasm [242, 243]. The Class II HDACs (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10) have sequence similarity to the yeast equivalent, Hda1 protein. The Class II HDACs are ubiquitously expressed but with enriched expression in specific tissues which varies depending on the Class II HDAC. Interestingly, these HDACs show mixed subcellular distribution, with all showing at least some cytoplasmic distribution, indicating a cytoplasmic role for all Class II HDACs. The divergence of the Class I HDACs and the Class II HDACs appears to have happened relatively early on in the evolutionary history of these proteins. In fact, because of their diversity, the Class II HDACs have since been further subdivided into subclasses; Class IIA (HDAC4, HDAC5, HDAC7 and, HDAC9) and Class IIB (HDAC6 and HDAC10). The Class III HDACs, also known as Sirtuins, (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7) have sequence similarity to the yeast equivalent Sir2 protein. Sirtuins are different from the classical HDACs in that they have two enzymatic activities: mono-ADP-riboseyltransferase and histone deacetylase. Another interesting characteristic is their cellular distribution, with SIRT1 and SIRT2 found in the nucleus and cytoplasm, SIRT3 in the nucleus and mitochondria, SIRT4 and SIRT5 found only in the mitochondria, SIRT6 only in the nucleus and SIRT7 in the nucleolus. In a similar fashion to the classical HDACs, sirtuins also have non-histone substrates. The sole member of the Class IV family, HDAC11, shares little sequence similarity with Class I and Class II HDACs, however, it possesses all 9 catalytic sites required for deacetylase activity [244]. HDAC11 exhibits enriched expression in the kidney, brain, heart, skeletal muscle and testis [221].
Figure 4. Classification of human HDACs. The HDACs are classified into 4 classes. The number of amino acid residues is depicted on the right of each protein. Enzymatic domains are shown in their respective colours. Note, nuc – nucleous, cyt – cytoplasm, mito – mitochondria. Adapted from [430].
1.8 Traditional physiological roles of HDACs

The most obvious function of HDACs is that they oppose the functions of the HATs, which is a crucial part of maintaining the dynamic equilibrium of protein acetylation/deacetylation. Deacetylation of histones and non-histone proteins induces chromatin conformational changes or can alter the activity of transcription factors resulting in significant changes in gene expression. A high-resolution mass spectrometry analysis identified at least 3,600 acetylation sites in 1,750 histone and non-histone proteins within the cell [245]. Therefore, the molecular changes induced by HDACs can have significant impacts on cellular function and as such, play important roles in health and disease.

1.8.1 HDACs indirectly control many post-translational modifications

The lysine ε-amino group is capable of receiving many different PTMs, including acetylation, methylation, ubiquitination, sumoylation, neddylation, propionylation, butyrylation, and crotonylation [246]. Therefore, acetylation of the ε-amino group excludes other modifications on the same residue. In this way, HDACs act indirectly to promote the regulation of other modifications which can drastically alter how a protein behaves within the cell. For example, acetylation is known to inhibit ubiquitination, which can lead to proteasome-mediated degradation of the ubiquitinated protein [247]. Consequently, HDACs can indirectly accelerate the degradation of proteins by exposing ε-amino group for ubiquitination. HDACs are also important regulators of histone and chromatin crosstalk through regulating histone acetylation and methylation. For example, acetylation of lysine 9 of histone 3 (H3K9) inhibits methylation at the same residue and also promotes methylation at the lysine 4 residue (H3K4) [248]. Subsequently, the combination of these modifications induces the chromatin structure to become more accessible, promoting transcription activation. Therefore, HDACs that deacetylate H3K9 inhibit H3K4 methylation, and ultimately repress transcription. The role of the different HDACs is summerized in Table 3.

1.8.2 HDACs control gene transcription

The traditional view of acetylation regulating transcriptional activity dictated that acetylation of core histones reducing DNA binding, making the DNA more accessible for transcription. Alternatively, histone deacetylation strengthens histone-DNA interactions, thereby repressing transcription [249]. However, recent evidence is now indicating a role for acetylation/deacetylation providing a specific docking surface for proteins. In other
words, acetylation of histones might induce interactions with transcriptional activators while deacetylation creates binding sites for transcriptional repressors. Interestingly, it appears that HDACs are capable of repressing transcription, independent of their deacetylase activity as determined in a recent study on HDAC5 and HDAC7 [250]. Furthermore, recent evidence shows that transcriptional repression may also be determined by the particular lysine residue that HDACs deacetylate. For example, deacetylation of H4K16 may result in global transcription repression, while deacetylation of H4K5, H4K8, or H4K12 alone has little effect, but in combination has a cumulative effect on transcription [251]. Finally, there is growing evidence that suggests HDACs may not only repress transcription, but can actually promote transcription of certain genes. For example, HDAC3 represses transcription when targeted to promoters but in contrast, it is also required for the transcription of at least one class of retinoic response elements [252, 253]. In support of this, cells derived from Hdac3 knockout mice show both upregulation and downregulation of gene expression [254]. Furthermore, gene expression profiles of cells treated with HDAC inhibitors compared to untreated cells showed similar levels of gene upregulation as the Hdac3 knockout derived cells [255]. It is possible that HDACs may downregulate transcription of transcriptional repressors, indirectly promoting gene expression. Another possibility is that HDACs may deacetylate and therefore, activate transcriptional activators or alternatively inhibit the activity of transcriptional repressors independent of modifying histones. Regardless, it is obvious that HDACs are clearly diverse in their role in controlling gene transcription, however the exact mechanisms of these activities remains to be determined.

1.8.3 The importance of HDACs in health and disease

Given the degree with which HDACs affect gene expression as well as their effects on protein function through non-histone deacetylation, it is unsurprising that HDACs have been implicated in almost every aspect of human health and disease. Most noteworthy are the roles HDACs have been shown to play in development, cancer, neurodegenerative diseases, immunological disorders, cardiac diseases and pulmonary diseases [221].

1.8.3a Class I

HDAC1 has been implicated in playing an essential role in cell cycle progression [256, 257], proliferation and differentiation [258, 259], adipogenesis [260], muscle development [231, 261], and autophagy [262]. HDAC1-null mice die before embryonic day 10.5 and display severe cardiac abnormalities [231, 259]. In addition, knockdown of hdac1 in zebrafish
results in a variety of lethal defects in skeletal and neuronal development [263-267]. HDAC1 has also been shown to play a role in intestinal metabolism through regulation of the levels of acetylation of certain inflammatory genes in order to minimise metabolic stress and an impaired response to oxidative stress. HDAC1 has also been linked to nutrient sensing and metabolism through activation of the adenosine monophosphate activated kinase [268].

HDAC2 has been defined as playing a major role in cell differentiation and proliferation [256], with HDAC2 knockout mice being only partially viable, depending on the knockout allele. Knockout of HDAC2 usually results in severe cardiac defects leading to cardiac hypertrophy [231]. Dysregulated HDAC2 expression has also been linked to several different cancers including gastric, colon and prostate cancer [269-271]. Additionally, HDAC2 activity has been reported to be important for normal brain development, which otherwise can result in perinatal lethality [272].

Loss of HDAC3 in the liver results in the disruption of lipid and cholesterol homeostasis leading to an accumulation of lipids and a decrease in glycogen storage [273]. Additionally, deletion of HDAC3 in cardiomyocytes has been shown to lead to a significant increase in lipid storage in the heart, resulting in massive cardiac hypertrophy [274]. HDAC3 has been shown to be important in promoting various cancers including pancreatic, colon, breast and ovarian [275-278].

HDAC8 has been identified as an important mediator of normal cranial development, where HDAC8-knockout mice die during the perinatal period as a result of skull instability leading to brain trauma [279]. HDAC8 is expressed in several cancer tissues, including colon, breast, lung and pancreas. Knockout of HDAC8 by RNAi, inhibits proliferation of human lung, colon and cervical cancer cell lines, whereas upregulation of HDAC8 promotes proliferation [280-282].

1.8.3b Class IIa

HDAC4 has been highly implicated for its role in the development of the skeleton [283]. Mice with a global deletion of HDAC4 die early on due to ectopic ossification of endochondral cartilage, which prevents expansion of the ribcage which prevents normal breathing. Furthermore, HDAC4 has been implicated in the pathogenesis of Huntington’s disease as well as other neurodegenerative diseases [284]. HDAC4 has also been shown to be associated with several forms of cancer including colonic and gastric cancer [285, 286].
HDAC5 is important in protecting against cardiac hypertrophy as a study showed that HDAC5 knockout mice develop cardiac hypertrophy with age in response to excess workload or neuronal signalling [287]. Cytoplasmic accumulation of HDAC5 is necessary for axon regeneration, as expression of an HDAC5 mutant which is only present in the nucleus, interferes with axon regeneration [288]. This is consistent with the role that HDAC5 is known to play in controlling memory function with the dysregulation of HDAC5 being linked to Alzheimer’s disease [289].

HDAC7 is highly expressed in developing thymocytes at the CD4+ and CD8+ double-positive stage [290]. More recently it was shown that deletion of HDAC7 results in a significant reduction in the survival or positive selection of single-positive CD4+ lymphocytes [291]. In addition to its role in T cell development, HDAC7 has also been shown to be important in the development of B lymphocytes [292]. It is believed that HDAC7 is an important transcriptional repressor of lineage-inappropriate genes in B lymphocytes, however the effects of this in vivo remain to be established.

In a similar fashion to HDAC5, HDAC9 has also been reported to be important for protecting against cardiac hypertrophy. In fact, a combined deletion of both HDAC5 and HDAC9 genes in mice show a propensity for lethal ventricular septal defects and thin-walled myocardium, both of which arise from abnormal growth and maturation of cardiomyocytes [287]. Recently, HDAC9 has also been linked to neuronal physiology and pathology. HDAC9 is expressed in the mouse cerebellar cortex during postnatal cortical development. During development of postnatal cortical neurons, HDAC9 is exported to the cytoplasm leading to the expression of the c-Fos gene which promotes dendritic growth [293]. In the context of neuronal pathology, the effects of HDAC9 depletion in mice remains to be determined. It has been reported that in patients with schizophrenia, HDAC9 is hemizygously deleted suggesting a link between HDAC9 and schizophrenia [294].

1.8.3c Class IIb

HDAC6 is a unique enzyme within the HDAC family owing to the fact that it possesses 2 catalytic domains. While HDAC6 knockout mice are viable, aberrant expression of HDAC6 has been linked to certain pathological conditions. In particular, abnormal HDAC6 expression has been linked to neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease and Huntington’s disease [295]. Overexpression of HDAC6 has also been implicated in tumorigenesis, cell survival and tumour metastasis [296-298].
HDAC10 has been reported to be involved in homologous recombination, melanogenesis, cell autophagy, cell cycle regulation and DNA mismatch repair [299-303]. Additionally, HDAC10 has been shown to be involved in a number of different carcinomas, a recent study showed that overexpression of HDAC10 promotes lung cancer growth. Conversely they showed that knockdown of HDAC10 induces G1 arrest and apoptosis in lung cells [304].

1.8.3d Class III

SIRT1 mainly regulates cellular metabolic patterns, while its own activity is influenced by nutrient availability, specifically, induction during under-nutrition [305]. SIRT1 performs a variety of functions, including mitochondrial biogenesis as well as catabolism of triglycerides and cholesterol in the liver, skeletal muscles and adipose tissue [306, 307]. Furthermore, it inhibits glycolysis while also activating gluconeogenesis and fatty acid oxidation in most tissues [308, 309]. Several studies have linked lack SIRT1 to an increase in susceptibility to diet-induced obesity [310]. SIRT1 has also been shown to be involved in carcinogenesis through its activity in promoting DNA damage repair, inhibiting chronic inflammation, downregulation of the HIF-1α and upregulation of another sirtuin – SIRT6 [311-313].

In humans SIRT2 is mainly active in the cytoplasm, where one of its main substrates is the α-tubulin in microtubules [314]. SIRT2 has similar effects to SIRT1 on carbohydrate and lipid metabolism by promoting gluconeogenesis and inhibiting adipocyte differentiation [315]. Knockout studies of SIRT2 reveal that SIRT2-deficient mice show increased prevalence and incidence of cancer [316]. Furthermore, SIRT2 also possesses anti-inflammatory functions through repression of nuclear factor KB (NF-KB) [317].

SIRT3 is located in the mitochondria and has a number of mitochondrial substrates including: complex I, complex III, manganese superoxide dismutase (MnSOD) and isocitrate dehydrogenase [318, 319]. The deacetylase activity of SIRT3 is known to increase the efficacy of the electron transport chain, preventing production of reactive oxygen species (ROS) from oxidative phosphorylation byproducts [320]. In addition, SIRT3 activates MnSOD, which facilitates the further removal of ROS from cells [321]. Deficiency of SIRT3 is characterized by an increase in ROS which induces DNA damage and activation of the HIF-1α transcription factor, thus it is believed that SIRT3 possesses cancer-preventative functions [322, 323].
SIRT4 is thought to regulate ATP homeostasis and to provide retrograde signalling from the mitochondria to the nucleus. SIRT4 activity within the mitochondria improves the efficacy of ATP synthesis by inhibiting the oxidative phosphorylation uncoupler – ANT2 [324]. During the cell response to DNA damage, SIRT4 activity enables the use of the glutamine-derived nitrogen atoms in the purine nucleotide synthesis which is essential for DNA repair [325]. Loss of SIRT4 promotes the accumulation of DNA damage through impairment of the DNA repair system. As such, SIRT4 knockout mice have increased susceptibility to cancers, in particular lung tumours [326].

SIRT5 promotes the urea cycle by activating carbamoylphosphate synthetase with SIRT5 deficient mice exhibiting elevated levels of ammonia in the blood [327]. SIRT5 has also been shown to be involved in decreasing cellular ROS concentrations by activating CU/Zn superoxide dismutase 1 [328].

SIRT6 plays a key role in mediating DNA repair and genomic stability by integrating the actions of DNA-damage signalling factors with recruitment of DNA repair enzymes, particularly during oxidative stress [329]. SIRT6 knockout mice die within 4 weeks due to severe metabolic disorders such as: loss of subcutaneous fat, lordokyphosis, colitis, lymphopenia, osteopenia and progressive hypoglycaemia [330]. Additionally, SIRT6 has been identified as a potent tumour suppressor through suppression of cancer metabolism [331]. In fact, overexpression of SIRT6 causes profound apoptosis in cancer cells but not in normal cells [332].

SIRT7 is a nuclear protein where it promotes rDNA transcription, particularly in proliferating cells [333]. Global SIRT7 depletion has been linked to premature aging, especially in certain tissues such as the backbone, white adipose tissue and the heart [334]. Contrastingly, elevated levels of SIRT7 have been associated with aggressive cancer phenotypes, metastatic disease and poor patient prognosis [335].

1.8.3e Class IV

HDAC11 is the sole member of the Class IV HDAC family and it shares similarities between both Class I and Class II HDACs [244]. HDAC11 has been defined as having an important role in immune cells. Elevated levels of HDAC11 have been observed in neutrophils as compared to other innate immune cells, and increased levels of HDAC11 correlate with more aggressive neutrophil phenotypes [336]. Depletion of HDAC11 in T
cells enhances their proliferation, inflammatory cytokine production and become less responsive to suppression by regulatory T cells [337].

### 1.9 Emerging role of HDACs in virus infection

While considerable research has looked into the role HDACs play in physiological diseases, emerging research is now looking into the role they play in infectious diseases, particularly viral infections. HDACs have been shown to have differential roles during viral infections which is dependent on the nature of the virus infection. During chronic infection HDACs appear to promote viral latency and thereby allowing the infection to persist whereas during an actively replicating viral infection, HDACs promote the antiviral immune response through promotion of the IFN immune response. Additionally, new research is being done by combining HDAC inhibitors with oncolytic viral therapy to enhance tumour cell killing. The role of HDACs in viral infections is summarised in Table 3.

#### 1.9.1 Role in chronic viral infections and promoting viral latency

The nature of chronic virus infections means that much of the life cycle of the virus is present in a latent stage, whereby no active virus replication is carried out. In this state, viruses can hide from the immune system, allowing the virus to persist within an infected individual, in some cases indefinitely. Under certain conditions the virus can switch from a latent infection to an active infection where new viral progeny is produced, often resulting in the infected individual becoming symptomatic and infectious. Numerous studies have reported on the role of HDACs in chronic viral infections as they are often portrayed as promoters of latency. Further research is being carried out looking into the role of using HDAC inhibitors combined with antiviral drugs in order to enhance the antiviral therapy [222].

After gaining entry into the host cell, the human immunodeficiency virus (HIV) carries out reverse transcription of its RNA and integrates the newly transcribed DNA into the infected cells genome. It is now well understood that epigenetic modifications and changes in chromatin structure directly govern viral promoter activity to influence viral latency and reactivation. In this regard, HDACs have been highly studied in their role in promoting latency of HIV. HDAC1, HDAC2 and HDAC3 are all known to be recruited by various transcription factors including COUP-TF interacting protein, c-promoting binding factor-1, c-myc and Sp1 to the HIV long terminal repeat (LTR) promoter [338-340]. Upon
recruitment to the LTR, these HDACs deacetylate histone proteins locally which leads to transcriptional silencing of viral genes.

During hepatitis B virus (HBV) infection of hepatocytes, the virus induces expression of many pro-survival genes often linked with tumour growth and metastasis. One of the key HBV proteins responsible for inducing the expression of these genes is the HBx protein. HBx is known to induce HDAC1 to form a complex with Sp1, resulting in the deacetylation of Sp1 and reduced transcription of insulin-like growth factor binding protein 3 (IGFBP3). The outcomes of these epigenetic changes promote cell survival and proliferation allowing chronic HBV infections to develop [341]. In addition, HBx induces a complex to form between HDAC1/2 and metastasis-associated protein 1 (MTA1) to stabilise HIF-1α which is believed to play an important role in angiogenesis and metastasis of HBV-associated hepatocellular carcinoma [342]. SIRT1 has been shown to be significantly upregulated during HBV infection and that SIRT1 enhances HBV core promoter activity through association with transcription factor Ap-1 which is important in promoting cell survival and inhibiting apoptosis [343]. Meanwhile depletion of SIRT1 has been shown to inhibit HBV replication [344]. SIRT2 has also been shown to be upregulated during HBV infection by the activity of HBx. This upregulation of SIRT2 has been shown to promote HBV transcription and replication [345].

Epstein-Barr virus (EBV) is a gammahepresvirus that establishes latent infection, predominantly in B lymphocytes. Class I HDACs have been implicated in maintaining EBV latency, with several studies demonstrating the use of pharmacological inhibitors of these HDACs inducing reactivation [346]. Furthermore, HDAC1, HDAC2 and HDAC3 have been found to be overexpressed in EBV-infected cells as compared to healthy cells [347].

Changes in chromatin structure have also been reported to be important in regulating the latency of herpes simplex virus (HSV). An early study using the general HDAC inhibitor trichostatin A (TSA) on latently infected neurons revealed that the activity of the viral immediate early promoter increased substantially [348]. More recently, studies have been able to demonstrate that the HSV immediate early regulatory protein ICP0 is important for stimulating the initiation of the lytic cycle through inhibiting HDAC activity. Specifically, ICP0 dissociates HDAC1 and HDAC2 from the CoREST/REST complex which is normally bound to the viral genome, inhibiting viral gene expression [349].

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Permissiveness for human cytomegalovirus (HCMV) infection is dependent on the state of cellular differentiation and is linked to the repression of the viral major immediate early promoter (MIEP). HDAC1 has been shown to promote HCMV latency by interacting with the viral immediate early (IE) protein IE86 which results in repression of MIEP [350]. Meanwhile, HDAC3 is the target of IE1 and IE2, which antagonize HDAC3 to promote viral replication [351]. Contrastingly, the sirtuins appear to play an antiviral role during HCMV infection as siRNA-mediated knockdown of each of the sirtuins promotes HCMV infection [352].

1.9.2 Role in acute viral infections and the innate antiviral response

During acute viral infections, HDACs are primarily involved in regulating the host innate antiviral response, as they can act as co-activators and co-repressors of gene transcription. HDACs are known to positively regulate Toll-like receptor responses by regulating transcription factor function [353]. Members of the interferon response element (IRF) family such as IRF7 can be acetylated within their DNA binding domain, which is associated with impaired DNA binding. Thus, it is believed that deacetylation of IRF family members is important for IRF nuclear translocation and DNA binding, however the specific HDACs that are responsible for this remain unknown [354]. HIF-1α is another pro-inflammatory transcription factor that has been shown to be regulated via interactions with HDAC1, HDAC3, HDAC4, HDAC6 and HDAC7 to positively regulate its function [355, 356]. Another transcription factor which is involved in the antiviral response is NF-KB. NF-KB can be deacetylated within the nucleus which promotes its nuclear export, attenuating its activity [357]. In addition, HDAC activity has been implicated in regulating the type I IFN response. Acetylation of the IFNα receptor leads to decreased association with IFNα, however this is reversed by the deacetylase activity of HDAC3 which enhances IFNα binding [358]. STAT proteins have been observed to directly interact with HDACs and inhibition of HDAC activity leads to hyperacetylation of STAT [359]. Several studies have revealed that HDAC activity is required for STAT-dependent gene expression. In particular, during the antiviral immune response, IFN-induced STAT1 and STAT2 signalling is significantly impaired after general HDAC inhibition [360]. Importantly, during infection, many viruses are able to actively target these HDACs as a means to antagonise their antiviral function.

During Japanese Encephalitis Virus (JEV) infection HDAC3 mediates the antiviral response by deacetylating NF-KB to promote its activation. In response, HDAC1,
HDAC2 and HDAC3 are all downregulated during infection after 12 and 24 hours of infection with JEV which corresponds with a decrease in NF-KB activation [361]. In contrast, HDAC6 appears to promote JEV replication as treatment with specific HDAC6 inhibitors but not general HDAC inhibitors reduced JEV replication [362].

During adenovirus infection HDAC1 activity inhibits virus replication through some unknown mechanism. However, adenovirus encodes an early gene product known as Gam1 which binds to HDAC1 and inactivates it [363].

HDAC5 has been shown to be an important antiviral factor that restricts vaccinia virus, likely through the promotion of many ISGs. In turn, the vaccinia virus early protein C6 targets HDAC5 for proteasomal degradation [364]. Furthermore, the use of the general HDAC inhibitor TSA has been linked with enhanced vaccinia virus growth, both in vitro and in vivo [365].

Many of the HDACs have been reported to be involved in restricting influenza virus replication. HDAC1 and HDAC2 have both been shown to modulate the innate immune response to restrict IAV replication. However, they themselves are targeted by IAV to antagonise their antiviral function [225, 226]. HDAC6 is also known to restrict IAV in several ways. Firstly, it interferes with the trafficking of viral components destined for assembly through deacetylation of acetylated microtubules [79]. Secondly, it promotes the innate immune response by deacetylating RIG-I which enhances its viral-RNA sensing activities [366]. Depletion of HDAC6 impairs the cellular immune response to influenza and HDAC6 knockout mice are much more susceptible to influenza infection [367]. Finally, a recent study showed that HDAC6 impairs the activity of the IAV PA protein in two ways. HDAC6 deacetylates PA which suppresses viral RNA polymerase activity and subsequently viral replication. HDAC6 also induces the ubiquitination of PA which induces its degradation via the proteasomal pathway [368]. In order to minimise the antiviral potential of HDAC6, IAV induces its degradation via caspase 3 activity [369]. HDAC8 has also been reported to play a role in IAV infection as expression of the micro RNA miR-21-3p actively downregulates HDAC8 expression leading to enhanced IAV replication [370]. All members of the class III HDAC family have been shown to have anti-IAV properties as the knockdown of each member of this family promoted IAV replication [352]. Recently, HDAC11 has been identified as part of the host innate anti-IAV immune response. Depletion of HDAC11 by RNAi resulted in greater IAV replication and decreased ISG expression during infection. Furthermore, IAV infection
induced a significant reduction in HDAC11 mRNA during infection, likely as a means to counteract its role in promoting ISG expression [224].

