Identification of the host histone deacetylase 1 and 2 as novel anti-influenza virus factors

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Dedicated to my beloved master
Bhagawan Sri Sathya Sai Baba,
and my family.
Abstract

Influenza virus continues to pose serious medical and economic challenges to global public health as novel influenza viruses regularly emerge in human population. A universal influenza virus vaccine is not available yet and currently available influenza type- and subtype-specific vaccines need regular updating. Therefore, antiviral drugs are the first line of defence against a novel influenza virus. However, currently-approved anti-influenza drugs target viral components and influenza has mutated those components to acquire the drug resistance. Therefore, there is a need to develop alternative, effective, and long-lasting antiviral strategies to overcome the continuously emerging novel and drug-resistant influenza viruses in humans. One approach is to selectively target specific interactions of influenza virus with host pro-viral and anti-viral factors to inhibit virus replication. This strategy will less likely induce the viral resistance.

This PhD project was designed to determine the role of host histone deacetylase (HDAC) 1 and 2 in the infection of influenza A virus (IAV), the most significant influenza virus. The rationale of this project was based upon the recent discovery in our lab indicating that host histone deacetylases (HDACs) are potentially a family of novel anti-IAV factors. HDACs catalyse the deacetylation of a variety of cytoplasmic and nuclear proteins, consequently regulating diverse cellular processes. Mammalian HDACs have been divided into four classes. The HDAC1 and 2 belong to class I, and are the first and second discovered HDACs, respectively. Earlier, our lab demonstrated that HDAC6, a class II HDAC, possesses an anti-IAV property and IAV downregulates HDAC6 activity to potentially undermine its antiviral function. Based on these findings, we proposed the hypothesis that class I HDACs also have similar properties. HDAC1 is a prototypic member of class I and HDAC2 has about 86% amino acid sequence similarity with HDAC1. Hence, we investigated the role of HDAC1 and 2 in IAV infection using