1.9.3 Role in oncolytic viral infection

HDAC inhibitors have been recognised for their ability to weaken the cellular anti-viral immune response by impairing IFN and IFN-inducible gene expression. Therefore, several studies have looked into using HDAC inhibitors in combination with oncolytic viral therapy in order to suppress the immune system in tumour cells to improve viral replication and ultimately destruction of the tumour cells.

Vesicular stomatitis virus (VSV)Δ51 is a naturally occurring VSV variant which contains a deletion in the M gene, which renders the virus unable to counteract the IFN response in cells [371]. Therefore, VSVΔ51 replication and lytic activity can only occur in cancer cells with defective IFN responses, however some cancer cells possess residual IFN activity capable of restricting VSVΔ51 growth. In order to overcome this constraint, two HDAC inhibitors, vorinostat and MS-275 have been used in combination with VSVΔ51 in order to inhibit IFN-inducible gene expression. Both inhibitors were able to enhance growth of VSVΔ51 and promote the intrinsic apoptotic pathway, resulting in a synergistic induction of cancer cell death. These effects were accompanied by vascular shutdown, resulting in reduced blood flow within the tumour mass [372]. These inhibitors were shown to interfere with NF-KB signalling, suppressing the IFN-response and subsequently promoting VSV replication and apoptosis [373].

Otuski et al. carried out a study using the HSV-1 oncolytic variant rQNestin34.5 in combination with the HDAC inhibitor valporic acid in glioma-derived cell lines [374]. rQNestin34.5 contains the RL1 gene which encodes the viral virulence factor ICP34.5 which is under the control of the glioma-specific nestin promoter [375]. The study revealed that pre-treatment with valporic acid suppressed transcription of antiviral ISGs such as STAT1, PKR and promyelocytic leukemia (PML), which was sufficient to enhance HSV gene expression, replication and cytotoxicity. Another study used the general HDAC inhibitor TSA to enhance HSV replication and oncolytic activity of another HSV variant, R849 which lacks the virulence factor ICP34.5. Here, TSA treatment resulted in the enhanced activation of NF-KB as well as upregulation of p21 which induced G1 cell cycle arrest to inhibit tumour cell growth [376].
The combination therapy of using oncolytic adenoviruses and HDAC inhibitors has become an attractive strategy for improving oncolytic viral therapy. Ad5 is the most commonly used oncolytic virus variant which infects host cells through the cellular coxsackievirus and adenovirus receptor (CAR) and α-integrin which mediate cell attachment and internalization respectively. However, in cancer cells CAR is often expressed at lower levels which impairs viral binding and consequently hampers the anti-tumour efficacy of Ad5. Recently, a number of HDAC inhibitors have been reported to increase the infectivity of AD5 by promoting the expression of CAR and α-integrin in various cancer cell lines [377-383]. Furthermore, this induced expression of CAR and α-integrin appears to preferentially occur in cancer cells which minimises potential off-target effects with Ad5 infecting normal cells [378, 379, 381].

Vaccinia virus has also been shown to have enhanced oncolytic activity in combination with HDAC inhibitor treatment. TSA was found to be the most potent HDAC inhibitor by increasing vaccinia virus replication and tumour cell killing activity [365]. While pre-treatment with IFN protected cancer cells from vaccinia virus infection, TSA treatment attenuates this protection and rescues vaccinia virus infectivity. Interestingly, TSA did not counteract the IFN response in normal cells, highlighting the specificity for cancer cells. Several cancer cell types have been tested with TSA co-treatment with vaccinia virus including lung and colon cancer cells [384, 385].
Table 3. Role of HDACs in health, disease and virus infection

<table>
<thead>
<tr>
<th>HDAC</th>
<th>Role in Health and Disease</th>
<th>Role in Virus Infection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC2</td>
<td>Cell proliferation, cancer development, brain development</td>
<td>HIV and EBV latency, JEV and IAV innate immune response</td>
<td>[226, 256, 269, 272, 338, 347, 361]</td>
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<tr>
<td>HDAC3</td>
<td>Cholesterol homeostasis, cancer development</td>
<td>HIV, EBV and HCMV latency, JEV innate immune response</td>
<td>[273, 275]</td>
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<tr>
<td>HDAC4</td>
<td>Skeletalgenesis, neurodegenerative diseases, cancer development</td>
<td>HIV, EBV, MHV68 and HSV latency, HBV, HCV and VSV innate immunity</td>
<td>[283, 284, 285, 338, 417, 418, 420, 423, 426]</td>
</tr>
<tr>
<td>HDAC5</td>
<td>Cardiac growth, axon regeneration</td>
<td>Vaccinia virus innate immune response</td>
<td>[287, 288, 364]</td>
</tr>
<tr>
<td>HDAC6</td>
<td>Neurodegenerative diseases, cancer development</td>
<td>IAV immunity</td>
<td>[79, 295, 296, 366, 367, 368]</td>
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<tr>
<td>HDAC7</td>
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<td>HDAC8</td>
<td>Cranial development, cancer development</td>
<td>IAV replication</td>
<td>[279, 280, 370]</td>
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<td>HDAC11</td>
<td>Immune cell proliferation</td>
<td>IAV innate immune response</td>
<td>[224, 336, 337]</td>
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1.10 Aim of Thesis

This research set out to uncover the role of class IIa HDAC, HDAC4 in the context of IAV infection which has not previously been reported. Given the previous work done in our lab where we have identified the anti-IAV role of several other HDACs, we hypothesized that HDAC4 is also an anti-IAV host factor. The first results chapter (Chapter II) aims to identify if HDAC4 possesses an inherent antiviral function using RNAi and HDAC4 overexpression studies to determine the effect on IAV replication. Chapter III then goes on to explore the antiviral function of HDAC4 in greater detail to understand the mechanism by which HDAC4 is able to exert its antiviral potential through regulation of the innate immune response. Finally, Chapter IV looks at how IAV targets HDAC4 to dysregulate its antiviral function by inducing degradation of the HDAC4 polypeptide and downregulation of the mRNA.
CHAPTER II - RESULTS:

THE ANTIVIRAL PROPERTIES OF

HISTONE DEACETYLASE 4
2.1 Introduction

HDAC4 is a member of the class IIa in HDAC family which are characterised by a large N-terminal extension with conserved binding sites for the transcription factor MEF2 and the chaperone protein 14-3-3. HDAC4 is ubiquitously expressed with enriched expression in the brain, heart and skeletal tissues [386, 387]. HDAC4 has been shown to play a central role in the formation of the skeleton and is highly expressed in pre-hypertrophic chondrocytes \textit{in vivo}. Mice with a global deletion of HDAC4 gene die during the perinatal period due to abnormal and premature endochondral ossification which prevents expansion of the rib cage and leads to an inability to breathe [283]. While HDAC4 is most characterised for its role in bone development, it is also known to play an important role in other tissues and its aberrant expression is linked to a number of health complications including heart disease, neuronal degeneration and cancer [388].

2.1.1 Physiological functions of HDAC4

HDAC4 regulates the expression of genes involved in cell growth, survival and proliferation, with abnormal expression resulting in aberrant physiology and death. HDAC4 is most well characterized for its role in regulating chondrocyte hypertrophy and endochondral bone formation through inhibiting the activity of MEF2C and Runx2 [283, 389]. MEF2C regulates the expression of extracellular matrix proteins including collagen type X alpha 1 and vascular endothelial growth factor which are both important for the late stages of chondrocyte development [390-392]. Runx2 is important in the expression of the secreted growth factor Indian hedgehog which is important in enhancing chondrocyte proliferation, especially during endochondral ossifications [393, 394]. In the absence of HDAC4, transcriptional activation of MEF2C and Runx2 is uncontrolled leading to excessive bone formation. Thus, HDAC4 is able to delay the expression chondrocyte hypertrophy and thereby control the timing and extent of ossification of endochondral bones through regulation of Runx2 and MEF2C.

HDAC4 also plays an important role in muscle development through the regulation of MEF2C, particularly in cardiac muscle growth. HDAC4 binds directly to and represses MEF2 function, thereby controlling the diversification of mesoderm cells into cardiomyoblasts through inhibition of GATA4 and Nkx2-5 expression [395]. Inhibition of HDAC4 activity induces the specification of mesodermal cells into cardiac-muscle lineage, as GATA4, Nkx2-5, MEF2C and cardiac alpha-actin are all upregulated [395]. The repression of these genes through the MEF-2/HDAC4 complex is relieved through CaMK
phosphorylation of HDAC4, inducing its translocation into the cytoplasm [396]. An overabundance of CaMK expression induces cardiac hypertrophy as cardiomyocytes undergo hypertrophic growth due to increased MEF2C activity [397]. A number of cardiovascular disorders arise as a result of cardiac hypertrophy including myocardial infection, arterial hypertension and altered contractility.

HDAC4 expression is also highly enriched in the brain and nervous tissue where it is predominantly expressed in the cytoplasm [398]. Depletion of HDAC4 in the forebrain of mice results in the impairment of memory, behavioural learning and long-term synaptic plasticity [399]. Additionally, overabundance of HDAC4 within the nucleus of neurons affects gene transcription profile of the central synapsis, directly affecting information processing in the brain [400]. Abnormalities of HDAC4 expression have been linked with several neurological disorders in human patients too. HDAC4 locus has been observed to be mutated in patients with brachydactyly mental retardation (BDMR) syndrome which is characterised by intellectual disabilities, development delays and behavioural abnormalities [401]. In patients with ataxia telangiectasia, a neurodegenerative disease, HDAC4 is highly localized to the nucleus of neurons [402]. In the nucleus, HDAC4 interacts with the transcription factors MEF2A and cAMP response element binding protein. This results in altered gene expression programs associated with neuronal degeneration [403]. Additionally, HDAC4 has been implicated in the pathogenesis of Huntington’s disease (HD) where HDAC4 associates with huntingtin and co-localises with cytoplasmic inclusions. The reduction of HDAC4 in HD mouse models delayed the formation of cytoplasmic aggregates and rescued neuronal synaptic function [404].

2.1.2 HDAC4 and cancer

HDAC4 has also been shown to be involved in the development of several forms of cancer. Increased HDAC4 expression has been linked to promotion of cell growth and proliferation of various cancers, including gastric cancer, ovarian cancer, lung cancer, head and neck cancer, colon cancer, glioblastoma and oesophageal carcinoma [286, 405-409]. This occurs through nuclear accumulation of HDAC4 which is linked to repression of many genes, in particular p21, which is important in promoting cell cycle arrest. Additionally, the downregulation of a number of different miRNAs that specially target HDAC4 has also been found in many different cancer types [408, 410]. Furthermore, inhibition of HDAC4 has been shown to make certain cancer types more sensitive to anti-cancer drugs. For example, inhibition of HDAC4 has been found to decrease HIF-1
transcriptional activity resulting in the reduction of resistance to docetaxel chemotherapy in prostate and liver cancers [411]. Additionally, increased HDAC4 expression has been identified in patients with platinum-resistant ovarian cancer [412]. Furthermore, elevated levels of HDAC4 have also been identified in bladder tumour tissues as compared to healthy bladder tissues [413].

2.1.3 The role of HDAC4 in virus infection

HDAC4 has been reported to be involved in several different latent viral infections, the most characterised of which is HIV-1. HDAC4 has been shown to be important in maintaining HIV-1 latency. Both the downregulation of HDAC4 and the pharmacological inhibition of HDAC4 are shown to promote reactivation of HIV-1 transcription and reverse transcriptase activity [338, 414-416]. HDAC4 has also been shown to be involved in maintaining the latency of several gammaherpesvirus, such as EBV. It has been proposed that HDAC4 inhibits the expression of the EBV BZLF1 gene which is an important transcription factor that mediates the switch from latency to the productive cycle. The inhibition appears to be induced through recruitment of HDAC4 to the BZLF1 gene promoter via DNA-bound MEF2 proteins [417]. Additionally, HDAC4 has been shown to inhibit the expression of the murine gammaherpesvirus 68 (MHV68) gene, gene 50, which is involved in the switch from latent gene expression to lytic replication. Downregulation of HDAC4 in murine macrophages infected with MHV68 was sufficient to promote active viral replication [418]. Human papillomavirus is able to manipulate tumour angiogenesis through dissociation of HDAC4 from HIF-1α via its E7 oncoprotein [419]. HDAC4 also plays a role in herpes simplex virus 1 (HSV-1) infection and appears to be an important target to mediate the switch from the latent cycle to lytic cycle. The HSV-1 gene ICP0 has been shown to interact with, and localises HDAC4 to the cytoplasm, alleviating its transcription repressive properties [420]. Interestingly, HDAC4 appears to be an important gene in the active replication of HSV-1, as cells lacking HDAC4 produce significantly less HSV-1 than normal expressing cells [421]. Contrastingly, a recent study showed that HDAC4 enhances the type I IFN response during infection with HSV-1 and vaccinia virus (VACV) infection. Mechanistically, HDAC4 interacts with STAT2 and is then recruited to IFN-stimulated response element (ISRE)-containing promoters. As a means to counter this type I IFN signalling, VACV C6 protein interacts with HDAC4 to induce its degradation via the proteasomal pathway [422].
While HDAC4 appears to benefit chronic viral infections, its role is reversed during actively replicating viral infections whereby it inhibits virus replication. For example, in cells infected with HBV, the expression of miR-548 is upregulated and targets HDAC4 to decrease its expression. HDAC4 is believed to inhibit HBV through deacetylation of the HBV covalently closed circular DNA (cccDNA) which subsequently inhibits cccDNA transcription. Therefore, HBV replication is promoted through the downregulation of HDAC4 via miR-548 [423-425]. Hepatitis C virus (HCV) also targets HDAC4 transcript expression through the activity of its core protein, as a means to block HDAC4-induced IFN signalling. Additionally, it has been shown that ectopic expression of HDAC4 promotes resistance in hepatocellular carcinoma cells to infection with VSV through enhancement of the IFN response [426]. Finally, a recent study showed that influenza promotes the expression of miR-22 in primary bronchial epithelial cells (pBECs) which goes on to downregulate the expression of HDAC4 [427].

Previously, it has been reported that other HDACs play an antiviral role during infection with IAV. At least one member of each class has been reported to possess anti-IAV properties, including HDACs, 1, 2, 6, all of the sirtuins, and the sole member of the class IV family, HDAC11 [79, 224-226, 352]. Therefore, the purpose of this chapter is to determine if, like the other previously reported HDACs, HDAC4 possesses antiviral properties in the context of IAV infection. The outcomes from this chapter reveal that HDAC4 indeed possesses anti-IAV properties.
2.2 Results

In order to determine the potential antiviral role of HDAC4 in IAV infection, 2 approaches were used. The first was to use siRNA to knockdown HDAC4 expression and determine if this enhanced IAV replication. The second approach was to determine if overexpression of HDAC4 from a plasmid inhibited IAV replication.

2.2.1 Efficiency of HDAC4-targeting siRNA and its effect on IAV infection

First, we wanted to optimize a concentration of siRNA to effectively knockdown HDAC4 expression in human lung alveoli (A549) cells. Therefore, different concentrations (1, 5, 10, 20 nM) of siRNA targeting human HDAC4 and 20 nM of a non-targeting control (CT) siRNA were transfected in A549 cells. After 72 hours, the total cell lysates were prepared and resolved by SDS-PAGE. The level of HDAC4 polypeptide was measured by western blotting (WB) and protein disulphide isomerase (PDI) was detected as a loading control as its levels are unaffected during IAV infection [225, 226]. Each of the different concentrations tested resulted in almost complete depletion of the HDAC4 polypeptide which was barely detectable by WB (Figure 5A). Protein bands were visualized by Odyssey Fc imaging system (Li-COR) and the HDAC4 and PDI bands were quantified using Image Studio Lite V5.2 software (Li-COR). HDAC4 was normalized to PDI and the normalized amount of HDAC4 in the control siRNA-treated sample was considered 100% for comparisons to the HDAC4 siRNA-treated samples. The lowest concentration (1 nM) used was sufficient to reduce the HDAC4 polypeptide level by 98% (Figure 5B). Additionally, this siRNA concentration had a negligible cytotoxic effect as compared to the control siRNA (Figure 5C). This led us to use 1 nM of HDAC4-targeting siRNA for all subsequent experiments to deplete HDAC4 expression.

Additionally, we wanted to confirm that the knockdown of HDAC4 had no substantial effect on the ability of IAV to infect the HDAC4-depleted cells. Here, cells were transfected with control siRNA and HDAC4-targeting siRNA. The cells were then infected at 1.0 MOI for 8 hours after which, immunofluorescent staining of viral NP was done. Briefly, cells were fixed in 4% paraformaldehyde (Sigma) and then were permeabilized using 0.2% TritonX-100. An antibody targeting viral NP was then added, followed by a fluorescent secondary antibody. Finally, Hoechst dye (Invitrogen) was added to stain the nucleus. The cells were observed using fluorescent microscopy in order to determine the percentage of IAV-infected cells. To determine this, the total number of NP positive cells was divided by the total number of cells present in the field of view. This
number was then compared between HDAC4-depleted cells and control cells. Here we were able to determine that approximately 70% of HDAC4-depleted cells and approximately 72% of the cells treated with the control siRNA were infected, thus, indicating that the knockdown HDAC4 had no significant effect on the efficiency of virus infection (Figure 6A-B).
Figure 5. Knockdown efficiency and cytotoxic effect of HDAC4-targeting siRNA. (A) A549 cells were transfected with indicated concentrations of non-targeting control (CT) siRNA or targeting (HD4) siRNA for 72 hours. Total cell lysates were prepared and HDAC4 (140 kDa) and protein disulphide isomerase (PDI) (57 kDa) polypeptide levels were measured by WB. (B) The HDAC4 and PDI bands from the 1 nM sample in panel (A) blot were quantified using Image Studio Lite software (li-Cor). The level of HDAC4 in each sample was then normalized with the corresponding PDI levels. Finally, the normalized level of HDAC4 in CT siRNA transfected cells was considered 100% to compare its level in HD4 siRNA transfected cells. (C) A549 cells were transfected with Lipofectamine (LF) only or in a complex with CT or HD4 siRNA. After 72 hours, the viability of the cells was determined by MTT assay. Then, the viability of LF only transfected cells was considered 100% to compare the viability of CT and HD4 siRNA transfected cells. Error bars represent means ± standard errors of the means of three independent experiments (B) or technical replicates (C). P value calculated using unpaired t test. MW, molecular weight.
Figure 6. Equal numbers of cells were infected in both CT and HD4 siRNA treated cells. 
(A) A549 cells were transfected with 1 nM of CT siRNA or HD4 siRNA for 72 hours. Cells were then infected with PR8 at an MOI of 1.0. After 8 hours the cells were fixed, permeabilized, and then stained with mouse anti-viral NP followed by Alexa 488-conjugated rabbit anti-mouse IgG antibody. DNA binding dye Hoechst was used for nuclear staining and then analysed by fluorescent microscopy. (B) Total numbers of NP positive cells were counted in HD4 and CT siRNA-treated cells. This number was then divided by the total number of cells counted in the same field using the Hoechst nuclear stain to count individual cells. Percentage of infected cells were then compared between CT and HD4 siRNA-treated cells. Error bars represent means ± standard errors of the menas of 3 independent experiments. P value calculated using unpaired t test.
2.2.2 The knockdown of HDAC4 expression promotes IAV infection

Next, we wanted to determine if, like the other previously reported HDACs, HDAC4 possesses antiviral property against IAV. To achieve this, we depleted A549 cells of HDAC4 using the transfection conditions that were optimized above (Figure 5). Here, the cells were transfected with control or HDAC4 siRNA for 72 hours and were then infected with influenza virus strain A/PR/8/34 (H1N1) (hereafter referred to as PR8) at 1.0 multiplicity of infection (MOI) for 24 hours. Following this, the culture media was collected to be titrated by plaque assay in order to determine the amount of infectious virus progeny released in HDAC4-depleted cells versus cells expressing HDAC4 at normal levels. Meanwhile, RNA from the cell lysates was extracted in order to analyse the level of viral mRNA expression. Additionally, some cell lysates were harvested for WB analysis in order to confirm the successful knockdown of HDAC4. The outcome from the plaque assay revealed that IAV was able to replicate more efficiently in cells depleted of HDAC4 as these cells produced approximately 2.6 fold more infectious progeny than the control siRNA-treated cells (Figure 7A). Furthermore, in HDAC4-depleted cells, the mRNA levels of the viral NP gene was found to be 1.9-fold higher and the M gene was 3.9-fold higher compared to the control siRNA-treated cells (Figure 7B). WB analysis of the cell lysates revealed that the knockdown of HDAC4 was successful, but also revealed that in control siRNA-treated infected cells, HDAC4 polypeptide appeared to be downregulated (Figure 7C). This phenotype is similar to what we have previously reported for some other HDACs [224-226, 369] and will be further explored in Chapter IV. The intracellular viral NP polypeptide expression was also quantified and found to be 1.9 fold greater in HDAC4-depleted cells (Figure 7D).

Next, we wanted to determine how the depletion of HDAC4 effects the growth kinetics of IAV. A549 cells were transfected with control and HDAC4 siRNA as described previously and were then infected with PR8 at an MOI of 0.1. Here, a lower MOI was used in order to reduce the level of dysregulation of HDAC4 during IAV infection in control siRNA-treated cells. Theoretically, this would allow HDAC4 to exert its antiviral potential for a longer period of time and therefore result in a greater observable difference in viral replication between control and HDAC4 siRNA-treated cells. The culture medium and infected cells were harvested separately at 0, 6, 12 and 24 hours post-infection. The culture media was divided into two parts, one part was analysed by WB to measure the total virus progeny release, while the other part was titrated by plaque assay to measure the infectious virus progeny release. The infected cell lysates were harvested and analysed by WB in order
to confirm the successful depletion of HDAC4 polypeptide. Knockdown of HDAC4 was confirmed and once again the IAV-induced dysregulation was observed with a noticeable decrease between 6 and 12 hours of infection (Figure 8A). Additionally, a greater difference in infectious virus release was measured where HDAC4-depleted cells produced approximately 4.4 fold more infectious virus than control siRNA-treated cells (Figure 8B). Furthermore, after 6, 12 and 24 hours of infection, the cells transfected with HDAC4-targeting siRNA released more total virions (replicative competent and incompetent virus in the culture medium) than the cells transfected with control siRNA (Figure 8C).

Lastly, we infected A549 cells depleted of HDAC4 expression with influenza A/California/07/2009(H1N1) strain (henceforth referred to as CA09) to determine if a recent clinical isolate would also replicate to higher titres in HDAC4 depleted cells. Again, A549 cells were transfected with either control or HDAC4 siRNA for 72 hours, following which, the cells were infected with CA09 at 1.0 MOI for 24 hours. Once again the culture media was collected to be titrated by plaque assay to determine the infectious virus release while the cell lysates were harvested for total RNA to analyse the viral gene mRNA expression. Consistent with the previous results, cells depleted of HDAC4 produced 2.7 fold more infectious viral progeny compared to cells expressing normal levels of HDAC4 (Figure 9A). The mRNA level of viral NP and M and cellular actin mRNA was measured by qPCR and the threshold cycle (Ct) values were determined. The Ct values of the viral genes were normalized using the actin value. The mRNA level of the viral mRNA in CT siRNA-treated cells was considered 1 fold for comparison with HD4 siRNA-treated cells. This analysis revealed that the mRNA expression of the viral genes NP and M was increased by 1.8 fold and 3.1 fold respectively in HDAC4-depleted cells (Figure 9B).
Figure 7. Depletion of HDAC4 promotes PR8 replication. (A-D) A549 cells were transfected with 1 nm of CT siRNA or HD4 siRNA for 72 hours. Cells were then infected with PR8 at an MOI of 1.0 for 24 hours, after which, the culture media and the cells were harvested separately. (A) The culture media was titrated on MDCK cells by plaque assay in order to determine the total infectious virus released. Then, the amount of virus released from CT siRNA-treated cells was considered 1 fold to compare the amount of virus released from HD4 siRNA-treated cells. (B) The total RNA content from infected CT and HD4 siRNA-treated cells was processed and the levels of viral NP, M and cellular actin mRNA were detected by qPCR. Then, the NP and M mRNA levels were normalized with corresponding actin mRNA levels. Finally, the normalized levels of each viral mRNA in CT siRNA-treated cells were considered 1 fold to compare their levels in HD4 siRNA-treated cells. (C) Total cell lysates of uninfected (UNI) and PR8-infected (INF) cells transfected with CT or HD4 siRNA were prepared and HDAC4, PDI and viral NP (56 kDa) polypeptides were detected by WB. (D) The NP and PDI bands in panel (C) blots were quantified and normalized as was done previously. Then, the normalized level of NP in CT siRNA-treated cells was considered 1 fold to compare its levels in HD4 siRNA-treated infected cells. Error bars represent means ± standard errors of the means of three independent experiments. P value calculated using unpaired t test. MW, molecular weight.
Figure 8. PR8 replication kinetics are enhanced in HDAC4-depleted cells. (A-D) A549 cells were transfected with 1nm of CT or HD4 siRNA for 72 hours. Cells were then infected with PR8 at an MOI of 0.1 and the cells and culture medium were harvested separately at 0, 6, 12 and 24 hours post-infection. (A) Total cell lysates were prepared and resolved via SDS-PAGE, and HDAC4, PDI and viral NP were detected by WB. (B) The culture media was titrated on MDCK cells by plaque assay in order to determine the amount of infectious virus progeny released. (C) Total culture media from CT and HD4 siRNA-treated cells were concentrated by trichloroacetic acid precipitation, and the levels of viral HA (68 kDa) and viral NP were compared by WB. Error bars represent means ± standard errors of the means of three independent experiments. P value calculated using unpaired t test. MW, molecular weight.
Figure 9. CA09 replication is enhanced in HDAC4-depleted cells. (A-B) A549 cells were transfected with 1 nm of CT siRNA or HD4-targeting siRNA for 72 hours. Cells were then infected with CA09 at an MOI of 1.0 for 24 hours and then the cells and culture media were harvested separately. (A) The culture media was titrated on MDCK cells by plaque assay in order to determine the amount of infectious virus progeny released. Then, the amount of virus released from CT siRNA-treated cells was considered 1 fold to compare the amount of virus released from HD4 siRNA-treated cells. (B) The total RNA content from infected CT and HD4 siRNA-treated cells was processed and the levels of viral NP, M and cellular actin mRNA were detected by qPCR. Then, the NP and M mRNA levels were normalized with corresponding actin mRNA levels. Finally, the normalized levels of each viral mRNA in control siRNA-treated cells were considered 1 fold to compare their levels in HDAC4 siRNA-treated cells. P value calculated using unpaired t test. Error bars represent means ± standard errors of the means of 3 independent experiments.
2.2.3 Overexpression of HDAC4 inhibits IAV replication

The knockdown of HDAC4 indicated that endogenously expressed HDAC4 plays an antiviral role during IAV infection. IAV replication and total virion release was observed to be greater in A549 cells depleted of HDAC4. Therefore, the next step was to determine if transfection of a plasmid containing the human HDAC4 gene would have an opposite effect and inhibit IAV replication.

First of all, the transfection conditions were optimized in order to ascertain a suitable plasmid concentration and lipofectamine 2000 (LF) volume for use in subsequent experiments. A549 cells were transfected with either an empty plasmid (peGFP) or an HDAC4-expressing plasmid (HD4-GFP) (~149 kDa) with various ratios of plasmid concentrations (1, 2 and 3 µg) and LF volumes (1, 2 and 3 µL). After 48 hours the cell lysates were harvested and analysed by WB. It was found that the lowest concentration of plasmid required for sufficient HDAC4 expression was with 1 µg of DNA combined with 3 µL of LF (Figure 10A). Next, we wanted to compare the expression capabilities of a second HDAC4 expressing plasmid (HD4-FLAG) (~120 kDa) with the previously used HD4-GFP plasmid. A549 cells were transfected with either 1 µg or 2 µg of peGFP plasmid or two HDAC4 plasmids using 3 µL of LF for 48 hours. Once again the cell lysates were harvested for analysis by WB which revealed that there was no major difference in the level of HDAC4 expression between the two plasmids (Figure 10B). However, we chose HD4-FLAG for all subsequent overexpression experiments because the HDAC4 polypeptide is of a similar size as the endogenous HDAC4 on an SDS-PAGE. Furthermore, the FLAG tag is fused to the C-terminus of the HDAC4 gene whereas the HDAC4 antibody recognises the N terminus of HDAC4. This allows for detection of HDAC4 from both ends of the polypeptide, which could be useful for future analysis into the dysregulation of HDAC4 by IAV.

Next, we wanted to determine if, like endogenously expressed HDAC4, overexpressed HDAC4 is also severely dysregulated by IAV. To do this, A549 cells were transfected with either HD4-GFP or HD4-FLAG using the aforementioned transfection conditions for 48 hours. Next, the cells were either infected with PR8 at 1.0 MOI or were left uninfected for 24 hours, following which the cell lysates were harvested for WB analysis. Interestingly, even in cells overexpressing HDAC4, IAV infection was able to significantly decrease the levels of HDAC4 polypeptide as compared to uninfected cells (Figure 11A). Because the effect on HDAC4 polypeptide overexpression was so profound in A549 cells, we decided...
to try another cell line, human embryonic kidney 293T (HEK293T) cells. Therefore, HEK293T cells were transfected with both HD4-GFP and HD4-FLAG in exactly the same manner as was performed in the A549 cells. After 48 hours, the cell lysates were prepared and analysed by WB, which revealed that, unlike A549 cells, HEK293T cells maintained HDAC4 overexpression, even in the presence of IAV infection (Figure 11B). Detection of intracellular viral NP revealed that IAV was able to successfully replicate in these cells. Lastly, we wanted to determine the efficiency of transfection between A549 and HEK293T cells by fluorescent microscopy. Both cell lines were transfected with HD4-FLAG using the same transfection conditions as used previously. After 48 hours of transfection, the cells were stained as described above using an HDAC4 antibody and visualised using immunofluorescence. This revealed that HEK293T cells were transfected at far greater efficiency than A549 cells (Figure 11C-D) and thus HEK293T cells were chosen for subsequent HDAC4 overexpression experiments.

To determine if ectopically-expressed HDAC4 inhibits IAV infection, HEK293T cells were transfected with either the empty plasmid, pcDNA3.1(-) or HD4-FLAG plasmid, using the transfection conditions described previously. After 48 hours of transfection, the cells were infected with PR8 for 24 and 48 hours at either 0.1 or 1.0 MOI. Some of the cells were harvested for WB analysis to confirm the overexpression of HDAC4 and that HDAC4 polypeptide expression was maintained throughout the course of infection (Figure 12A). Meanwhile, the culture media was harvested for plaque assay analysis to determine infectious virus release. Compared to cells transfected with pcDNA3.1(-) plasmid, HDAC4 overexpressing cells infected with PR8 at 0.1 MOI released 49% and 46% less infectious virus progeny after 24 and 48 hours of infection respectively (Figure 12B). Similar results were obtained in HEK293T cells infected with PR8 at 1.0 MOI. HDAC4 overexpressing cells released 42% less infectious viral progeny at 24 hours post-infection and 46% less at 48 hours post-infection as compared to cells expressing normal HDAC4 levels (Figure 12C). Finally, to determine if the levels of infectious virus progeny measured correlated to the level of virus replication inside the cells, a qPCR was performed exactly as above on RNA extracted from the cell lysates infected with PR8 at 1.0 MOI. The qPCR analysis revealed that the mRNA level of the viral NP and M genes were decreased by 41% and 50% respectively, when normalized to the actin mRNA level and 48% and 44% respectively, when normalized to 18SRNA level (Figure 12D).
Figure 10. Transfection efficiency of HDAC4-expressing plasmids. (A) A549 cells were transfected with either peGFP or HD4-GFP using the indicated concentrations of plasmid DNA and volumes of LF for 24 hours. Total cell lysates were then prepared and HDAC4 and PDI were detected by WB. (B) A549 cells were transfected with 1 or 2 µg of an empty pcDNA3.1(−) plasmid (pc), HD4-GFP or pcDNA3.1(−) plasmid containing HDAC4 (HD4-FLAG) using 3 µL of LF for 24 hours. Total cell lysates were then prepared and HDAC4 and PDI were detected by WB.
Figure 11. HDAC4 overexpression is enhanced in HEK293T cells compared to A549 cells. A549 cells (A-B) or HEK293T cells (C-D) were transfected with 1 µg of HD4-GFP or HD4-FLAG with 3 µL of LF for 24 hours. (A and C) The cells were then infected with PR8 at 1.0 MOI or left uninfected for 24 hours. Total cell lysates were prepared and HDAC4, PDI and viral NP were detected by WB. (B and D) Cells transfected with the HD4-FLAG plasmid were fixed with 4% paraformaldehyde. Next, 0.2% TritonX-100 was added to permeabilize the cells. Next HDAC4 antibody was added, followed by anti-rabbit secondary Alexa flour 594 dye. The cells were then viewed and imaged under an inverted fluorescence microscope (Olympus) at magnification of 10X. Data is representative of a single experiment. MW, molecular weight.
Figure 12. Overexpression of HDAC4 restricts IAV replication. (A-D) HEK293T cells were transfected with 1 µg of either pcDNA3.1(−) (pc) or HD4-FLAG (HD4) with 3 µL of LF for 24 hours. The cells were then infected with PR8 at 1.0 MOI (A and B) or 0.1 MOI (C and D). The cell lysates and culture media were harvested separately at 0, 24 and 48 hours post-infection. (A) Total cell lysates were prepared and HDAC4, PDI and viral NP were detected by WB. The culture media was titrated on MDCK cells by plaque assay in order to determine the amount of infectious virus progeny released at 24 hours (B) and 48 hours (C) post-infection. Then, the amount of virus released from pc-transfected cells was considered 100% to compare the amount of virus released from HD4-transfected cells. (D) The total RNA content from 24 hour infected pc- and HD4-transfected cells was processed and the levels of viral NP and M and host cell actin and 18SRNA mRNA were detected by qPCR. Then, the NP and M mRNA levels were normalized with corresponding actin and 18SRNA mRNA levels. Finally, the normalized levels of each viral mRNA in pc-transfected cells were considered 100% to compare their levels in HD4-transfected cells. Error bars represent means ± standard errors of the means of 3 independent experiments. P value calculated using unpaired t test. MW, molecular weight markers.
2.3 Discussion

The data presented in this chapter demonstrate that HDAC4 plays an antiviral role during IAV infection. While others have previously reported anti-IAV roles for other members of the HDAC family, the role of HDAC4 in IAV infection has not been described [79, 224-226, 352]. Here, we show that depletion of HDAC4 in A549 cells results in an increase in IAV replication (Figure 7, 8, 9). Additionally, we show that overexpression of HDAC4 from a plasmid inhibits IAV replication in HEK293T cells (Figure 12). The observations were confirmed by analysing several parameters of IAV replication; extracellular (total and infectious) virion release and intracellular viral mRNA and protein levels. This was confirmed using two IAV H1N1 strains, the lab-adapted strain, PR8 (Figure 7), and the clinically relevant strain, CA09 (Figure 9). Ultimately, IAV growth was found to be 2-3 fold higher in HDAC4-depleted cells when infected at 1.0 MOI. However, one could argue while statistically significant, this is only a modest increase in virus growth. This discrepancy is made more apparent when comparing the effect of other HDACs on IAV growth. For example, HDAC11-depleted cells have been shown to produce up to 1-log more infectious IAV virions when compared to cells expressing normal levels of HDAC11 [224]. However, a likely and simple explanation is due to the profound downregulation of the HDAC4 polypeptide in IAV-infected cells. In fact, the antagonism of HDAC4 by IAV was found to be so severe that in A549 cells overexpressing HDAC4, the levels of HDAC4 polypeptide were reduced to barely detectable levels after 24 hours of infection (Figure 11A). Hence, in control siRNA-treated IAV-infected cells, its expression is reduced to similar levels seen in the HDAC4-depleted cells. Therefore, HDAC4 has less time to assert its antiviral effect and the resulting difference observed in virus growth cannot be as profound. Furthermore, when we infected cells with 0.1 MOI we observed decreased HDAC4 antagonism, but a greater difference in virus growth between HDAC4-depleted cells and control cells (Figure 8B). Therefore, the decreased level of HDAC4 antagonism seen at 0.1 MOI indicates that HDAC4 is able to assert its antiviral effect for a longer period of time in the control cells. In addition, a recent study further supports this argument where overexpression of HDAC4 induced resistance to infection by VSV, but only with infection at low titres [426]. Furthermore, both the HDAC4 mRNA and polypeptide have been shown to be highly unstable with their respective half-lives being approximately 4 and 8 hours [429]. Finally, functional redundancy has been observed between different HDACs within the same class and even outside classes [430]. Therefore, in the absence of HDAC4, other HDACs could partially compensate for its antiviral role.
and hence could be contributing to the modest change observed in viral growth in HDAC4 depleted cells. The combination of all of these factors suggest that HDAC4 restricts IAV replication but is rapidly targeted and dysregulated by IAV, potentially as a means to inhibit its antiviral potential.

Some important considerations to take into account when interpreting this data is that for the depletion of HDAC4, only one siRNA was used. In order to gain greater confidence in the result, multiple siRNAs should be utilised in order account for potential off-target effects. However, the overexpression data presented in Figure 12 does somewhat alleviate this issue. Also, the varying knockdown efficiency of different siRNAs used might correlate with the effect on viral replication as reported with HDAC11 where 3 different siRNAs were used [224]. Additionally, use of an siRNA-resistant HDAC4 plasmid in combination with an HDAC4-targeting siRNA would also give greater confidence in these results.

Another important experiment that could have been performed was to use an HDAC4-specific inhibitor and determine its effect on IAV replication. General HDAC inhibitors such as TSA, which inhibit the activity of multiple HDACs including HDAC4 have been used previously and have been shown to promote the replication of different viruses including IAV [225, 226]. An obvious choice for an HDAC4-specific inhibitor would be tasquinimod, as this would indicate the importance of the deacetylase activity of HDAC4 in its antiviral role [521]. Finally, because HDAC4 can shuttle between the cytoplasm and the nucleus, it would be interesting to determine how the subcellular distribution of HDAC4 changes during IAV infection. This might provide some clues as to how and where HDAC4 is able to exert its anti-IAV function.
CHAPTER III - RESULTS:

THE ANTIVIRAL MECHANISM OF

HISTONE DEACETYLASE 4
3.1 Introduction

The outcomes from Chapter II revealed that host HDAC4 is playing an antiviral role during IAV infection. Therefore, the next step was to determine how HDAC4 exerts its antiviral function. Previous studies on HDAC4 have revealed that it plays an important role in regulating the innate immune response, specifically through enhancement of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signalling pathway. Furthermore, other HDACs namely HDAC1, 2 and 11 have also been implicated in the innate immune response during IAV infection [224-226]. Based upon this understanding, an obvious next step was to explore the effect of HDAC4 manipulation on the IAV-induced interferon signalling pathway.

3.1.1 The innate immune response to IAV infection

When IAV first infects a host cell, conserved products of viral replication known as pathogen-associated molecular patterns (PAMPs) are recognised by host pathogen recognition receptors (PRRs). PRRs include RIG-I and Toll-like receptors (TLRs) and are able to distinguish certain characteristics present on the IAV RNA that are not shared by cellular RNAs such as regions of dsRNA or the presence of a 5′-triphosphate group [172, 173]. Following recognition of IAV PAMPs, RIG-I is activated and its caspase and recruitment domains (CARDs) are exposed. The CARD is then dephosphorylated or ubiquitinated by E3 ligases which triggers downstream signalling at the mitochondrial membrane [431]. Transcription factors including IRF3, IRF7 and NF-KB are activated promoting production of type I IFNs and inducing expression of ISGs [432].

Type I IFNs are key mediators of innate antiviral immune responses and have 3 major functions to facilitate this. Firstly, they induce an antiviral state in infected cells as well as neighbouring cells, which limits further spread of infectious agents [433]. Secondly, they enhance innate immune responses through promotion of antigen presentation and natural killer cell functions while inhibiting pro-inflammatory pathways and cytokine production [432]. Thirdly, they augment the development of high-affinity antigen-specific T and B cell responses [434]. IFNα and IFNβ are the most well characterised type I IFNs and are the first signalling molecules of the IFN response which culminates in the activation of hundreds of ISGs that are able to restrict viral infection by targeting nearly every stage of the virus life cycle. The response begins with the binding of type I IFN to the IFNα receptor (IFNAR) which is composed of IFNAR1 and IFNAR2 subunits. This binding then activates JAK1 and tyrosine kinase 2 (TYK2) which go on to phosphorylate STAT1
and STAT2. Tyrosine-phosphorylated STAT1 and STAT2 dimerize and translocate into the nucleus where they form a complex with IFN-regulatory factor 9 (IRF9) to form a trimolecular structure called IFN-stimulated gene factor 3 (ISGF3). ISGF3 then binds to IFN-stimulated response elements (ISREs) which then directly activates the transcription of ISGs. STAT1 can also form homodimers which translocate into the nucleus and bind to gamma-activated sequences to induce pro-inflammatory genes [435].

3.1.2 HDAC4 and the innate immune response

Several studies have demonstrated the role that HDAC4 plays in regulating the innate immune response. Multiple groups have reported on the role HDAC4 plays in promoting STAT1 activation to promote the innate immune response. The first study revealed that in cisplatin-resistant cancer cells HDAC4 physically interacts with STAT1 which allows STAT1 to become activated. Further they showed that overexpression of HDAC4 promotes STAT1 phosphorylation which allows STAT1 to translocate into the nucleus allowing expression of STAT1-inducible genes [412]. Other studies have since reported similar findings on the role of HDAC4 in promoting STAT1 activation in other cell lines and that STAT1 activity promotes HDAC4 expression [436, 437]. A recent further study supported these findings in hepatocellular carcinoma cells, whereby depletion of HDAC4 decreased STAT1 phosphorylation and as a result decreased type I IFN signalling. Consequently, these HDAC4-depleted cells were more susceptible to VSV infection. Meanwhile, ectopic expression of HDAC4 enhanced STAT1 activation and type I IFN expression, resulting in impaired VSV replication [426]. STAT2 has also been shown to be positively regulated by HDAC4 activity as a recent study revealed that HDAC4 coprecipitates with STAT2 and is recruited to IFN-stimulated response element (ISRE)-containing promoters following IFN induction. In the absence of HDAC4, STAT2 binding to these promoters is greatly reduced, leading to enhanced replication of HSV-1 and vaccinia virus [422].

Conversely, other studies have reported that HDAC4 plays a role in suppressing the innate immune response. Upon being dephosphorylated, HDAC4 localizes to the nucleus where it represses the innate immune response in several ways. Firstly, it deacetylates NF-KB p65, which results in the repression of many inflammatory genes including TNF-α, IL-6, IL-1β, and IL-12 [438]. Secondly, HDAC4 binds with LXRα and promotes its sumoylation allowing LXRα to bind nuclear pSTAT1 in a trimeric complex with HDAC4. This complex prevents phosphorylated STAT1 (pSTAT1) from binding to the IRF1 promoter
preventing IFN production [439]. More recently, a study showed that cytoplasmic HDAC4 can also interfere with the innate response by preventing the phosphorylation of IRF3 and thereby decrease type I IFN production. HDAC4 outcompetes IRF3 as the substrate of the kinases TBK1 and IKKε, which normally phosphorylates IRF3 to promote its activation [421].

Previously, it has been reported that HDAC1, 2 and 11 all play a role in promoting the innate immune response during IAV infection [224-226]. Furthermore, HDAC4 itself has been shown to play roles in enhancing and inhibiting the innate immune response under various conditions [436-438]. Taking the outcomes from Chapter II into account, the purpose of this chapter was to determine if HDAC4 inhibits IAV replication by enhancing the innate immune response.
3.2 Results

3.2.1 Depletion of HDAC4 impairs IFN mRNA expression

In the light of the involvement of HDAC4 in innate immune response discussed above, we wanted to determine the role of HDAC4 in IAV-induced host innate antiviral response. For this, A549 cells were transfected with CT or HD4 siRNA as in Chapter II (Figure 5). After 72 hours, the cells were infected with PR8 at an MOI of 1.0 for 6 hours. Additionally, a control group of CT and HD4 siRNA-treated cells were harvested for WB analysis to confirm the knockdown of HDAC4. Following this, the cell lysates were prepared and the total RNA was extracted and converted to cDNA for analysis by qPCR. WB analysis of the uninfected cells revealed that the knockdown of HDAC4 was successful (Figure 13A). The mRNA level of IFNα, IFNβ, IFNγ and actin was measured by qPCR and the ct values were determined. The ct values of the IFNs were normalized using the actin value. The mRNA level of the IFNs in CT siRNA-treated cells was considered 100% for comparison with HD4 siRNA-treated cells. There was a significant 73.8% and 83% decrease in the expression of IFNα and IFNβ respectively, in HDAC4-depleted cells compared with the control cells. However, there was no significant change in the expression of IFNγ (Figure 13B).
Figure 13. HDAC4 depletion decreases IFNα and IFNβ mRNA expression during IAV infection. A549 cells were transfected with 1 nM of CT or HD4 siRNA for 72 hours. (A) Total cell lysates were prepared and HDAC4 and PDI polypeptide levels were measured by WB. (B) The siRNA treated cells were infected with PR8 at 1.0 MOI for 6 hours. The levels of IFNα, IFNβ, IFNγ and actin mRNA were detected by qPCR. The levels of IFNα, IFNβ or IFNγ were normalised with the levels of the corresponding actin mRNA. Finally, the normalised mRNA level of each gene in CT siRNA-treated cells was considered 100% to compare its level in HD4 siRNA-treated cells. Error bars represents means ± standard errors of the means of three repeats. P value calculated using unpaired t test. MW molecular weight.
3.2.2 The depletion of HDAC4 inhibits IAV-induced STAT1 signalling

The above results revealed that HDAC4 plays a role in the IAV-induced IFN signalling pathway. Therefore, the next step was to determine whether HDAC4 is involved in promoting the phosphorylation of STAT1. For this, A549 cells were transfected with CT siRNA or HD4 siRNA and then infected with PR8 at 1.0 MOI. After 0, 6, 12 and 24 hours, cell lysates were prepared and the levels of HDAC4, phosphorylated STAT1 (pSTAT1) (87 kDa), total STAT1 (tSTAT1) (87 kDa), PDI and viral NP were detected by WB. The tSTAT1 and PDI were used as loading controls and viral NP was detected as an infection marker. The analysis revealed that there was a noticeable decrease in pSTAT1 level in the cells transfected with HD4 siRNA compared to the cells transfected with CT siRNA, particularly at 12 and 24 hours post-infection (Figure 14A). In order to quantify the decrease in pSTAT1 levels, the intensity of pSTAT1 and tSTAT1 bands were quantified using the Image Studio Lite Software (Version 7.0, LI-COR). The amount of pSTAT1 was normalised with the corresponding tSTAT1 amount. Then, the normalised amount of pSTAT1 at each time point in CT siRNA-treated cells was considered 100% to compare its amount in HD4 siRNA-treated cells. This revealed that there was a significant 54.3% and 61.9% decrease in pSTAT1 levels in HDAC4-depleted cells at 12 and 24 hours post infection respectively (Figure 14B).
Figure 14. HDAC4 is important for IAV-induced phosphorylation of STAT1. A549 cells were transfected with 1 nM of CT or HD4 siRNA for 72 hours. Cells were then infected with PR8 at an MOI of 1.0 and the cells were harvested separately at 0, 6, 12 and 24 hours post-infection. (A) Total cell lysates were prepared and resolved via SDS-PAGE, and the levels of HDAC4, pSTAT1 (91/84 kDa), tSTAT1 (91/84 kDa), PDI and viral NP were detected by WB. (B) The intensity of the pSTAT1 and tSTAT1 bands was quantified using the Image Studio Lite software (LI-COR). The levels of pSTAT1 was then normalised with the corresponding tSTAT1 levels. Finally, the normalised level of pSTAT1 at each time point in CT siRNA-treated cells was considered 100% to compare its amount in HD4 siRNA-treated cells (6, 12, and 24h). Error bar represents means ± standard errors of the means of three independent experiments. P value calculated using unpaired t test. MW, molecular weight.
3.2.3 Depletion of HDAC4 impairs expression of ISGs: IFITM3, viperin and ISG15

The above observations indicated that HDAC4 is playing a role in regulating the innate immune signalling pathway in IAV-infected cells. Therefore, the next step was to investigate the effect of decreased IFNα and IFNβ mRNA expression as well as the impaired phosphorylation of STAT1 in HDAC4-depleted cells. Hence, the mRNA expression of various ISGs (IFITM1, IFITM2, IFITM3, ISG15, viperin, cholesterol-25-hydroxylase (CH25H), effector mitochondrial antiviral signalling protein (MAVS), TRM22, MX1 and OAS3) was investigated in HDAC4-depleted cells, because their previously reported roles in IAV infection [435, 440, 441]. While the mRNA expression of most of the genes tested did not appear to change in the absence of HDAC4, ISG15 mRNA expression decreased by 88% and viperin mRNA expression decreased by 70.2% in cells depleted of HDAC4 compared to HDAC4 expressing cells (Figure 15).

In addition, we wanted to determine if a similar phenotype could be observed at the polypeptide level of several of the ISGs (IFITM3, viperin and ISG15) in HDAC4-depleted cells. Here, IFITM3 was used as a negative control. To assess this, A549 cells were transfected with CT or HD4 siRNA and were then infected with PR8 at 1.0 MOI. After 0, 6, 12 and 24 hours of infection, cell lysates were prepared and the polypeptide levels of HDAC4, IFITM3, viperin and ISG15 were measured by WB. HDAC4 was confirmed to be knocked down prior to virus infection and remained depleted throughout the entire time course. Interestingly, the polypeptide expression of both IFITM3 and viperin were massively decreased in HDAC4-depleted cells, whereas ISG15 expression initially decreased in HDAC4-depleted cells but seemed to recover after 24 hours of infection (Figure 16A). Analysis of three independent experiments revealed that in HDAC4-depleted cells, IFITM3 polypeptide expression decreases significantly by 49.3% at 12 hours post infection and 59.7% at 24 hours post-infection (Figure 16B). HDAC4 depletion results in viperin polypeptide expression to decrease by 70% and 76.7% at 12 and 24 hours post-infection, respectively (Figure 16B). Interestingly, in cells depleted of HDAC4, ISG15 polypeptide expression initially decreases by 56.7% at 12 hours post infection. However, after 24 hours, the expression of ISG15 recovers back to similar levels observed in the control (Figure 16C).
Figure 15. Depletion of HDAC4 impairs mRNA expression of ISG15 and viperin during IAV infection. A549 cells were transfected with 1 nM of CT or HD4 siRNA for 72 hours following which the cells were infected with PR8 at 1.0 MOI for 6 hours. The mRNA levels of actin and the indicated ISGs were detected by qPCR and normalised and presented as was done in figure 12. Error bars represents means ± standard errors of the means of three repeats. P value calculated using unpaired t test.
Figure 16. Depletion of HDAC4 impairs ISG polypeptide expression. (A) A549 cells were transfected with 1 nM of CT siRNA or HD4-targeting siRNA for 72 hours. Cells were then infected with PR8 at an MOI of 1.0 and the cells were harvested separately at 0, 6, 12 and 24 hours post-infection. Total cell lysates were prepared and resolved via SDS-PAGE, and HDAC4, IFITM3 (15 kDa), viperin (42 kDa), ISG15 (18 kDa), PDI and viral NP were detected by WB. The IFITM3 (B), viperin (C) or ISG15 (D) bands were quantified and normalized to the corresponding PDI bands. Then, the normalized levels of IFITM3, viperin and ISG15 at each time point in CT siRNA-treated cells was considered 100% to compare their levels in HD4 siRNA-treated cells at the respective time points. Error bars represent means ± standard errors of the means of three independent experiments. P value calculated using unpaired t test. MW, molecular weight.
3.2.4 Overexpression of HDAC4 promotes IAV-induced innate antiviral signalling

The above results demonstrated that depletion of HDAC4 impairs IAV-induced pSTAT1 activation and the downstream signalling of antiviral ISGs at both mRNA and polypeptide levels. Therefore, the next step was to determine if the opposite effect could be observed when overexpressing HDAC4, whereby the IAV-induced immune response would be enhanced. As was done above, the effect of HDAC4 overexpression was examined in the context of IFN mRNA expression. First, HEK293T cells were transfected with HD4-FLAG plasmid or the empty vector pcDNA3.1(-) for 24 hours. Following this, some of the cells were harvested for WB analysis in order to confirm the overexpression of HDAC4 (Figure 17A). Meanwhile, the remaining cells were infected with PR8 at 1.0 for 6 hours, after which, the cells were harvested and total RNA was extracted. The mRNA expression of IFNα, IFNβ, IFNγ and actin was measured by qPCR in HD4-FLAG transfected cells and compared to pcDNA3.1(-) transfected cells. Consistent with the knockdown data, IFNα mRNA expression increased by 3.3 fold in cells overexpressing HDAC4 during IAV-infection. Consistent with the knockdown data, IFNγ mRNA expression was not affected in response to HDAC4 overexpression (Figure 17B). Interestingly, IFNβ mRNA did not appear to be affected by the overexpression of HDAC4 despite its decreased expression in the absence of HDAC4.

The above data indicated a clear link between HDAC4 expression and IFNα mRNA expression. Therefore, it was likely that the mRNA level of one or more of the downstream ISGs would also be promoted in HDAC4 overexpressing cells. HEK293T cells were transfected with HD4-FLAG or pcDNA3.1(-) exactly as was performed above. The cells were then infected for 6 hours with PR8 at 1.0 MOI, after which, the cells were harvested for RNA. The mRNA levels of IFITM1, IFITM2, IFITM3, ISG15, viperin, CH25H, MAVS, TRM22 and actin were analysed by qPCR. The IAV-induced mRNA expression of the ISGs in HDAC4 overexpressing cells was compared with cells expressing normal levels of HDAC4. Once again, the mRNA levels of most of the ISGs observed did not significantly alter in response to the overexpression of HDAC4. However, ISG15 mRNA expression increased significantly by 8.2 fold in HDAC4 overexpressing cells. Likewise, viperin mRNA expression increased significantly by 3.66 fold in cells overexpressing HDAC4 (Figure 18). The complementary data obtained from the knockdown experiments indicate that the mRNA expression of both of these genes is regulated by HDAC4.
Next, we wanted to determine if the overexpression of HDAC4 affected the polypeptide expression of these ISGs in a complementary pattern to the HDAC4 knockdown data. Therefore, the IAV-induced activation of STAT1 and subsequent polypeptide expression of the downstream ISGs in HDAC4 overexpressing cells was compared to cells expressing normal levels of HDAC4. Here, HEK293T cells were transfected with HD4-FLAG or pcDNA3.1(-) for 24 hours. Next, the cells were infected with PR8 at 1.0 MOI and the cells were harvested at 0, 6, 12 and 24 hours post infection. The cell lysates were prepared and HDAC4, pSTAT1, tSTAT1, viperin, ISG15, PDI and viral NP were detected by WB. tSTAT1 and PDI were used as loading controls and viral NP as an infection marker. Consistent with the above mRNA data (Figure 15), the overexpression of HDAC4 appeared to induce an opposite effect on the activation of STAT1 and polypeptide expression of the examined ISGs. Phosphorylation of STAT1 was increased in cells overexpressing HDAC4 while the polypeptide expression of viperin and ISG15 increased, at least at 24 hours post infection (Figure 19). Interestingly, STAT1 phosphorylation induction was greatest at 0 hours, suggesting that plasmid transfection alone is sufficient to induce the innate immune response.
Figure 17. Overexpression of HDAC4 promotes IFNα mRNA expression. HEK293T cells were transfected with either an empty pcDNA3.1(-) or HD4-FLAG plasmid using 1 μg of DNA and 3 μL of LF for 24 hours. (A) Cells were harvested and total cell lysates were prepared. The samples were run on a 10% SDS-PAGE and HDAC4 and PDI were detected by WB. (B) Cells were infected with PR8 at 1.0 MOI for 6 hours, following which the levels of IFNα, IFNβ, IFNγ and actin mRNAs were detected by qPCR. Then, the IFNα, IFNβ and IFNγ levels were normalized to the corresponding actin mRNA levels. Finally, the normalized levels of each IFN mRNA in pcDNA plasmid transfected cells was considered 1-fold to compare their levels to HD4-FLAG transfected cells. Error bars represent means ± standard errors of the means of three repeats. P value calculated using unpaired t test. MW, molecular weight.
Figure 18. HDAC4 overexpression increases ISG15 and viperin mRNA expression. HEK293T cells were transfected with 1µg pcDNA3.1(−) or HD4-FLAG with 3µL of LF for 24 hours. The cells were then infected with PR8 at 1.0 MOI for 6 hours. Next, the mRNA levels of actin and the indicated ISGs were detected by qPCR and normalised and presented as was done in the previous figure. Error bars represent means ± standard errors of the means of three repeats. P value calculated using unpaired t test.
Figure 19. Overexpression of HDAC4 promotes phosphorylation of STAT1 and expression of viperin and ISG15 during IAV infection. HEK293T cells were transfected with 1 µg of either pcDNA3.1(-) or HD4-FLAG with 3 µL of LF for 24 hours. The cells were then infected with PR8 at 1.0 MOI and the cells were harvested following 0, 6, 12 and 24 hours post infection. Total cell lysates were prepared and HDAC4, pSTAT1, tSTAT1, viperin, ISG15, PDI and viral NP were detected by WB. Data is representative of a single experiment. MW, molecular weight.
3.2.5 HDAC4 enhances STAT1 activation and IFITM3 expression, independent of IAV

Based upon the above data where IFNα mRNA expression was decreased in HDAC4-depleted cells and increased in HDAC4 overexpressing cells, we wanted to determine whether HDAC4 is involved in the IFNα mediated antiviral response. For this, A549 cells were transfected with CT or HD4 siRNA for 72 hours. Next, the cells were treated with IFNα (10 IU/mL) and the cells were harvested after 0, 2, 4 and 6 hours of treatment. Total cell lysates were prepared and HDAC4, pSTAT1, tSTAT1 and PDI polypeptide levels were analysed by WB. Under these conditions, pSTAT1 levels appeared rapidly within the first 2 hours of treatment then subsided to barely detectable levels by 6 hours post-treatment. Consistent with the previous observations on the role of HDAC4 and the activation of STAT1, the IFNα-induced phosphorylation of STAT1 in HDAC4 depleted cells was impaired (Figure 20A). The initial phosphorylation of STAT1 at 2 hours post-treatment is similar between CT and HD4 siRNA-treated cells. However, in the HDAC4-depleted cells, the level of STAT1 phosphorylation diminishes rapidly when compared to cells expressing normal levels of HDAC4. In fact, by 6 hours post-treatment, there is a significant 50.6% decrease in STAT1 phosphorylation in HD4 siRNA-treated cells as compared to CT siRNA-treated cells (Figure 20B).

Additionally, the IFNα-induced expression of IFITM3, viperin and ISG15 were investigated and compared in HD4 siRNA-treated cells to CT siRNA-treated cells. A549 cells were transfected with CT or HD4 siRNA for 72 hours, after which, the cells were treated with IFNα (10 IU/mL). The cells were harvested at 0, 2, 4, 6 hours post-treatment and total cell lysates were prepared for each time point and HDAC4, IFITM3, viperin, ISG15 and PDI were detected by WB. Detection of HDAC4 revealed that the knockdown was successful, however, both viperin and ISG15 were undetectable even 6 hours post-treatment. IFITM3 was detectable even without IFNα induction due to its basal expression. Consistent with the previously observed results, IFNα-induced expression of IFITM3 was decreased in cells depleted of HDAC4 (Figure 21A). At 6 hours post-treatment, the expression of IFITM3 polypeptide in HDAC4 depleted cells was reduced by 50.1% when normalized with PDI (Figure 21B).

Collectively these results demonstrate that HDAC4 plays a role as part of the host innate antiviral response against IAV, through activation of STAT1, and promotion of the downstream ISGs including IFITM3, viperin and ISG15.
Figure 20. IFNα-induced phosphorylation of STAT1 is impaired in HDAC4–depleted cells. A549 cells were transfected with 1 nM of CT or HD4 siRNA for 72 hours, following which the cells were treated with IFNα (10 IU/mL). (A) After 0, 2, 4 and 6 hours of treatment the cells were harvested and the levels of HDAC4, pSTAT1, tSTAT1 and PDI polypeptides were analysed by WB. The knockdown of HDAC4 was confirmed at the 0h time point where no polypeptide can be detected in the HD4 sample. (B) The intensity of the pSTAT1 and tSTAT1 bands was quantified using Image Studio Lite software (LI-COR). The levels of pSTAT1 was then normalised with the corresponding tSTAT1 levels. Finally, the normalised level of pSTAT1 at each time point in CT siRNA-treated cells was considered 100% to compare its amount in HD4 siRNA-treated cells. Error bars represents means ± standard errors of the means of three biological replicates. P value calculated using unpaired t test. MW, molecular weight.
Figure 21. IFNα-induced expression of IFITM3 is decreased in HDAC4-depleted cells. A549 cells were transfected with 1 nM of CT or HD4 siRNA for 72 hours, following which the cells were treated with IFNα (10 IU/mL). (A) After 0, 2, 4 and 6 hours of treatment the cells were harvested and the levels of HDAC4, IFITM3 and PDI polypeptides were analysed by WB. The knockdown of HDAC4 was confirmed at the 0h time point where no polypeptide can be detected in the HD4 sample. (B) The intensity of the IFITM3 and PDI bands was quantified using Image Studio Lite software (LI-COR). The levels of IFITM3 was then normalised with the corresponding PDI levels. Finally, the normalised level of IFITM3 at the 6h time point in CT siRNA-treated cells was considered 100% to compare its amount in HD4 siRNA-treated cells. Error bars represents means ± standard errors of the means of three biological replicates. P value calculated using unpaired t test. MW, molecular weight.
3.6 Discussion

In chapter II, we were able to demonstrate that host HDAC4 plays an antiviral role in IAV infection. The aim of this chapter was to elucidate the mechanism by which HDAC4 is exerting its antiviral function. The data presented here indicate that HDAC4 plays an important role as a component of type I interferon signalling.

HDAC4 regulates the type I interferon signalling pathway at several levels. In IAV-infected HDAC4-depleted cells, the mRNA level of IFNα is reduced, whereas in HDAC4-overexpressing cells, the level is enhanced (Figure 13, 17). It should be noted that we assumed IFN expression was induced as a result of IAV infection as we did not actually confirm the induction by using an uninfected control. Regardless, this is a reasonable assumption to make given the link between viral infection and IFN expression. Interestingly, this data directly contrasts with a study which reported that HDAC4 decreases type I IFN production during virus infection by preventing phosphorylation of IRF3 [421]. However, in this study, only the expression of IFNβ was examined and only in response to infection with Sendai virus, VSV and HSV-1. Furthermore, the results were obtained in HEK293T cells, which are not natural infection models of these viruses or IAV. Given the diversity of heterogeneous phenotypes within mammalian cell lines, it is possible that HDAC4 might both promote and inhibit the antiviral response against different viruses in different cell lines. In addition, another Class II HDAC, HDAC6, promotes the phosphorylation of IRF3 and type I IFN production during RNA virus infection, including IAV [366]. Being from the same class, HDAC4 and HDAC6 might possess similar functionality and appear to be involved in similar cellular processes [367]. Nevertheless, it would be interesting to determine how the depletion and overexpression of HDAC4 effects the phosphorylation of IRF3 during IAV infection and how this correlates with downstream type I IFN production.

This data also reveals that HDAC4 is important in promoting the phosphorylation of STAT1 during IAV infection. Decreased HDAC4 expression corresponded with a decrease in IAV-induced STAT1 phosphorylation (Figure 14). Subsequently, the downstream effect of decreased STAT1 phosphorylation was observed in the decreased polypeptide expression of ISGs – IFITM3, ISG15 and viperin (Figure 16), all of which have been previously reported to restrict the IAV infection [441]. Consistently, overexpression of HDAC4 promoted STAT1 phosphorylation and ISG expression during
IAV infection. However, the transfection of large DNA structures such as the plasmids that were used here induced the innate immune response. This is evident as STAT1 was phosphorylated even before infection with IAV (Figure 19). Regardless, the polypeptide levels of pSTAT1, viperin and ISG15 were all found to be increased in HDAC4 overexpressing cells as compared to control cells (Figure 19). Others have previously reported that HDAC4 physically interacts with STAT1 to promote its phosphorylation and subsequent nuclear import [412, 426, 436]. However, none of these studies observed this phenomenon in the context of IAV infection and only 1 study focused on the HDAC4-STAT1 interaction in response to a virus infection –VSV. In this study they were able to show that HDAC4 depletion decreased STAT1 phosphorylation and type I IFN signalling while overexpression of HDAC4 activated STAT1 inducing resistance to VSV infection [426]. Additionally, HDAC4 is also known to activate STAT2 during infection with HSV-1 and vaccinia virus to promote type I IFN signalling [422]. Furthermore, it has been established that HDACs 1, 2, 3, 8 and 11 all play a role in promoting STAT1 activation under various conditions [224-226, 442, 443].

In direct contrast with the data presented here, several studies have reported that HDAC4 negatively regulates the innate immune response. Recently, 2 findings showed that HDAC4 depletion inhibits VSV and HSV-1 replication [421, 508]. However, neither study reported the effect of HDAC4 overexpression on the growth of these viruses. Furthermore, a different cell type, Hep-2 cells were used which have been shown to have differential effects on HSV-1 replication where knockdown of STING inhibited HSV-1 replication where in other cell lines STING depletion promotes HSV-1 replication [444]. Another group reported that nuclear localized HDAC4 present prevents phosphorylated STAT1 from binding to its target promoters by forming a complex with LXRα, HDAC4 and pSTAT1 [439]. Interestingly, this inhibitory activity is a result of HDAC4 sumoylation and not its deacetylase activity. This is in line with the data showing HDAC4 regulates STAT1 in the cytoplasm through its deacetylase activity which promotes STAT1 phosphorylation and translocation into the nucleus to induce the type I IFN response. Thus, it seems that HDAC4 can play a dual role in both promoting and inhibiting the type I IFN signalling pathway. However, as mentioned above, this is highly dependent upon the cell line and conditions under which the type I IFN pathway is stimulated. Even so, in the context of IAV infection, it is apparent that HDAC4 promotes type I IFN signalling in order to restrict viral replication. The targeting of HDAC4 by IAV observed here and in another
study [427] provides further evidence towards the importance of HDAC4 as an anti-IAV host factor.

We also investigated the effect on the JAK/STAT pathway in HDAC4-depleted cells after stimulation with IFNα. Here we found that the JAK/STAT signalling cascade was impaired in HDAC4-depleted cells, indicating that the antiviral role of HDAC4 is not specific to IAV, but a general response to viral infection (Figure 20). Here, phosphorylation of STAT1 was greatest at 2 hours post-treatment, indicating that IFN-α-induced induction of STAT1 phosphorylation occurs sooner than this. Interestingly, the level of pSTAT1 is only slightly less in the HDAC4-depleted cells, however the levels diminish at a faster rate when compared to the HDAC4-expressing cells (Figure 20B). This is potentially due to the acetylation-phosphorylation switch previously observed in STAT1 signalling [445]. The acetylation of phosphorylated STAT1 at lysine residues K410 and K413 induce a conformational change in the structure of phosphorylated STAT1. This exposes the phosphorylated tyrosine residue Y701 to the phosphatase T cell protein (TCP45) which then dephosphorylates STAT1 [513, 514]. Upon being dephosphorylated, STAT1 becomes inactive and translocates to the nucleus where it remains in a latent state [445]. Deacetylation of STAT1 by HDACs then allows STAT1 to become phosphorylated once more, allowing it to translocate back into the nucleus to promote the expression of immune genes (Figure 33). Hence, in HDAC4-depleted cells, STAT1 is dephosphorylated at a faster rate (Figure 16) and in cells overexpressing HDAC4, STAT1 remains phosphorylated for longer (Figure 19). However, it still remains to be determined if HDAC4 actively deacetylates STAT1 as this has not been experimentally shown here. Furthermore, there is still some uncertainty within the literature regarding the acetylation-phosphorylation switch of STAT1 as 1 study as suggested that STAT1 is not regulated by such a mechanism [515]. Nevertheless, it is clear that further investigations into the interaction between HDAC4 and STAT1 are necessary in order to understand how HDAC4 affects STAT1 phosphorylation in IAV-infected cells. Regardless, the downstream response to STAT1 phosphorylation had a consistent effect on the expression of the STAT1-regulated ISGs – viperin, ISG15 and IFITM3.

However, there was an interesting discrepancy observed between the levels of IFITM3 mRNA and polypeptide in IAV-infected, HDAC4 depleted cells. Here, the mRNA of IFITM3 was unchanged in response to HDAC4 knockdown (Figure 15), however, there was an obvious decrease in the polypeptide expression as compared to normal expressing
cells (Figure 16). A possible explanation for this is that HDAC4 does not promote the mRNA expression of IFITM3 but rather, indirectly enhances the stability of the IFITM3 polypeptide. The stability of the IFITM3 polypeptide is known to be mediated by the E3 ubiquitin ligase NEDD4 [446]. Ubiquitination of IFITM3 is primarily mediated by NEDD4 and results in the degradation of the IFITM3 polypeptide. As such, mutation of the ubiquitination sites on IFITM3 or knockdown of NEDD4 results in an increase in IFITM3 stability [446, 447]. Previously, it has been shown that some members of the HDAC family including the Class IIa HDAC, HDAC5, repress NEDD4 gene expression [448, 449]. Therefore, it is possible that HDAC4 may also play a similar role in repressing NEDD4 expression and in its absence, NEDD4 expression is increased, leading to increased degradation of the IFITM3 polypeptide. Examining the effect of NEDD4 expression in response to HDAC4 knockdown and overexpression would provide insight into this potential mechanism of IFITM3 regulation. An alternative explanation could be that the primers used to detect IFITM3 mRNA could be giving a false reading given that these primers were not tested in uninfected cells. Testing these primers in uninfected cells and using different primers to detect IFITM3 might resolve this discrepancy. Another limitation of the experiments performed pertains to the lack of mock infected cells in the experiments examining the mRNA levels of different ISGs in response to IAV infection (Figure 13, 15, 17, 18). The lack of mock infected controls for these experiments means that there is no discernible induction of these ISGs as a result of IAV infection. Furthermore, because the data is presented as percentages, it means the observations could be a result of background readings as opposed to an actual effect on the ISG mRNA expression in response to HDAC4 expression. This might further elucidate the inconsistency between the IFITM3 mRNA and polypeptide expressions. It might also be prudent to use antibodies to probe for any of the other ISGs whose mRNA levels did not change in response to HDAC4 knockdown or overexpression.
CHAPTER IV - RESULTS:

THE ANTAGONISM OF HOST HISTONE DEACETYLASE 4 BY INFLUENZA A VIRUS
4.1 Introduction

The data presented in chapter II and III clearly demonstrate that host HDAC4 possesses anti-influenza properties and exerts its antiviral effect through being a component of host innate immune response against IAV. While obtaining these data, it became apparent that during the course of IAV infection, the polypeptide expression level of HDAC4 was being dramatically reduced. In fact, after 24 hours of infection with 1 MOI, HDAC4 polypeptide would often be undetectable. This level of antagonism indicated that IAV might be targeting HDAC4 to decrease its expression within the cell in order to disrupt its antiviral function, thereby allowing the virus to replicate more efficiently. Therefore, the purpose of this chapter is to identify the mechanism(s) that IAV employs to dysregulate HDAC4 expression so effectively. Previous studies have shown that IAV dysregulates the polypeptide expression of other HDACs (HDAC1, 2 and 6) during the course of infection and this appears to be important in promoting viral replication. However, none of these HDACs appear to be antagonized as profoundly as has been observed here with HDAC4. This chapter explores the precise nature of HDAC4 dysregulation as a result of IAV infection including identifying the importance of viral dose and strain as well as the overall dysregulation kinetics over time. Furthermore, it looks into the role of some the common host mechanisms that regulate protein expression in the context of IAV-induced HDAC4 dysregulation. Finally, this chapter aims to identify the specific IAV gene(s) that are responsible for inducing the dysregulation of HDAC4 during infection.

4.1.1 IAV mechanisms of host manipulation that promote virus replication

Many of the IAV proteins play a multifunctional role during infection where they are able to interact with specific host proteins in order to dysregulate the antiviral response and influence the cell physiology to be optimal for virus replication. NS1 is the most well characterised IAV protein in this regard and has been shown to be involved in host innate immune defence, host and viral mRNA expression, apoptosis, viral RNA splicing and morphogenesis. NS1 is able to bind with the host cell double-stranded RNA sensors such as protein kinase R (PKR), RIG-I, and 2'-5' oligoadenylate synthetase [450-454]. The binding of NS1 to these sensors interferes with their function and prevents them from activating the innate immune response. Furthermore, NS1 is also able to interact with some of the regulators of these sensors such as the protein activator EIF2AK2, TRIM25 and NF90 [455-457]. For example, NS1 binds to the coiled-coil domain of TRIM25, blocking TRIM25-mediated activation of RIG-I [456]. NS1 also plays a role in suppression of
cellular mRNA nuclear export and subsequent expression, including the expression of type I interferon genes. NS1 achieves through interaction with poly(A)-binding protein II (PABII) as well as the host export machinery, thereby blocking the nuclear export of fully processed host mRNAs [39, 458]. Additionally, NS1 can also manipulate the cell cycle progression and delay virus-induced apoptosis through activation of the phosphoinositide 3-kinase (PI3K)/AKT serine/threonine kinase 2 (AKT) pathway and interaction with p53 [459, 460]. This prolongs the period of time which the cell is able to produce viral progeny and prolong the infection cycle. Conversely, NS1 has been shown to interact with heat shock protein 90 and alpha tubulin to promote apoptosis [461, 462]. Finally, NS1 promotes viral replication, RNA splicing and viral morphogenesis through utilization of several host factors. Translation of viral mRNA is enhanced by NS1 through interaction with eIF4GI and PABPI. Regulation of viral M1 RNA splicing is also mediated by NS1-binding protein, as it forms a complex with heterogeneous nuclear ribonucleoproteins [463]. In the late stages of infection, NS1 is believed to involved in the morphogenesis of the viral particle by binding with the Staufen protein [464, 465].

IAV NP is also known to interfere with cellular responses and suppresses the immune response. NP is able to interact with the DExD-box helicase 39B and high mobility group box 1 (HMGB1) which promotes viral RNA synthesis and viral replication. Additionally, NP has been found to interact with the chaperone protein heat shock protein 40 (Hsp40). This interaction is thought to prevent the phosphorylation of PKR and the subsequent activation of the downstream antiviral IFN response [44, 466, 467].

The IAV RdRp is the key enzyme that drives the virus replication cycle and consists of three individual polypeptides, PB1, PB2 and PA. While the primary role of the RdRp is to carry out the transcription of viral genes, it has also been shown to antagonise cellular processes too. For example, RdRp can inhibit RNA Pol II transcription by inducing the degradation of Pol II via the ubiquitin-proteasome system [468]. Additionally, it is believed that because of its cap-snatching activity, RdRp can promotes the degradation of nascent RNAs through the activity of host exonucleases such as Xrn1 and Xrn2 [469].

The PB1 gene segment also encodes a variant of the PB1 protein known as PB1-F2 which is encoded by an alternate (+1) reading frame within the Pb1 gene. PB1-F2 has been described as another IAV protein that is involved in blocking the host immune response. PB1-F2 is able to interact with MAVS and other components of the RIG-I/MAVS system which leads to a decreased production of cytokines such as IFN-β, IL-6, IL-8, and IL-1β.
Additionally, PB1-F2 is able to interfere with some of the downstream pathways of the RIG-I/MAVS including the IRF3, the NF-KB and the TBK1 pathways to further dysregulate the IFN signalling [473-475].

PA-X is a recently discovered protein that is derived as a result of ribosomal frameshifting during translation and has been well characterised for its role in host shutoff activity through host RNA degradation. PA-X has been found to preferentially destabilise host mRNA synthesised by cellular Pol II, which is responsible for transcribing all host mRNA and many non-coding RNAs in the cell. This preferentially targeting of RNA transcribed by Pol II means that viral RNAs are spared from PA-X mediated degradation as these are transcribed by the viral RdRp. Pol II-transcribed mRNAs undergo PA-X-mediated endonucleolytic cleavage which then allows the host 5'-3'-exonuclease Xrn1 to complete the degradation of the RNA [48].

4.1.2 Mechanisms of cellular HDAC4 regulation

Appropriate regulation of HDAC4 is highly important for healthy development, therefore, it is unsurprising that there several layers of regulation within the cell that control when, and where HDAC4 is expressed. MicroRNAs (miRNAs) are a class of regulatory RNAs that have been shown to modulate HDAC4 expression which include miR-1, miR-29, miR-140, miR-155, miR-200a, miR-206, and miR-365 in a variety of different cell types [388]. These miRNAs target GC regions in the 3' UTR region of the HDAC4 mRNA and thereby repress its expression.

HDAC4 is able to shuttle between the cytoplasm and the nucleus in response to various stimuli [476]. This change in HDAC4 subcellular distribution has been identified as a key way in which the cell is able to rapidly modulate HDAC4-controlled gene expression. Post-translational modifications have been identified has key regulators that determine where HDAC4 localises, which can have a profound effect on a number of cellular responses. Recent evidence has shown that HDAC4 is subject to phosphorylation, sumoylation, ubiquitination as well proteolytic cleavage, all of which control HDAC4 distribution and function. Phosphorylation/dephosphorylation is an important regulatory mechanism of HDAC4 as it efficiently and rapidly couples the repression of HDAC4 to environmental signals. Phosphorylation of HDAC4 is carried out by calcium/calmodulin-dependent protein kinase (CaMK), extracellular signal-regulated kinases 1 and 2 (ERK1/2), protein kinase A (PKA) and glycol synthase kinase 3 (GSK3). HDAC4 contains phosphoserine binding sites that, upon being phosphorylated, allow for binding with the 14-3-3 chaperone.
protein. This interaction occurs within in the nucleus and results in the escort of HDAC4 into the cytoplasm leading to the activation of HDAC4-target genes [477-479]. CaMK phosphorylation of HDAC4 promotes myogenesis through disruption of MEF2-HDAC4 complexes and induction HDAC4 nuclear export [480]. Phosphorylation by PKA induces the proteolytic cleavage of HDAC4 at tyrosine 207 by some unknown protease which leads to a nuclear accumulation of the generated N-terminal fragment of HDAC4 capable of selectively inhibiting MEF2, but not other HDAC4 target genes [481]. Additionally, GSK3 can phosphorylate HDAC4 at position 298 and 302 to induce proteasome-mediated degradation which shown to be important in regulating random cell motility [482]. HDAC4 is also known be dephosphorylated by protein phosphatase 2A (PP2A) at multiple serine residues on the polypeptide including at the 14-3-3 binding sites. This induces its nuclear import and subsequent reinstatement of its transcriptional repression activity [483]. Sumoylation has also been reported to be important for the enzymatic functionality of HDAC4. Specifically, SUMO-1 recognises HDAC4 at a single lysine residue (lysine599) which does not affect the subcellular distribution, but seems to be important in promoting the transcriptional repression and enzymatic activities of HDAC4 [484, 485]. Finally, direct proteolytic cleavage of HDAC4 has been shown to be a highly important mechanism for regulating HDAC4 activity and promoting apoptosis. Caspase-mediated cleavage of HDAC4 polypeptide has been shown to occur at aspartic acid 289 which generates a small N terminal fragment. This N-terminal fragment possesses a nuclear localization sequence and after cleavage is translocated into the nucleus where it inhibits transcription, induces cell death and strongly represses MEF2C [429, 486-489].
4.2 Results

4.2.1 HDAC4 polypeptide expression is downregulated in a cell line independent manner

In order to further understand the IAV-induced downregulation of HDAC4 polypeptide, several experiments were performed. Firstly, the effects of varying IAV doses was explored to determine if there was a correlation between IAV dose and HDAC4 antagonism. A549 cells were infected with PR8 at an MOI of 0.5 or 5.0 for 24 hours. The total cell lysates were prepared and HDAC4, actin and viral NP were detected by WB. The protein bands were visualised using the Odyssey Fc imaging system (LI-COR) and the HDAC4 and actin bands were quantified using Image Studio Lite software. HDAC4 was normalized to actin, then the normalized amount of HDAC4 in the uninfected sample was considered 100% in order to compare it to the infected, 0.5 and 5.0 MOI samples. The WB analysis revealed that the IAV-induced downregulation of HDAC4 was a dose-dependent response, with greater HDAC4 antagonism occurring at higher doses (Figure 22A). Quantitation of 3 independent experiments revealed the IAV-induced antagonism of HDAC4 reduced the polypeptide by 36.5% at 0.5 MOI and 52% at 5.0 MOI when compared to uninfected cells harvested at the same time (Figure 22B).

Additionally, IAV-induced antagonism of the HDAC4 polypeptide was also analysed in MDCK cells. Despite these cells not being physiologically relevant, MDCK cells are conducive to IAV infection and have been extensively used to study IAV biology. MDCK cells were infected for 24 hours with PR8 at either 0.5 or 5.0 MOI or were left uninfected. Following this, the cells were harvested and whole cell lysates were analysed by WB. Once again, WB analysis revealed a dose-dependent downregulation of the HDAC4 polypeptide after IAV infection. Furthermore, the antagonism was enhanced in MDCK cells as compared to A549 cells (Figure 23A). Quantitation of 3 independent experiments revealed the HDAC4 polypeptide was downregulated by 57.9% and 86.8% after infection at 0.5 and 5.0 MOI respectively, as compared to uninfected cells (Figure 23B).
Figure 22. IAV downregulates HDAC4 polypeptide level in A549 cells. A549 cells were infected with PR8 at 0.5 MOI or 5.0 MOI. (A) After 24 hours, the UNI and infected (0.5 and 5.0) cells were harvested, total cell lysates were prepared, and HDAC4, actin (42 kDa) and viral NP polypeptides were detected by WB. (B) The HDAC4 and actin bands in panel (A) blot were quantified using Image Studio Lite software (LI-COR). The levels of HDAC4 in each sample was then normalized with the corresponding actin levels. Finally, the normalized level of HDAC4 in UNI sample was considered 100% to compare its level in infected (0.5 or 5.0) cells. Error bars represent means ± standard errors of the means of 3 independent experiments. P value calculated using unpaired t test. MW, molecular weight.
Figure 23. IAV downregulates HDAC4 polypeptide level in MDCK. MDCK cells were infected with PR8 at 0.5 MOI or 5.0 MOI. (A) After 24 hours, the UNI and infected (0.5 and 5.0) cells were harvested, total cell lysates were prepared and HDAC4, actin and viral NP polypeptides were detected by WB. (B) The HDAC4 and actin bands in panel (A) blot were quantified using Image Studio Lite software (LI-COR). The levels of HDAC4 in each sample was then normalized with the corresponding actin levels. Finally, the normalized level of HDAC4 in UNI sample was considered 100% to compare its level in infected (0.5 or 5.0) cells. Error bars represent means ± standard errors of the means of 3 independent experiments. P value calculated using unpaired t test. MW, molecular weight.
4.2.2 IAV-induced antagonism of HDAC4 polypeptide is strain-independent

Next, we wanted to determine if the IAV-induced antagonism of HDAC4 is strain-independent. Here, A549 cells were infected for 24 hours with the clinically relevant strain CA09 (at 0.5 or 5.0 MOI) or another lab-adapted strain, WSN (at 1.0 or 3.0 MOI). The cells were then harvested and whole cell lysates were used to detect the polypeptide expression of HDAC4, actin and viral NP by WB. Consistent with the previous observations using PR8, both CA09 (Figure 24A) and WSN (Figure 24B) downregulated the HDAC4 polypeptide expression in a dose-dependent manner.

Additionally, a further experiment was performed using UV-irradiated IAV to ascertain if a productive infection is required to antagonise the HDAC4 polypeptide. For this, A549 cells were infected for 24 hours with either UV-irradiated PR8, or ‘live’ PR8, or left uninfected. UV-irradiation inactivates the genetic material of the virus, rendering it unable to replicate. However, the virus still retains its antigenic nature, capable of eliciting and immune response [490]. WB analysis revealed that the HDAC4 polypeptide expression in cells infected with UV-irradiated virus was comparable in uninfected cells (Figure 24C). Absence of viral NP confirmed that the UV-irradiated virus was unable to replicate.
Figure 24. Downregulation of HDAC4 polypeptide is strain-independent. (A and B) MDCK cells were infected for 24 hours with (A) CA09 (at MOI of 0.5 or 5.0) or (B) WSN (at MOI 1.0 or 3.0). The UNI and infected (0.5, 1.0, 3.0 and 5.0) cells were harvested, total cell lysates were prepared and HDAC4, actin and viral NP polypeptides were detected by WB. (C) MDCK cells were infected with ‘live’ (INF) or UV-irradiated (INF_UV) PR8 at an MOI of 0.5 for 24 hours. Total cell lysates were prepared, and HDAC4, actin and viral NP polypeptides were detected by WB. Data is representative of a single repeat. MW, molecular weight.
4.2.3 IAV-induced antagonism of the HDAC4 polypeptide is time-dependant

The above results were observed 24 hours post-infection, however, the antagonism of HDAC4 was likely occurring much earlier. Therefore, we wanted to understand the antagonism kinetics of the HDAC4 polypeptide over the 24-hour infection period. Due to the greater level of IAV-induced antagonism of HDAC4 observed above, MDCK cells were chosen for these experiments. For this, MDCK cells were infected with PR8 at 1.0 MOI and were harvested after 2, 6, 12 and 24 hours of infection. The total cell lysates were prepared and resolved on a 7-12% gradient SDS-PAGE and HDAC4, PDI and viral NP were detected by WB (Figure 25A). Intriguingly, use of a gradient gel revealed 2 smaller HDAC4 fragments of approximately 90 kDa and 30 kDa in size. These fragments first appeared around 12 hours post-infection and intensified at 24 hours. This indicated that the IAV-induced antagonism of HDAC4 was occurring as result of the polypeptide undergoing proteolysis. Previously, it has been reported that another class II HDAC, HDAC6 is also cleaved during IAV infection as a result of the activity of caspase 3 [369]. Additionally, the first signs of HDAC4 cleavage occur around the same time that viral NP is cleaved, which is also cleaved by caspase 3 [79]. Therefore, the same samples were probed with caspase 3 antibody to determine if the inactive form (Cas3-FL) was being cleaved into the active form (Cas3-CL) at a similar time. Interestingly, WB analysis revealed a significant accumulation of the Cas3-CL by 12 hours post infection, indicating that activated caspase 3 could be cleaving the HDAC4 polypeptide. Visualisation of HDAC4 full length polypeptide revealed that IAV-induced antagonism was most profound within the 12- to 24-hour time period. The HDAC4 and PDI protein bands from panel (A) blot were quantified using Image Studio Lite software (LI-COR). Full length HDAC4 was normalized to the corresponding PDI. Then, the normalized amount of HDAC4 at the 2-hour time point was considered 100% for comparisons to the 6, 12 and 24-hour time points. This analysis revealed no significant change in HDAC4 polypeptide level at 6 hours and widely variable levels at 12 hours post-infection. However, at 24 hours postinfection, there was a significant 82% reduction in HDAC4 full length polypeptide (Figure 25B).
Figure 25. IAV-induced HDAC4 polypeptide downregulation occurs in a time-dependent manner. (A) MDCK cells were infected with PR8 at an MOI of 1.0, and harvested after 2, 6, 12 and 24 hours. Total cell lysates were prepared and resolved on a 7-12% gradient SDS-PAGE, and HDAC4, PDI, viral NP, full length caspase 3 (Cas3-FL) (35 kDa) and cleaved caspase 3 (Cas3-CL) (17 kDa) were detected by WB. Arrows indicate the HDAC4 polypeptide cleavage products. MW lane was combined with the rest of the lanes after removing an unwanted middle lane. (B-D) The intensity of the full length HDAC4 (B), NP (C), and Cas3-CL (D) bands in panel (A) were quantified and then normalized with the corresponding PDI levels or Cas3-FL for (D). Finally, the normalized level of HDAC4, NP or Cas3-CL in the 2-hour time point (2 h) was considered 100% or 1 fold to compare the amount to the other time points. The HDAC4 blot is the upper and lower half of the same blot as is the caspase 3 blot. Error bars represent means ± standard errors of the means of 3 independent experiments. P value calculated using unpaired t test. MW, molecular weight.
4.2.4 Lysosomally-associated caspase 3 cleaves HDAC4 polypeptide in IAV-infected cells

The above data revealed the IAV-induced antagonism of HDAC4 involved proteolytic cleavage of the HDAC4 polypeptide. Therefore, the next step was to identify the degradation pathway responsible for the IAV-induced cleavage of the HDAC4 polypeptide. In mammalian cells two main pathways exist that facilitate the degradation of polypeptides; the lysosome-mediated and proteasome-mediated pathways. The lysosome pathway is the major pathway involved in the turnover of cytosolic proteins. Lysosomes are acidic membrane-bound vesicles which contain a variety of hydrolytic enzymes used to break down macromolecules within the cell. The proteasome pathway is able to degrade both cytosolic and nuclear associated proteins. This pathway involves the tagging of unwanted or damaged proteins through the small protein called ubiquitin. Upon being tagged, the proteasome, a large protein complex, is able to recognise and subsequently degrade the protein. Previously, our lab has shown that during IAV infection, both HDAC1 and HDAC2 polypeptides are degraded via the proteasome pathway [225, 226]. Therefore, it is likely that the proteasome pathway is degrading the HDAC4 polypeptide during IAV infection. However, as mentioned above, caspases are also likely candidates. In order to identify which of these pathways might be involved, we perturbed each using the lysosome inhibitor NH₄Cl and the proteasome inhibitor MG132. Additionally, we used a caspase 3 inhibitor (Cas3-I) to determine if caspase 3 was involved. For this, half of a set of MDCK cells were infected with PR8 at 1.0 MOI for 24 hours while the other half were left uninfected. After 1 hour, the culture media was replaced with SF-MEM containing NH₄Cl (10 mM), MG132 (20 µM) or Cas3-I (40 µM) as these concentrations of the inhibitors have been shown to inhibit the respective pathways [491, 492]. Additionally, an untreated mock set of cells were infected or left uninfected exactly as described above. After 24 hours, the cells were harvested and whole cell lysates were prepared the levels of HDAC4, PDI, viral NP and caspase 3 were detected by WB. The analysis revealed the treatments of NH₄Cl and Cas3-I rescued the full length HDAC4 polypeptide in IAV-infected cells. Additionally, the recovery of the HDAC4 polypeptide in the Cas3-I samples coincided with the inhibited activation of caspase 3 in infected cells. Caspase 3 activation was determined by conversion of the inactive, full length form (Cas-FL) into the active, cleaved form (Cas-CL). Further, caspase 3 activation in NH₄Cl-treated infected cells was partially inhibited (Figure 26A). Compared to 81% reduction of the full length HDAC4 polypeptide level in mock-treated cells, there was only a 54% and a 46% reduction in cells
treated with NH₄Cl and Cas3-I, respectively. In other words, HDAC4 polypeptide levels recovered from 19% to 46% and 54% in NH₄Cl and CAS3-I-treated infected cells, respectively (Figure 26B). Interestingly, MG132 treatment actually enhanced HDAC4 cleavage in infected cells and even induced cleavage in uninfected cells (Figure 26A). Quantitation of the infected, MG132-treated cells revealed a 94.4% reduction in the HDAC4 full length polypeptide (Figure 26B). The effect of MG132 treatment on HDAC4 polypeptide levels, both in uninfected and infected cells, further indicated caspases are responsible for cleaving HDAC4. MG132 treatment is known to induce apoptosis (hence caspase activation) in mammalian cells [509].

The above data indicated that caspase 3 cleaves HDAC4 during IAV infection, however, the caspase inhibitor used is also known to inhibit the other executioner caspases; 6 and 7. This meant that other caspases might also play a role in the IAV-induced cleavage of HDAC4. Caspase 3, 6 and 7 are the executioner caspases that are involved in apoptotic-mediated protein cleavage. The other members of the caspase family are known as the initiator caspases, which are upstream activators of the executioner caspases. The initiator caspases cleave the procaspase form of the executioner caspase into a smaller, activated form. Therefore, we wanted to identify specific which executioner caspase(s) cleaves HDAC4 during IAV infection so we decided to conduct a knockdown screen of the executioner caspases. Here, two sets of A549 cells were transfected with 10 nM of CT siRNA or siRNA targeting caspase 3 (Cas3), caspase 6 (Cas6) or caspase 7 (Cas7) for 72 hours. Then, the first set of cells were harvested immediately and the total RNA was extracted to confirm the knockdown of each caspase via qPCR. Meanwhile, the second set of cells were either infected for 24 hours with PR8 at 1.0 MOI or were left uninfected. The cells were then harvested and total cell lysates were prepared and HDAC4, PDI and viral NP were detected by WB. The WB analysis revealed that caspase 3 knockdown recovered the full length HDAC4 polypeptide in infected cells, whereas knockdown of caspase 6 and caspase 7 did not (Figure 27A). qPCR analysis of the uninfected caspase siRNA-treated cells revealed that all 3 caspases were successfully knocked down. Specifically, there was a 90.6%, 96.1% and 94.6% reduction in caspase 3, 6 and 7 mRNA expression, respectively (Figure 27B).

Knockdown of the executioner caspase revealed that only caspase 3 was involved in the IAV-induced cleavage of HDAC4. Therefore, a further knockdown experiment was performed solely on caspase 3, in order to quantify the recovery of the full length HDAC4 polypeptide in caspase 3 depleted, IAV-infected cells. In addition, the effect of caspase 3
depletion on the HDAC4 cleavage products was also investigated. Again, A549 cells were treated with CT or Cas3 siRNA as described above. Next, an uninfected subset of the cells was harvested for WB to determine the level of caspase 3 polypeptide in CT and Cas3 siRNA-treated cells. Meanwhile, the other set of cells were infected for 24 hours with PR8 at 1.0 MOI, or were left uninfected. Cells were harvested and total cell lysates were prepared and HDAC4, actin, viral NP and caspase 3 were detected by WB. Again, the analysis revealed that caspase 3 was sufficiently depleted in the cas3 siRNA-treated cells. Additionally, the full length HDAC4 polypeptide was restored in caspase 3 depleted, IAV-infected cells. However, the HDAC4 cleavage products remained in the Cas3 siRNA-treated cells, albeit at decreased levels as compared to CT siRNA-treated cells. Interestingly, in the Cas3 siRNA-treated cells, the HDAC4 cleavage product (denoted c) disappeared entirely, however a new product (denoted a) appeared slightly above cleavage product b (Figure 28A). Quantitation of the full length HDAC4 bands revealed that, consistent with the previous observations, knockdown of caspase 3 expression rescued the full length HDAC4 polypeptide in IAV-infected cells. Specifically, IAV infection induced a 77% reduction in the full length HDAC4 polypeptide level in CT siRNA-treated cells, whereas there was only a 29.4% reduction in Cas3 siRNA-treated cells. In other words, the full length HDAC4 polypeptide level in infected cells depleted of caspase 3, recovered to 70.6% from its level at 23.5% in infected cells expressing normal levels of caspase 3 (Figure 28B).
Figure 26. NH₄Cl and caspase inhibitor treatments recover full length HDAC4 polypeptide in infected cells. (A) MDCK cells were infected with PR8 at an MOI of 1.0 and, after removing the virus inoculum, were treated with NH₄Cl (10 mM), MG132 (20 µM), or caspase 3 inhibitor (Cas3-I; 40 µM). After 24 hours, the uninfected (UNI) and infected (INF) cells were harvested, total cell lysates were prepared, and HDAC4, PDI, viral NP, Cas3-FL and Cas3-CL were detected by WB. The arrows and letters indicate the different cleavage products detected. (B) The intensity of the full length HDAC4 bands were quantified and then normalized to the corresponding PDI levels. The normalized levels of HDAC4 for each treatment in the UNI cells was considered 100% to compare its amount to the respective treatment in the INF cells. The HDAC4 blot is the upper and lower half of the same blot as is the caspase 3 blot. Error bars represent means ± standard errors of the means of 3 independent repeats. P value calculated using unpaired t test. MW, molecular weight.
Figure 27. Caspase 3, not caspase 6 or 7 cleaves HDAC4 polypeptide in IAV-infected cells.

(A) A549 cells were transfected in duplicates with 10 nM of CT, caspase 3 (Cas3), caspase 6 (Cas6) or caspase 7 (Cas7) siRNA for 72 hours. One set of the cells were infected with PR8 at an MOI of 1.0. After 24 hours the cells were harvested and HDAC4, PDI and viral NP were detected by WB.

(B) To confirm the knockdown of caspase 3, 6 and 7, the second set of the cells in panel (A) were processed to detect levels of caspase 3, 6 or 7 and actin mRNAs by qPCR. Then, the levels of caspase 3, 6 or 7 mRNA in CT siRNA-treated cells and caspase siRNA-treated cells was normalized to the corresponding actin mRNA levels. Finally, the normalized level of caspase 3, 6 or 7 mRNA in CT siRNA-treated cells was considered 100% to compare its level in the corresponding caspase siRNA-treated cells. (C) The intensity of the full length HDAC4 bands in panel (A) blot were quantified and then normalized with the corresponding PDI levels. Finally, the normalized level of HDAC4 in CT siRNA-treated UNI cells was considered 100% to compare its levels in CT, Cas3, Cas6 and Cas7 siRNA-treated cells. Error bars represent means ± standard errors of the means of 3 independent experiments. P value calculated using unpaired t test. MW, molecular weight.
Figure 28. Knockdown of caspase 3 expression allows retention of full-length HDAC4 polypeptide in infected cells. (A) A549 cells were transfected with 10 nM of CT siRNA or cas3 siRNA for 72 hours. Half of the cells were left uninfected (UNI) while the other half were infected (INF) with PR8 at an MOI of 1.0. After 24 hours, both sets of cells were harvested and HDAC4, actin, viral NP and caspase-3 were detected by WB. Detection caspase 3 confirmed that the knockdown was successful. (B) The intensity of the full length HDAC4 bands in panel (A) blot were quantified and then normalized with the corresponding actin levels. Finally, the normalized level of HDAC4 in CT siRNA-treated UNI cells was considered 100% to compare its levels in CT siRNA-treated INF cells and Cas3 siRNA-treated INF cells. The HDAC4 blot is the upper and lower half of the same blot as is the caspase 3 blot. Error bars represent means ± standard errors of the means of 3 independent experiments. P value calculated using unpaired t test. MW, molecular weight.
4.2.5 The IAV protein PA-X is involved in the downregulation of HDAC4

We also wanted to identify which IAV gene(s) were involved in the IAV-induced antagonism of HDAC4. As mentioned above, the IAV genome consists of 8 gene segments, each of which encodes at least 1 protein. IAV encodes several non-structural genes which aid in viral replication by inhibiting certain host signalling pathways, particularly those involved in the immune response. However, studies have also revealed that some of the structural genes, namely viral NP and viral HA, can also dysregulate certain host responses [466, 493]. Therefore, to identify which IAV gene(s) are responsible for the antagonism of HDAC4, we performed an RNAi screen targeting 4 viral gene segments: NP, NS, PA, and PB1, using the previously described siRNAs [494]. To accomplish this, 50 nM of siRNAs targeting NP, NS, PA, and PB1 and the CT siRNA were delivered into A549 cells for 24 hours. The cells were then infected for 24 hours with PR8 at 1.0 MOI. Next, half of the cells were harvested for total RNA content to confirm the knockdown, while the other half were harvested and the total cell lysates were prepared. HDAC4, PDI and viral NP were detected by WB in order to determine the effect of viral gene knockdown on HDAC4 polypeptide expression. Additionally, the culture media was titrated by plaque assay to examine the effect of viral gene knockdown on IAV replication. The WB analysis revealed that knockdown of the PA gene segment was sufficient to rescue the level of the full length HDAC4 polypeptide in infected cells. However, despite the recovery in the full length polypeptide, HDAC4 cleavage fragments still remained (Figure 29A). In addition, quantification of the full length HDAC4 bands in the PA siRNA-treated samples revealed the recovery was only partial. Specifically, compared to the 74.1% reduction in the full length HDAC4 polypeptide level in CT siRNA-treated cells, there was only a 39.9% reduction in PA siRNA-treated cells. In other words, the level of full length HDAC4 polypeptide in PA siRNA-treated, infected cells, recovered to 65.8% from its level at 25.9% in CT siRNA-treated infected cells (Figure 29B). The depletion of the various viral genes was confirmed by qPCR, specifically, NP was depleted by 79.6%, NS1 by 24.6%, PA by 94.8% PA-X (encoded on the PA gene segment) by 94.2% and PB1 by 86% (Figure 29C). Additionally, plaque assay of the culture media revealed that the NP and PA siRNAs caused an approximate 1 log reduction in virus titre, whereas the NS and PB1 siRNAs had minimal effect (Figure 29D).

The recovery of the full length HDAC4 polypeptide observed in cells transfected with PA siRNA indicated that one of the proteins encoded on this segment is involved in the antagonism of HDAC4. From the known proteins encoded on PA, we wondered if PA-X
was the protein involved in the antagonism of HDAC4. The recently discovered IAV protein PA-X is expressed as a result of ribosomal frameshifting during translation of the PA gene segment [495]. It is an RNA endonuclease and identified as playing an important role in promoting host shutoff activity through selective degradation of host mRNA. Therefore, to determine if PA-X was inducing the antagonism of HDAC4, we transfected a plasmid containing the PA-X gene into A549 cells. After 48 hours, the cells were harvested and total cell lysates were prepared and HDAC4 and PDI were detected by WB. Consistent with the above PA knockdown data, expression of PA-X alone was able to downregulate the full length HDAC4 polypeptide in A549 cells. However, despite the downregulation of the full length HDAC4 polypeptide in PA-X transfected cells, no HDAC4 cleavage fragments could be detected (Figure 30A). Importantly, we have previously determined that even in the presence of caspase inhibitor, PA-X expression alone is still able to downregulate HDAC4 polypeptide expression, suggesting PA-X is dysregulating HDAC4 through an alternative alternate mechanism [522]. Quantitation of the full length HDAC4 across 3 independent experiments revealed that compared to the cells transfected with an empty plasmid, there was a 73.3% reduction in the HDAC4 polypeptide level in cells transfected with PA-X plasmid (Figure 30B). Finally, we were able to determine the successful expression of PA-X from the measuring the absolute copy number. This was estimated by comparing its ct value to a standard curve calibrator, made with same plasmid (Figure 30C).

Because PA-X was not involved in inducing the cleavage of the HDAC4 polypeptide, we decided to investigate if PA-X was antagonising HDAC4 at the mRNA level instead. For this, A549 cells were infected for 24 hours with PR8 at an MOI of 0.5 or 5.0, after which the cells were processed to isolate the total RNA content. The quality and quantity of the isolated RNA was analysed by measuring the 260/280 and 260/230 ratios using a Nanodrop reader spectrophotometer (ThermoScientific). Next, cDNA was synthesised and used as a template to measure the level of HDAC4 and viperin mRNA by qPCR using SYBR green chemistry. The ct values obtained were analysed using the relative quantitation method as described above, using the reference gene actin. The analysis revealed that IAV infection caused a significant 78.9% and 96.2% reduction in HDAC4 mRNA level at 0.5 and 5.0 MOI, respectively (Figure 31A). Here, viperin, which was greatly increased in the infected samples, was used as an infection marker (Figure 31B).

The above data indicated that IAV antagonises HDAC4 not only at the polypeptide level, but also at the mRNA. Next, we wanted to determine if PA-X was responsible for the
antagonism of the HDAC4 mRNA. In order to investigate this, A549 cells were transfected with CT siRNA or PA siRNA for 24 hours. Following this, the cells were infected for 24 hours with PR8 at 1.0 MOI. The cells were then harvested for total RNA and processed to measure the levels of HDAC4 and actin mRNA and 18SRNA by qPCR. The level of HDAC4 mRNA in cells transfected with PA siRNA was significantly recovered as compared to cells transfected with CT siRNA. Specifically, when normalizing to the actin levels, the level of HDAC4 mRNA in the PA siRNA-treated cells recovered to a significant 58.3% from its level at 8.7% in CT siRNA–treated cells. Similarly, when normalized to 18SRNA, the levels recovered from 69.4% in PA siRNA-treated cells to 7.2% in CT siRNA-treated cells (Figure 32A). Finally, in order to confirm that PA-X was antagonising the HDAC4 mRNA, we expressed PA-X from a plasmid in A549 cells and subsequently analysed the level of HDAC4 mRNA by qPCR. Consistent with the above observations, PA-X expression alone was sufficient to reduce the level of HDAC4 mRNA in A549 cells. When normalized to actin mRNA and 18SRNA levels and compared to cells transfected with empty plasmid, there was a 43.8% and 57.6% decrease, respectively, in HDAC4 mRNA level in the cells transfected with PA-X (Figure 32B).
Figure 29. Knockdown of viral PA gene expression recovers full length HDAC4 polypeptide (A-C) A549 cells were transfected in duplicate with CT siRNA or siRNA targeting viral NP, NS, PA or PB1 for 24 hours. The cells were then infected with PR8 at an MOI of 1.0 for 24 hours. (A) One set of the cells were harvested and HDAC4, PDI and viral NP were detected by WB. (B) The intensity of the HDAC4 and PDI bands was quantified using the Image Studio Lite software (LI-COR). The levels of HDAC4 was then normalized with the corresponding PDI levels. Finally, the normalized level of HDAC4 in CT siRNA-treated UNI cells was considered 100% to compare its levels in CT siRNA-treated INF cells and the viral gene siRNA-treated INF cells. (C) To confirm the knockdown of the different IAV genes, the second set of cells total RNA was processed to measure the levels of viral NP, NS1, PA, PA-X, PB1 and cellular actin mRNA by qPCR. Then, the levels of the respective IAV gene mRNA in CT siRNA-treated cells and the analogous IAV gene siRNA-treated cells were normalized with corresponding actin mRNA levels. Finally, the normalized levels of the viral gene mRNA in CT siRNA-treated cells were considered 100% to compare their levels in the respective IAV gene siRNA-treated cells. (D) The culture media from (A) was titrated by plaque assay to determine the effect of the different IAV gene siRNAs on the replication efficiency of the virus by measuring the titres of the released viral progeny. The HDAC4 blot is the upper and lower half of the same blot. Error bars represent means ± standard errors of the means of 3 independent experiments. P value calculated using unpaired t test. MW, molecular weight.
Figure 30. IAV PA-X expression alone is sufficient to downregulate HDAC4. (A-C) A549 cells were transfected in duplicates with either lipofectamine 2000 (LF), 1 µg of empty plasmid pcDNA3.1(−) (pc) or 1 µg of plasmid expressing PA-X (PX) for 48 hours. (A) The first set of cells were harvested and HDAC4 and PDI polypeptides were detected in total cell lysates by WB. (B) The intensity of the HDAC4 and PDI bands in panel (A) blot were quantified using the Image Studio Lite software (LI-COR). The levels of HDAC4 was then normalized with the corresponding PDI levels. Finally, the normalized level of HDAC4 in pc-transfected cells was considered 100% to compare its levels in PX-transfected cells. (C) To confirm the overexpression of PA-X, the second set of cells were processed to determine the PA-X mRNA by qPCR using the PA-X plasmid as a calibrator. For this, PA-X mRNA was detected in PX-transfected cells and serial 10-fold dilutions of PA-X plasmid. The PA-X mRNA in serial dilutions were calculated using the plasmid DNA concentration and the molecular weight. Then, ct value versus copy number standard curve was generated, which was then used as a reference to calculate the PA-X mRNA in PX-transfected cells. The HDAC4 blot is the upper and lower half of the same blot. Error bars represent means ± standard errors of the means of 3 independent experiments. P value calculated using unpaired t test. MW, molecular weight.
**Figure 31. IAV reduces HDAC4 mRNA level.** A549 cells were infected with PR8 at either 0.5 MOI or 5.0 MOI. After 24 hours of infection the levels of HDAC4 (A), viperin (B) and actin mRNA levels were detected by qPCR. Then the levels of HDAC4/viperin were normalized with the corresponding actin mRNA levels. Finally, normalized levels of HDAC4 (A) or viperin (B) mRNA in the uninfected (UNI) samples was considered 100% (A) or 1-fold (B) to compare their levels in the infected cells. Error bars represent means ± standard errors of the means of 3 independent experiments. P value calculated using unpaired t test.
Figure 32. IAV protein PA-X downregulates HDAC4 mRNA. (A) A549 cells were transfected with 10 nM of CT or PA siRNA for 24 hours. The cells were subsequently infected with PR8 at 1.0 MOI for 24 hours. The levels of HDAC4, actin and 18S ribosomal (18S RNA) RNA were detected by qPCR. Then, the levels of HDAC4 mRNA in all samples were normalized with corresponding actin mRNA or 18S RNA levels. Finally, the normalized levels of HDAC4 mRNA in CT siRNA-treated UNI cells were considered 100% to compare its levels in CT siRNA-treated INF cells and PA siRNA-treated cells. (B) A549 cells were transfected with 1 µg of pc plasmid or 1 µg of PX plasmid for 48 hours. The mRNA levels of HDAC4, actin and 18S RNA were detected by qPCR. Then, the levels of HDAC4 mRNA in all samples were normalized with the corresponding actin mRNA or 18S RNA levels. Finally, the normalized level of HDAC4 mRNA in pc-transfected cells was considered 100% to compare its levels in PX-transfected cells. Error bars represent means ± standard errors of the means of 3 independent experiments. P value calculated using unpaired t test.
4.7 Discussion

While investigating the anti-IAV role of HDAC4 in chapters II and III, we noticed that HDAC4 expression was severely diminished in IAV-infected cells. Therefore, the aim of this chapter was to identify the mechanism(s) by which IAV dysregulates HDAC4.

Here, we demonstrated that the IAV-induced antagonism of HDAC4 polypeptide occurs in both A549 and MDCK cells in a time-dependent and H1N1 strain-independent manner (Figure 22, 23, 24, 25). It is well understood that in order to replicate effectively within host cells, IAV manipulates the expression and activity of host factors involved in its replication. It is likely, given its anti-IAV role established above, that HDAC4 is targeted by IAV in order to circumvent its antiviral potential. In further support of this, the IAV-induced dysregulation of other HDACs which are also antiviral, has been well documented [79, 224-226]. However, the mechanisms by which IAV dysregulates each of these HDACs is distinct. For example, HDAC1 and 2 are degraded via the proteasome pathway, HDAC6 is degraded by caspases and HDAC11 mRNA is profoundly depleted in IAV-infected cells.

This indicates that IAV antagonises the various HDACs based upon the characteristics of the genes themselves.

Therefore, we explored the IAV-induced antagonism of HDAC4 further and found that the observed antagonism was a result of the proteolytic cleavage of the polypeptide into smaller fragments. At approximately 12 hours post-infection, two cleavage products appear which also coincides with the activation of caspase 3 and the cleavage of viral NP (Figure 25). Viral NP is cleaved by caspases and is important for its function in the late stages of infection [510]. Additionally, the cleavage of HDAC4 by caspase 2 and caspase 3 has been previously reported, both of which produce fragments of a similar size to the fragments seen here [429, 483, 488, 489]. Incidentally, these studies report the cleavage of HDAC4 can be achieved via non-viral induced apoptosis. Additionally, another Class II HDAC, HDAC6, has already been shown to be cleaved by caspase-3 during IAV infection [369], adding further support to the notion that caspases are responsible for the IAV-induced cleavage of HDAC4. Therefore, IAV, which is known to induce apoptosis in mammalian cells, is able to create an environment which is favourable for the degradation of the HDAC4 polypeptide in infected cells. Consequently, both HDAC4 degradation and proteolytic caspase-3 activation was significantly reversed in IAV-infected cells upon treatment with a caspase-3 inhibitor (Figure 26). Furthermore, depletion of the
executioner caspases revealed that only caspase 3, but not caspase-6 or caspase-7, was responsible for the cleavage of the HDAC4 polypeptide in IAV-infected cells (Figure 27). However, despite partially restoring the full-length HDAC4 polypeptide, some of the cleavage fragments remained, indicating that another mechanism(s) is involved in the IAV-induced cleavage of HDAC4. Indeed, when cells were treated with NH₄Cl, a potent lysosomal pathway inhibitor, the HDAC4 polypeptide was also partially recovered (Figure 26). However, some cleavage fragments remained which were different to the fragments appearing in the caspase inhibitor treated cells. This indicated that HDAC4 is cleaved by the activity of at least two distinct pathways during IAV infection and perhaps by the activation of other proteases. Due to the partial recovery seen in lysosomal inhibitor-treated cells, a likely family of host proteases involved in the IAV-induced cleavage of HDAC4 are the lysosomal-associated proteases; cathepsins. In fact, a recent study showed that HDAC4 is cleaved by cathepsin H in a heterologous system [496]. In addition to this, the nature of the HDAC4 degradation seen here is very similar to the lysosome-mediated degradation of host cortactin polypeptide in IAV-infected cells [497]. Thus, it is quite likely that lysosomes are closely connected with the apoptotic pathway in IAV-infected cells. Interestingly, lysosomes have been shown to be involved in the induction of both intrinsic and extrinsic apoptotic pathways in heterologous systems [498-501]. Another mechanism by which HDAC4 is regulated which was not explored here, is by the activity of protein kinase A (PKA). It has been shown that PKA is able to interact directly with HDAC4 but not with any of the other class IIa HDACs as they lack the appropriate binding site. Once PKA binds to HDAC4, an unknown protease is then able to cleave HDAC4 at amino acid 201, which generates a small N terminal fragment of similar size to what is observed here [481]. These findings demonstrate the complex nature by which HDAC4 is regulated within cells, both under normal and abnormal conditions. It is also interesting to note that different viruses antagonise HDAC4 utilising different mechanisms. For example, similar to IAV, the HCV core protein degrades HDAC4 polypeptide via proteasome-independent pathway [426]. Contrastingly, a recent study showed that the vaccinia virus C6 protein utilises the proteasome pathway to degrade HDAC4 polypeptide [422]. Even so, it is apparent that capsase-3 is involved in the IAV-induced cleavage of HDAC4, however, a thorough investigation is needed to identify other proteases that might also play a role.

The ability of IAV to antagonise plasmid-expressed HDAC4 in A549 cells but not HEK293 cells is an interesting observation (Figure 11). Examining the effect of endogenous HDAC4 in HEK293 cells might reveal that these cells are resistant to IAV-
induced HDAC4 antagonism, possibly due to the lack of a component that is essential for the antagonism. Alternatively, HEK293 cells transfect highly efficiently (Figure 11D), therefore, the apparent lack of antagonism could be due to the overabundance of HDAC4 to A549 cells which transfect much less efficiently (Figure 11B). Nevertheless, it would be interesting to determine the effect IAV infection has on endogenous HDAC4 in HEK293 cells.

In addition, we also identified that HDAC4 mRNA is significantly downregulated in IAV-infected cells (Figure 31). Using an RNA interference screen we found that IAV-infected cells transfected with the siRNA targeting PA exhibited a partial recovery in the full length HDAC4 polypeptide (Figure 29). An important limitation for this experiment was that no uninfected controls using these siRNAs were used. This could potentially mean that the effects seen on HDAC4 polypeptide expression could be a result of off-target effects from the siRNA. Additionally, one could make the argument that this partial recovery could be due to impact on viral replication as the PA gene segment encodes the PA gene which is a vital gene for viral replication. While it is true that these siRNAs impair IAV replication as others have reported [494] and as we observed here, it is important to note that none of the other siRNAs affected HDAC4 expression. This is especially relevant in regards to the siRNA targeting the structural gene NP, which had a similar effect on viral growth as the PA siRNA. Interestingly, the knockdown of both PA and PB1 resulted in significantly reduced NP cleavage (Figure 29A). As mentioned previously this cleavage is known to be carried out via the activity of host caspase 3 [79]. It is likely that deleterious effects of the siRNA on viral replication means that the apoptotic pathway is not as profoundly activated in these cells compared to NS siRNA, which is only partially effective. Even so, despite the reduced NP cleavage in the PB1 siRNA-treated cells, HDAC4 antagonism remains strong, indicating that perhaps partial activation of caspase 3 is sufficient to reduce HDAC4 polypeptide expression. It would be interesting to determine the amount caspase activation in IAV-infected cells treated with the different IAV siRNAs in order to clarify this anomaly. Additionally, we overexpressed PA-X and found its expression alone was sufficient to downregulate HDAC4 mRNA (Figure 32). PA-X is a recently discovered protein and is highly conserved in the IAV genome including the strains PR8 and CA09 that were used here [511, 512]. Recently, it has been shown that IAV PA-X is able to selectively target host mRNA transcribed by the RNA Pol II complex for degradation by the activity of the host exonuclease XrnI [48]. Additionally, a recent study reported that IAV downregulates HDAC4 mRNA in primary bronchial epithelial
cells [427]. However, in this case IAV promotes the expression of miR-22 which targets HDAC4 mRNA to decrease its expression. In addition, 2 other studies showed that HDAC4 mRNA is also antagonised during HBV infection. HBV promotes the expression of 2 miRs, miR-548ah and miR-1, which target HDAC4 mRNA to inhibit deacetylation of the cccDNA which ultimately enhances viral replication [423, 425]. Potentially, the combined HDAC4 characteristics: its antiviral activity and the relatively short half-life of its mRNA, make it an ideal candidate for viruses to target.

It is interesting to note that the knockdown of PA-X did not completely recover the HDAC4 mRNA levels. Likewise, the overexpression of PA-X could not downregulate HDAC4 mRNA to similar levels seen in IAV-infected cells. A likely possibility is that IAV employs multiple mechanisms to dysregulate gene expression of the host cell. As mentioned previously, others found that IAV upregulates the expression of certain miRNAs such as miR-22 which is known to target HDAC4 [427]. It would be interesting to see if blocking this miRNA (and others known to target HDAC4) using an antisense miRNA would be sufficient to partially restore the HDAC4 mRNA expression in IAV-infected cells. Additionally, we did completely investigate the role of NS1 (one of the most characterised IAV virulence factors) in the role of HDAC4 antagonism. The siRNA we used only partially inhibited NS1 expression. Remaining NS1 could have been sufficient to downregulate HDAC4 mRNA, especially in the context of the short halflife of HDAC4 mRNA which is less than 4 hours [429]. Furthermore, we did not examine the effect of plasmid expressed NS1 on HDAC4 expression.
CHAPTER V:

CONCLUSIONS AND

FUTURE PERSPECTIVES
5.1 Conclusions

Like all viruses, IAV must utilise much of the host cell machinery in order to complete its replication cycle. Understanding how this interaction between host and virus is an important step to aid in the development of effective antiviral therapies. This is of particular importance in regards to IAV due to the lack of a universal vaccine and the rise in drug resistant strains [152]. The research presented here is the first evidence demonstrating the antiviral role of host HDAC4 in IAV infection. Thus, HDAC4 joins the list of other HDACs that are important in promoting an effective antiviral response to IAV. In addition, HDAC4 itself is severely antagonised during IAV infection, likely as means for the virus to inhibit its antiviral activity.

5.1.1 The antiviral role of HDAC4

Depletion of HDAC4 via RNAi resulted in enhanced IAV growth kinetics (section 2.2.2), while overexpression of HDAC4 inhibited IAV replication (section 2.2.3). The effect of HDAC4 depletion on IAV replication was observed using two different H1N1 strains – the lab adapted PR8 strain (Figure 7) and the clinically relevant strain CA09 (Figure 9). Furthermore, the effect on viral replication was observed at multiple levels including intracellular and extracellular NP polypeptide expression, infectious virus release via plaque assay and intracellular viral gene mRNA expression.

Mechanistically, HDAC4 was found to play a role in regulating the innate immune response during IAV infection. The mRNA expression of IFNα (Figure 13) and the two ISGs; ISG15 and viperin (Figure 15) were decreased in HDAC4-depleted cells. Conversely, the opposite was observed during HDAC4 overexpression, where the mRNA levels of IFNα (Figure 17), ISG15 and viperin (Figure 18) were all increased as compared to cells expressing normal levels HDAC4. Furthermore, the kinetics of STAT1 phosphorylation was delayed in HDAC4-depleted cells (Figure 14) which translated negative effect on the polypeptide expression of certain ISGs, namely IFITM3, viperin and ISG15 (Figure 16). Again, the opposite effect was observed in cells overexpressing HDAC4, whereby STAT1 phosphorylation was increased, as was the polypeptide expression of viperin and ISG15 (Figure 19). STAT1 is a transcription factor involved in promoting the expression of ISGs during infection with an invading pathogen. Upon cytokine stimulation, JAK activates STAT1 by phosphorylating it at serine and tyrosine
residues. Upon being phosphorylated, STAT1 can then translocate to the nucleus where it can induce the expression of many ISGs important in enhancing the immune response and inhibiting viral replication [435]. Interestingly, STAT1 acetylation of the lysine residues K410 and K413 within DNA binding domain inhibit DNA binding and facilitates the dephosphorylation of STAT1 [513]. The acetylation of these lysine residues induces a structural change in phosphorylated STAT1, ultimately exposing the phosphorylated tyrosine residue Y701 to the phosphatase TCP45 [514]. Others have also reported that STAT1 is regulated by a phosphorylation-acetylation switch, whereby STAT1 acetylation acts a memory mark indicating previously activated STAT1 [445]. Upon being acetylated, phosphorylated STAT1 is then able to become dephosphorylated via the activity of TCP45 and is transported to the cytoplasm where it remains in an inactive and latent state. Only by the activity of HDACs can STAT1 be deacetylated, allowing it to become activated once again to stimulate expression of immune genes. In the context of IAV infection, HDAC4 might be able to indirectly activate STAT1 by inducing its deacetylation. This would allow STAT1 to become phosphorylated once again, thus reactivating it where it can translocate back to the nucleus to promote the expression of innate immune genes (Figure 33).

However, another study has reported that STAT1 is not under the control of a phosphorylation-acetylation switch [515]. However, the conditions used in those studies varied from the ones used here, and under certain circumstances the acetylation-phosphorylation switch of STAT1 might not present at all. The conflicting information on the link between acetylation and phosphorylation of STAT1 leads to some uncertainty behind the exact mechanism of how the expression of HDAC4 can affect the phosphorylation of STAT1 during IAV infection. It is of interest to note that the phosphorylation of neither STAT2 nor STAT3 are reported to be affected by acetylation in the same way that STAT1 is [513]. Therefore, if phosphorylation of these STATs is affected by HDAC4 expression in a similar fashion to STAT1, it would suggest a different mechanism is in play. HDAC4 might directly activate the innate immune response. HDAC4 can localise within the nucleus or the cytoplasm, meaning that HDAC4 can coprecipitate with STAT1 and act directly on different ISG promoters. During HSV-1 infection, HDAC4 is needed for the recruitment of STAT2 to various ISG promoters and likely plays a similar role during IAV infection [422].

The effect on STAT1 phosphorylation by HDAC4 was also observed when using IFNα treatment as an innate immune stimulator (Figure 20). Although others have already identified the antiviral role of HDAC4 in other virus infections, this result solidifies the
role of HDAC4 as general antiviral host factor. Therefore, HDAC4 appears to be an important activator of the innate immune response through its deacetylase activity and perhaps through its non-deacetylase activity too.
Figure 33. Proposed model of how HDAC4 promotes STAT1 phosphorylation to mediate the antiviral response. Phosphorylated STAT1 is acetylated in the nucleus by different HAT enzymes. Acetylation induces conformational changes in STAT1, exposing phosphorylated tyrosine residue Y701 to the phosphatase TCP45. TCP45 dephosphorylates STAT1, resulting in its inactivation and translocation into the cytoplasm. HDAC4 is able to deacetylate STAT1, allowing it to become phosphorylated, where it localises to the nucleus where it can begin activation of STAT1-inducible genes including IFNα as well as the ISGs viperin, IFITM3, ISG15, and likely many more.
5.1.2 The IAV-induced antagonism of HDAC4

While HDAC4 was found to play a role in activating the innate response during IAV infection, the results here also demonstrate that IAV negatively targets HDAC4. This is likely as a means to inhibit its role in innate immune activation. Upon IAV infection with different H1N1 strains and using 2 different cell lines, we observed the HDAC4 polypeptide was significantly diminished (Figures 22-24). The HDAC4 polypeptide was found to be cleaved by caspase 3 at approximately 12 hours post-infection where several cleavage fragments appeared (Figures 25-26). The depletion of caspase 3 was sufficient to partially recover the full length HDAC4 polypeptide during IAV infection whereas caspase 6 and 7 knockdown had no effect on IAV-induced HDAC4 cleavage (Figures 27-28). However, despite the recovery of the HDAC4 full-length polypeptide in caspase 3 depleted cells, some of the cleavage fragments still appeared during IAV infection. Thus, other proteases must also be involved in the IAV-induced cleavage of HDAC4, although these are yet to be identified. The significance of the cleavage of HDAC4 during IAV infection remains to be determined, however, based upon what is already known about HDAC4 regulation, some hypotheses can be postulated. Others have previously reported on the cleavage of HDAC4 via caspases and other proteases in various systems, however none of these been reported during IAV infection [486-489]. A commonly reported HDAC4 cleavage site recognised by caspase 3 is at Asp 289 [489]. Cleavage at this site results in two fragments, an N terminal segment of about 34 kDa and C terminal fragment of approximately 97 kDa. The larger C terminal fragment is retained in the cytoplasm, however the N terminal fragment contains a NLS, which allows it to localize to the nucleus. This N terminal fragment has two commonly reported functions, the first is that it represses MEF2 gene transcription and the second is that has a strong proapoptotic effect [488]. In the context of IAV infection, the proapoptotic effect of the N terminal fragment is of obvious significance. While the traditional view of apoptosis is that is an important antiviral response by an infected cell to prevent further spread of a virus, more recent views have shown that some IAV genes promote apoptosis at the late stages of infection, likely as means to avoid host immune inflammatory responses due to uncontrolled cell death [523]. Furthermore, a study revealed that activation of caspase 3 and apoptosis is actually beneficial for IAV replication and promotes viral propagation [505]. Thus, the cleavage of HDAC4 could be a mechanism by which IAV is able to subvert the antiviral properties of HDAC4 and at the same time benefit from the proapoptotic effects of the N terminal fragment (Figure 34). Indeed, the repression of MEF2 by the cleaved N-terminal fragment
may also be important in the context of IAV infection as the MEF2 transcription factors are known to regulate several cellular processes including differentiation, proliferation, morphogenesis, apoptosis [506], as well as immune and metabolic activities [507]. Thus, the repression of MEF2 transcription factors nuclear localized HDAC4 may also create a more favourable environment for IAV replication, however, not much is known about the role MEF2 transcription factors during IAV infection. In addition to the IAV-induced proteolytic cleavage of the HDAC4 polypeptide, the HDAC4 mRNA is also targeted by IAV (Figure 31). It is likely the viral endonuclease PA-X was targets the HDAC4 mRNA for degradation as expression of PA-X alone was found to be sufficient to reduce HDAC4 mRNA levels while RNAi of PA during IAV infection recovered HDAC4 mRNA (Figure 32). The targeting of the HDAC4 mRNA by IAV has at least 2 benefits for viral replication. Firstly, it reduces the overall HDAC4 polypeptide level within the cell which is important for inhibiting STAT1 activation as alluded to earlier. Secondly, it reduces ribosomal load, thus allowing for enhanced translation of viral proteins and host proteins important in viral replication. The severity with which HDAC4 is targeted by IAV, both at the polypeptide level and the mRNA level, further emphasise its importance as an antiviral host factor.

In summary, this project follows in the wake of previous work done on the role of HDACs in IAV infection and was successful in determining that HDAC4 plays an antiviral role during IAV infection. Ever emerging drug-resistant strains of IAV and the lack of a universal vaccine emphasise the need to develop novel anti-IAV strategies. To aid in this endeavour, a deeper understanding of the molecular interplay between IAV and HDACs could aid in the development of such strategies. The findings presented here contribute to our understating of the molecular interplay between IAV and 1 of the HDACs, HDAC4.
Figure 34. Proposed model of IAV antagonism of HDAC4. The HDAC4 polypeptide is cleaved by caspase 3 at the Asp 289 residue. The N terminal fragment then localises to the nucleus via its NLS where it promotes apoptosis. Meanwhile, the C terminal fragment is retained in the cytoplasm and is further degraded by caspases or some other proteases. At the same time, the IAV PA-X protein induces the degradation of the HDAC4 mRNA.
5.2 Future directions

An important next step in this research is to further understand the nature between STAT1 phosphorylation and acetylation. Because of the conflicting body of knowledge in the literature surrounding the STAT1 phosphorylation-acetylation switch [445, 515], further work needs to be done to understand this phenomenon. Specifically, in the context of HDAC4 and IAV infection, mass spectrometry can be performed to determine how the levels of STAT1 acetylation change in response to HDAC4 depletion or overexpression and during IAV infection.

One of the most exciting areas for future research is to elucidate the nature of the IAV-induced cleavage of HDAC4. Using a site-directed mutagenesis approach would reveal exactly where the polypeptide is being cleaved by caspase 3. The most obvious site to mutate first would be the 289 aspartic acid residue as this has been previously reported to be cleaved by caspase 3 in response to various stimuli [429, 483, 488, 489]. Upon obtaining an HDAC4 mutant that is resistant to IAV-induced cleavage, many more experiments could be performed to determine the significance of HDAC4 cleavage. Firstly, the mutant would be useful in understanding how the cleavage of HDAC4 affects its antiviral capabilities. For example, determining if the innate immune signalling pathway is enhanced in cells expressing a cleavage-resistant HDAC4 mutant as compared to cells expressing wild type HDAC4 would be an obvious experiment to perform. This could be complemented by determining the effect on IAV replication in the presence of cleavage resistant HDAC4. In addition, overexpressing specific HDAC4 fragments might also reveal a potential role that these fragments play during IAV infection, providing further insight into the importance of the HDAC4 proteolytic cleavage. Perhaps it is more important to understand whether the IAV-induced cleavage of HDAC4 polypeptide occurs just as means to antagonise its antiviral function, or also plays a significant role in IAV replication. The N-terminal HDAC4 fragment derived as a result of caspase cleavage is quite stable and translocates to the nucleus where it binds to MEF2 transcription factor and represses its activity [481]. In addition, this N terminal fragment also induces the intrinsic apoptotic pathway in mammalian cells under various conditions [488, 489]. Evidently, both repressed host transcription and apoptosis are favourable conditions for IAV to proliferate [505]. It could be interesting to determine the effect that transfection of a plasmid containing only this N terminal fragment of HDAC4 might have in the context of IAV infection. Thus, further investigations into the significance of the HDAC4 cleavage
will reveal if IAV manipulates HDAC4 to antagonise its antiviral function, but also to promote its own replication.

It is well understood that the subcellular distribution of HDAC4 plays a major a factor in how HDAC4 functions [488, 489]. Using confocal microscopy to determine where HDAC4 localises during IAV infection might reveal how and where HDAC4 exerts its antiviral function. Furthermore, manipulating its distribution by mutagenesis of the NLS or NES would create distinct pools of HDAC4 that accumulate in the nucleus or cytoplasm. Determining the effect on IAV replication in cells expressing these distinct pools of HDAC4 might highlight the importance of HDAC4 shuttling in its role as antiviral host factor.

Given the traditional role assigned to HDAC4 as a deacetylase, it is important to determine what role this deacetylase activity has in promoting innate immune signalling. HDAC4 is known deacetylate the lysine residues at position 9, 14, 18 and 23 of histone 3 and 5, 8 12 and 16 of histone 4. The reversible acetylation of these residues mediates decondensation of the nucleosome structure, altering histone and DNA interactions, thus facilitating the binding of transcription factors [430]. Thus, using an HDAC4-specific inhibitor such as tasquinimod, and utilising RNA-seq might reveal how HDAC4 is able to regulate the cell transcriptome, both in infected cells and during infection with IAV. In addition, HDAC4 is known to deacetylate non-histone proteins in the cytoplasm including HIF-1α and MEKK2, both of which are known to be involved in regulating the immune response within cells [221]. Therefore, inhibiting the deacetylase activity of HDAC4 might reveal a role for deacetylation of these proteins during IAV infection.

It would be of particular interest to further elucidate the exact mechanisms by which HDAC4 promotes the innate immune response through STAT1. It is likely that HDAC4 not only promotes the innate immune response through enhancing STAT1 phosphorylation, but that it also binds to ISRE-containing promoters with STAT1 to enhance ISG expression. HDAC4 is known co-precipitate with STAT2 during HSV-1 and vaccinia virus infection to ISG promoters and is actually required for the binding of STAT2 to these promoters [422]. Therefore, a chromatin immunoprecipitation assay would reveal if HDAC4 does bind directly to the promoters of ISGs known to be important in inhibiting IAV replication. Furthermore, it would be interesting to determine if HDAC4 also precipitates with STAT1 and localises to the nucleus together in a complex as it has been shown with STAT2.
The IFN-α treatment experiments indicated that the HDAC4 antiviral role against IAV likely extends to other viral infections too. Indeed, others have already reported on the antiviral role that HDAC4 plays against other viruses. However, it would be interesting to determine if the antiviral mechanism of HDAC4 remains consistent between viruses through activating the innate immune pathway. Furthermore, it would be interesting to determine if other viruses actively target HDAC4 in a similar fashion to IAV in order to inhibit its antiviral function.

It could also be of potential interest to study the effects of the HATs in response to IAV infection. Given the findings that describe the anti-IAV role of HDACs presented here and in other studies, it is probable that HATs aid in IAV infection. Similar experiments to the ones presented here could be performed to determine if HATs promote IAV infection.

Finally, it would be interesting to perform in vivo experiments in HDAC4 knockout mice to see if the effect on IAV growth is comparable to cell culture. However, HDAC4 knockout mice exhibit a lethal phenotype and die within the first week of life due to abnormalities in skeletal physiology. Nevertheless, a conditional gen knockout method could be utilised here. Using a Cre-lox recombination system, the HDAC4 gene could be knocked out once skeletogenesis is complete and the lethal HDAC4 knockout phenotype can be mitigated.
CHAPTER VI:

MATERIALS AND METHODS
6.1 Cells and viruses

Human alveolar epithelial cells, A549, MDCK (Madin Darby Canine Kidney) and HEK293 (human embryonic kidney) cells were used. Influenza virus strains A/Puerto Rico/8/1934(H1N1) (PR8), A/California/07/2009(H1N1) (CA09) and A/Wisconsin/1934(H1N1) (WSN) were used.

6.2 Cell culture

For regular maintenance, cells were grown in complete minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS), 1% penicillin-streptomycin and 1% L-glutamine (Life Technologies) at 37°C and under 5% CO₂ atmosphere. For sub-culturing, the medium was removed, cells were washed twice with phosphate buffered saline (PBS) (Thermo Scientific) and were then incubated with trypsin-EDTA (Thermo Scientific) for 5-10 minutes at 37°C. Fresh medium was added and cells were split in a 1:3, 1:5 or 1:10 ratio.

6.3 Virus propagation

Influenza viruses were propagated in 10-day old embryonated chicken eggs, and titrated on MDCK cell monolayers by plaque assay as follows. Fertilized chicken eggs were incubated at 35°C under humidity. The eggs were examined under light every second day to check the development and viability of the embryo. On the 10th day, eggs were examined a final time to determine the location of the air sac cavity which was indicated using a pencil drawn on the egg shell. The marked area was first sterilized with 70% ethanol and then a small hole was punctured on the egg shell using an 18-gauge needle. 100 μL of virus inoculum containing 1000 PFU was inoculated into the eggs. The punctured area was then sealed off with tape and the eggs were incubated at 35°C for 48 hours. The embryos were then killed by placing the eggs in a cold room for 48 hours. Then, the tape was removed and the egg shell peeled away using sterile forceps, after which the chlorio-allantoic membrane was carefully removed. The virus-containing allantoic fluid was then harvested using a pipette and stored in a sterile 15 mL falcon tube. Care was taken not to disrupt the yolk sac or blood vessels which might contaminate the allantoic fluid. Viral titre of the
harvested samples was determined by performing the microplaque assay on MDCK cells (as described below).

6.4 Infection

Cell monolayers were washed twice with serum-free (SF)-MEM. The volume of virus stock required for the appropriate MOI was calculated and diluted in the appropriate volume of SF-MEM to make the viral infection media. 500 µL of the viral infection media then added to the cell monolayers for infection and incubated at 35°C for 1 hour. After the initial incubation period, the infection inoculum was removed and 1 mL of SF-MEM was added. For MDCK cells, the SF-MEM was supplemented with TPCK-trypsin (1 µg/mL) (Sigma-Aldrich) in order to aid the virus to continue multiple infection cycles. The initial infection inoculum was supplemented with the desired concentration of drugs where appropriate. MG132 (Calbiochem) and NH₄Cl (Sigma-Aldrich) were used for inhibiting proteasomal and lysosomal pathways, respectively.

6.5 UV irradiation of virus

To make the virus replication-deficient, the virus inoculum was irradiated with 1-2 mJ of UV light for 30 minutes prior to infection. UV light treatment results in the inactivation of the genetic material of the virus while maintaining the antigenic potential of the virus.

6.6 Plaque assay

The culture medium from infected cells was harvested and cell debris was removed by low-speed centrifugation. The media was divided into two separate constituents, the first was subjected to protein precipitation by trichloroacetic acid (TCA) (Calbiochem) while the second part was mixed with 0.3% BSA (Sigma-Aldrich) and titrated on MDCK cells. The microplaque assay was performed using confluent monolayers of MDCK cells which were infected with 10-fold serial dilutions of the culture medium. After 1 hour, the viral inoculum was removed and the cells were then overlaid with 2X SF-MEM containing 1µg.mL TPCK-trypsin and 0.8% Avicel (RC-581; FMC Biopolymer). After 24 hours of incubation, the overlay was removed and the cells were fixed with 4% formalin for 30 minutes. Subsequently, the cells were permeabilized with 0.5% TritonX-100 and 20 mM glycine. Cells were then stained with mouse anti-NP antibody (BEI resources, 1:1000) and
left overnight, followed by 3 washes with PBS (Thermo Scientific). Horseradish peroxidase-conjugated anti-mouse IgG antibody (Thermo Scientific, 1:1000) was then added to the cells and incubated for 1 hour at room temperature followed by 3 washes with PBS. Plaques were developed using 150 µL of KPL TrueBlue peroxidase substrate (Sera Care) for 20 minutes. Plaques were counted by eye using a dilution that contained between 25-250 plaques.

6.7 Trichloroacetic acid precipitation of viral proteins

Ice-cold TCA was mixed with culture medium at a final concentration of 20% and incubated on ice for 30 minutes. The supernatant was removed carefully and the resulting pellet was washed with ice-cold acetone followed by centrifugation at 20,000xg and 4°C for 15 minutes. The supernatant was then discarded and the pellet left to air dry overnight. The pellet was then suspended in 50 µL of SDS-PAGE sample buffer per pellet. Proteins were resolved by SDS-PAGE and viral NP and HA was detected by WB.

6.8 RNA interference

Pre-designed siRNA oligonucleotides targeting human HDAC4 gene – CAGCCAAGCUUCUGCAGCA, caspase 3 gene CAGCUUUCAUGAUUAGCAA and the siRNAs targeting individual influenza gene segments mentioned elsewhere [494] and a non-targeting MISSION control (Sigma Aldrich) were used for knockdown experiments. The lyophilized siRNA obtained from the supplier was reconstituted to obtain a master stock of 50µM and was further diluted to obtain working stocks of 1 µM and 10 µM. Finally, 1 nM of HDAC4 siRNA and 10 nM of caspase 3 siRNA was used to knockdown the expression of the respective genes. To achieve the knockdown of the target gene, OptiMEM (Invitrogen) medium was used for preparing the transfection complex. Desired concentrations of siRNA were added to 100 µL of OptiMEM. Simultaneously, 2 µL of RNAiMAX lipofectamine (Invitrogen) was added to 100 µL of OptiMEM. These were incubated at room temperature for 5 minutes, after which, the lipofectamine complex was added to the siRNA mix. This lipofectamine-siRNA mix was incubated at room temperature for 30 minutes in order to form the transfection complex. Meanwhile, during the incubation period, A549 cells were split and cells were counted using a haemocytometer in order to obtain a cell density of 200,000/mL. The A549 cells were
added to 200 µL of the transfection complex with the final volume reaching 1 mL through
the addition of complete MEM. The cells were then incubated at 37°C under 5% CO₂
atmosphere for 24-72 hours before infection or further processing.

6.9 Plasmid propagation and extraction

Human HDAC4 cloned in plasmid pcDNA3.1 (gifted by Eric Verdin, Addgene plasmid
#13821), human HDAC4 cloned in plasmid peGFP (gifted by Eric Verdin, Addgene
plasmid #45636), and influenza A virus PA-X (WSN) plasmid (gifted by Yoshiro
Kawaoka, The University of Tokyo, Japan) were amplified in *Escherichia coli* DH5α strain.
The bacterial swabs (obtained from Addgene) were spread onto LB agar (Calbiochem)
containing 50 µg/mL ampicillin and the plates were incubated overnight at 37°C.
Individual colonies were picked from the LB agar plate and inoculated into 25 mL
LB broth containing 50 µg/mL ampicillin (Sigma-Aldrich). Incubation was performed
overnight at 37°C with constant agitation (200 rpm). Plasmids were extracted using a
Qiagen midiprep plasmid extraction kit as per the manufacturers protocol. The DNA was
resuspended in 0.5 mL of sterile miliQ water and quantified using a Nanodrop
spectrophotometer (Thermo Scientific) and stored at -80°C.

6.10 Plasmid DNA transfection

Cells were grown to 80-90% confluency in a cell culture plate, and transfected with the
plasmids using Lipofectamine 2000 reagent (Invitrogen) following the manufactures
instructions. Briefly, 1 µg of plasmid DNA and 3 µL of Lipofectamine 2000 were diluted
separately in 100 µL of OptiMEM (Invitrogen), and incubated for 5 minutes at room
temperature. Following this, the Lipofectamine 2000 mix and the DNA mix were
combined and incubated together at room temperature for 30 minutes to allow the
formation of the DNA-Lipofectamine complex. This was then added to the cells in a 12
well plate and incubated at 37°C for 24-48 hours before infection or further processing.

6.11 Quantitative real-time PCR

Total RNA was isolated from the uninfected and infected cells using Nucleospin RNA
isolation kit (Macherey-Nagel) by following the manufactures instructions. The RNA was
then quantified using Nanodrop spectrophotometer (Thermo Scientific) and the purity of
the isolated RNA was analysed using the 260/280 ratio obtained (an absorbance in 1.8-2.0 range was considered acceptable). Following the successful isolation of the RNA, cDNA was then synthesised from 500 ng of total RNA using PrimeScript RT reagent kit (Takara) by following the manufactures protocol.

The quantitative real-time PCR was performed using SYBR select master mix (Invitrogen). Predesigned KiCqStart primers (Sigma Aldrich) for HDAC4, viperin, caspase 3, caspase 6, caspase 7 and interferon-stimulated genes were used to amplify the respective genes using quantitative real-time PCR (qPCR) (ViiA 6, Applied Biosystems). Custom-designed primers were also used to amplify 18S rRNA, Beta-actin, IAV NP gene, IAV NS gene, IAV PA gene, IAV PA-X gene and IAV PB1 gene (Table 1). All of these primers had been tested previously by our lab or others to confirm that each primer pair amplifies a single amplicon [224, 494]. Beta-actin and 18S rRNA were used as reference genes for normalising the expression of the genes of interest, whereas, viperin was used as an infection marker. The percentage or fold change in the mRNA levels of target genes was calculated using \( 2^{\Delta\Delta CT} \) method.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC4</td>
<td>5’-AAAAGAGACGAGATTGAGGAG-3’</td>
<td>5’-AGACAGACGAGACAAGAGAC-3’</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>5’-AAAGCCTGGAATGACATC-3’</td>
<td>5’-GCCATCATTTCACCACATTTC-3’</td>
</tr>
<tr>
<td>Caspase 6</td>
<td>5’-TGAAGAATTTCTCTGTCAC-3’</td>
<td>5’-AAGGATATTACCTCACCAGGG-3’</td>
</tr>
<tr>
<td>Caspase 7</td>
<td>5’-CTCAATGGAAGAGAGTTTC-3’</td>
<td>5’-AAGGCTATGGAAGAGAAAATG-3’</td>
</tr>
<tr>
<td>IFITM1</td>
<td>5’-CTACTCGTGAAAGTCTAGG-3’</td>
<td>5’-ATGAGGATGCCCAGAAATC-3’</td>
</tr>
<tr>
<td>IFITM2</td>
<td>5’-ACCTGATTCTTGATTCCTCC-3’</td>
<td>5’-ATTAGAAAGGTTGTTGAG-3’</td>
</tr>
<tr>
<td>IFITM3</td>
<td>5’-TGCCTCTACTGGAGAAGTCT-3’</td>
<td>5’-GCCATGAGATGAGCAGAAAT-3’</td>
</tr>
<tr>
<td>ISG15</td>
<td>5’-TGAAGCCTGGAATGACATC-3’</td>
<td>5’-GCCATGAGATGAGCAGAAAT-3’</td>
</tr>
<tr>
<td>Viperin</td>
<td>5’-CTTTTGCTGGGAAGCTCTTG-3’</td>
<td>5’-CAGCTGCTGCTTTCTCTCCT-3’</td>
</tr>
<tr>
<td>CH25H</td>
<td>5’-AGTCTAGCTACCTCAATAG-3’</td>
<td>5’-TAGAGTCAAGAATACAC-3’</td>
</tr>
<tr>
<td>MAVS</td>
<td>5’-CAAGGCCCCCATATTCACAG-3’</td>
<td>5’-TCTTCTTGCAAGAGCTGG-3’</td>
</tr>
<tr>
<td>TRM22</td>
<td>5’-ATGAAAGAGGAGAGAGTCTG-3’</td>
<td>5’-CTGGATCATCAGAACATAGCC-3’</td>
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<tr>
<td>MX1</td>
<td>5’-CAGGCTTTCTGGAATGACATC-3’</td>
<td>5’-CTTTCAATTTGTGAACCTG-3’</td>
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<tr>
<td>OAS3</td>
<td>5’-AGTGTTCCCTCACCAATGAG-3’</td>
<td>5’-ATGGATCATGATACAGAG-3’</td>
</tr>
<tr>
<td>18SRNA</td>
<td>5’-ATCGGGGATTGAGATGACAG-3’</td>
<td>5’-TCCTAAATACAGACACTG-3’</td>
</tr>
<tr>
<td>Actin</td>
<td>5’-GAGGATGCGCAAGAATCTCG-3’</td>
<td>5’-ATGATCTGGTCTGATCTC-3’</td>
</tr>
<tr>
<td>IAV NP</td>
<td>5’-AGAAATAAGAGAGAGTTTCTGCGGCTAGC-3’</td>
<td>5’-CATACCGGGCGGAACAAAGCGC-3’</td>
</tr>
<tr>
<td>IAV NS</td>
<td>5’-CAGGACATACTGATGAGGAT-3’</td>
<td>5’-GTTTCAGAGACTCAGACCTG-3’</td>
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<tr>
<td>IAV PA</td>
<td>5’-CTCTTATGCTTGAGCAGGCTAGG-3’</td>
<td>5’-CCGGAGAAAGCAAGCAAGACCCAG-3’</td>
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<td>IAV PA-X</td>
<td>5’-GCGACAATGCTCTCCAG-3’</td>
<td>5’-TTCAGGAGCTGCTGCTATG-3’</td>
</tr>
<tr>
<td>IAV PB1</td>
<td>5’-CGGATGATGAAGGATTGATTC-3’</td>
<td>5’-GACGTCTGAGGCTCTCAATGGTGGAAC-3’</td>
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</table>
6.12 Western blotting

At the desired time points, cells were scraped from the wells and pipetted into a 1.5 mL tube and centrifuged at 14,000 RPM for 30 seconds to pellet the cells. The supernatant was either stored or discarded and 10 µL of PBS was added to the cell pellet. The pellet was then resuspended in the PBS by vigorous vortexing. The cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% TritonX-100, and 1X protease-inhibitor cocktail (Roche). Protease-inhibitor cocktail was added just before cell lysis. 20 µg of protein was resolved on 10% or 15% Tris-glycine SDS-PAGE along with SeeBluePlus 2 pre-stained protein standards (Invitrogen) and transferred on to Protran Premium nitrocellulose membrane (GE Healthcare). Subsequently, membranes were probed with desired primary antibodies and relevant secondary antibodies at appropriate dilutions (Table 2). Proteins were then visualised by chemiluminescence and/or fluorescence. Images were acquired and protein bands were quantified on Odyssey Fc imaging system (Li-COR). Images were exported as TIFF files and compiled in Adobe Photoshop CC 2015 and Microsoft PowerPoint 2016. Raw signals for each band were calculated after background removal using the Li-COR software (Image studio 5.2). The signal from the protein of interest was normalised by dividing with PDI or Actin signal. Then, each sample was normalised with their corresponding control samples. The control sample was either kept as 1 for fold change or as 100% for percentage expression and the corresponding treated samples were calculated for fold change or percentage change, respectively.

An important limitation with the western blotting data is that no experiments were performed to check for the linearity of signal response for any of the target proteins or loading controls. In order to minimise error in western blot quantitation, experiments should have been performed for each detected protein to determine the range of sample loading that will produce a linear relationship between the amount of protein on the membrane and the band intensity recorded by the detector.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Manufacture</th>
<th>Cat #/Clone</th>
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<tr>
<td>HDAC4</td>
<td>1:1000</td>
<td>Cell Signalling</td>
<td>D15C3</td>
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<tr>
<td>Actin</td>
<td>1:10,000</td>
<td>Abcam</td>
<td>ab8227</td>
</tr>
<tr>
<td>IAV-NP</td>
<td>1:10,000</td>
<td>BEI</td>
<td>NR-19868</td>
</tr>
<tr>
<td>IAV HA</td>
<td>1:1000</td>
<td>Gift from St Jude</td>
<td>G-57</td>
</tr>
<tr>
<td>PDI</td>
<td>1:10,000</td>
<td>Sigma</td>
<td>P7496</td>
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<tr>
<td>Caspase 3</td>
<td>1:1000</td>
<td>Cell Signalling</td>
<td>8G10</td>
</tr>
<tr>
<td>Viperin</td>
<td>1:1000</td>
<td>Cell Signalling</td>
<td>D52TX</td>
</tr>
<tr>
<td>pSTAT1</td>
<td>1:1000</td>
<td>BD Biosciences</td>
<td>pY701</td>
</tr>
<tr>
<td>total STAT1</td>
<td>1:1000</td>
<td>BD Biosciences</td>
<td>610185</td>
</tr>
<tr>
<td>IFITM3</td>
<td>1:1000</td>
<td>Abcam</td>
<td>ab15592</td>
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<tr>
<td>ISG15</td>
<td>1:1000</td>
<td>Cell Signalling</td>
<td>F-9</td>
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<tr>
<td>Donkey anti-rabbit HRP</td>
<td>1:5000/1:2000</td>
<td>Thermo Scientific</td>
<td>626520</td>
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<tr>
<td>Goat anti-mouse HRP</td>
<td>1:5000</td>
<td>Thermo Scientific</td>
<td>A16023</td>
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</table>
6.13 Immunofluorescent microscopy

Cells were grown in a 24-well plate. After various treatments, the cells were washed carefully with PBS and then fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20 minutes at room temperature. Next, the cells were washed with PBS 3 times and then 500 µL of 0.2% Triton-X100 was added to permeabilize the cells for 10 minutes. This was then followed by 3 washes of PBS and then the cells were incubated with mouse-NP primary antibody (1:500) in 10% FBS for 1 hour at room temperature. Cells were then washed with PBS 3 times and then incubated with donkey anti-mouse Alexa flour 594 dye (1:500) for 1 hour at room temperature. The cells were washed again with PBS 3 times and then stained with Hoechst dye (Invitrogen) to identify the nucleus (1:5000) for 20 minutes at room temperature. Finally, the cells were washed 3 more times with PBS and the cells were examined under the fluorescent microscope (Olympus) at 10X magnification.

6.14 Statistical analyses

All statistical analyses were performed using Prism 7 (GraphPad). The p-values were calculated using unpaired t-tests.
Appendix I:
Recipes
Table 6: Composition of cell lysis buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCL (pH 7.4)</td>
<td>50 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>150 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>0.50%</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>0.50%</td>
</tr>
<tr>
<td>Triton-X</td>
<td>1%</td>
</tr>
<tr>
<td>Protease-inhibitor cocktail</td>
<td>1X</td>
</tr>
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Table 7: Composition of SDS-sample buffer

<table>
<thead>
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<th>Reagent</th>
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<tr>
<td>Tris-HCL (pH 6.8)</td>
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<td>SDS</td>
<td>2%</td>
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<tr>
<td>Glycerol</td>
<td>30%</td>
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<tr>
<td>Bomophenol blue</td>
<td>0.04%</td>
</tr>
<tr>
<td>1-mercaptoethanol</td>
<td>5%</td>
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Table 8: Composition of resolving and stacking gels

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Resolving Gels</th>
<th>Stacking Gel</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>10%</td>
<td>15%</td>
</tr>
<tr>
<td>MiliQ-water (mL)</td>
<td>3.9</td>
<td>2.9</td>
</tr>
<tr>
<td>Lower Tris (pH 8.8) (mL)</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Upper Tris (pH 6.8) (mL)</td>
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<tr>
<td>10% SDS (µL)</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>40% Acrylamide (mL)</td>
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<td>4</td>
</tr>
<tr>
<td>10% APS (µL)</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>TEMED (µL)</td>
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<td>Total (mL)</td>
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### Table 9: Composition of running buffer (1X)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
<th>Manufacturer</th>
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</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>192 mM</td>
<td>14.4</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Tris base</td>
<td>25 mM</td>
<td>3.02g</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1%</td>
<td>1g</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>MiliQ-water</td>
<td>Final volume up to 1L</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 10: Composition of transfer buffer (1X)

<table>
<thead>
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<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>192 mM</td>
<td>14.4</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Tris base</td>
<td>25 mM</td>
<td>3.02g</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Methanol</td>
<td>20% (vol/vol)</td>
<td>200 mL</td>
<td>LabServ</td>
</tr>
<tr>
<td>MiliQ-water</td>
<td>Final volume up to 1L</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 11: List of blocking buffers

<table>
<thead>
<tr>
<th>Blocking Reagent</th>
<th>Buffer</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% non-fat milk</td>
<td>PBS</td>
<td>All proteins</td>
</tr>
<tr>
<td>1% BSA</td>
<td>TBS</td>
<td>Phosphorylated proteins</td>
</tr>
<tr>
<td>0.1% Casein</td>
<td>TBS</td>
<td>Phosphorylated proteins</td>
</tr>
</tbody>
</table>
Appendix II:
Publications and Presentations
Publications


4. Rajesh Lamichhane, **Henry D Galvin**, Rachel F Hannaway, Sara M de la Harpe, Fran Munro, Joel A Tyndall, Andrea Vernall, John McCall, Matloob Husain and James E Ussher. *Type I interferons are important co-stimulatory signals during T cell receptor mediated MAIT cell activation*. European Journal of Immunology. (2019).

Influenza A virus-induced host caspase and viral PA-X antagonize the antiviral host factor, histone deacetylase 4

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Influenza A virus (IAV) effectively manipulates host machinery to replicate. There is a growing evidence that an optimal acetylation environment in the host cell is favorable to IAV proliferation and vice versa. The histone deacetylases (HDACs), a family of 18 host enzymes classified into four classes, are central to negatively regulating the acetylation level, hence the HDACs would not be favorable to IAV. Indeed, by using the RNAi and overexpression strategies, we found that human HDAC4, a class II member, possesses anti-IAV properties and is a component of host innate antiviral response. We discovered that IAV multiplication was augmented in HDAC4-depleted cells and abated in HDAC4-supplemented cells. Likewise, the expression of IFTM3, BST2, and viperin, some of the critical markers of host anti-IAV response was abated in HDAC4-depleted cells and augmented in HDAC4-supplemented cells. In turn, IAV strongly antagonizes the HDAC4 by down-regulating its expression both at the mRNA level via viral RNA endonuclease PA-X and at the polypeptide level by inducing its cleavage via host caspase-3 in infected cells. Such HDAC4 poly-peptide cleavage resulted in a ~30 kDa fragment that is also observed in some heterologous systems and may have a significant role in IAV replication.

Influenza virus is an ever-evolving human pathogen, and continues to be a concern for global public as well as animal health. Influenza virus impacts the global human population in several ways, namely by causing: 1) regular seasonal epidemics, which alternate in Northern and Southern hemispheres; 2) intermittent unpredictable pandemics; and 3) zoonotic outbreaks, which have become more frequent lately. All of these events result in significant morbidity and mortality as well as productivity and economic losses worldwide (1–5). The zoonotic outbreaks of newly-emerged avian influenza A virus (IAV) subtypes in humans result in unusually high mortality rates (4–5). Furthermore, they pose the threat of the emergence of a highly virulent pandemic IAV. These events are aided by the segmented nature of influenza virus RNA genome and constant circulation of influenza viruses in various clinical and reservoir hosts globally (6). Consequently, the individual infections and co-infections of each host with influenza virus gives rise to genetically diverse influenza progeny populations due to de novo mutations and genetic reassortments. Such rapid evolution of influenza viruses has precluded the development of a universal influenza virus vaccine and makes the annually formulated influenza vaccines only variably effective (7). This phenomenon also aided the rapid emergence of drug resistance. Consequently, half of the available anti-influenza virus drugs, i.e. adamantanes, have become practically obsolete and the other half, i.e. neuraminidase inhibitors prone to be ineffective over time (8).

All these influenza virus characteristics combined also mean that it will be practically impossible to eradicate, particularly the type A influenza viruses from nature. Therefore, there is an undeniable need to identify the missing links, both host and viral, that are critical for influenza virus multiplication and pathogenesis to aid the development of alternative, effective and long-lasting anti-influenza virus strategies. One such strategy is to identify and target the naturally occurring antiviral defenses already present in the host cell. These defenses play a pivotal role as part of the host antiviral response by targeting nearly every stage of virus life cycle. Influenza virus is potentially less likely to evolve rapidly against the host-directed therapies.

We have identified and characterized a role of multiple human histone deacetylases (HDACs) in IAV infection (9–12). The HDACs are a family of enzymes that were originally described to catalyze the deacetylation of acetylated histones (13). Now, a variety of nonhistone proteins, both cytoplasmic and nuclear are known to be the HDAC substrates (13). HDACs work in equilibrium with histone acetyltransferases to control the level of protein acetylation, a post-translational modification, and influence diverse biological processes like gene expression (14), protein trafficking (15), and the innate immune response (16). Consequently, the imbalance in protein acetylation due to aberrant function of HDACs or histone acetyltransferases contributes to multiple human diseases, such as cancer (17), neurodegeneration (18), and infection (19, 20). Since our first observation in 2009 (21), an important role of host acetylation machinery in IAV infection is also emerging (22–25).
Oral presentations

1. **Henry D Galvin** and Matloob Husain. *Influenza A virus-employs multiple mechanisms to antagonise the antiviral host factor histone deacetylase 4*. 38th annual meeting American Society for Virology, Minneapolis, Minnesota, USA, July 2019.

Poster presentations


Appendix III:
Abbreviations
NEP  Nuclear export protein
ng  Nano gram
NF-KB  Nuclear factor-kappa B
NLS  Nuclear localization signal
NP  Nucleoprotein
NS  Non-structural protein
PA  Polymerase acid
PB1  Polymerase basic 1
PB2  Polymerase basic 2
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PFA  Paraformaldehyde
PFU  Plaque forming unit
PI3K  Phosphatidylinositol 3-kinase
Pol  Polymerase
PRR  Pathogen recognition receptor
qPCR  Quantitative real-time PCR
RNA  Ribonucleic acid
RNP  Ribonucleic protein
Rpm  Revolutions per minute
SA  Sialic acid
siRNA  Small interfering RNA
STAT  Signal transducers and activators of transcription
UV  Ultraviolet
vRNA  Viral RNA
WB  Western blot
WHO  World Health Organisation
References


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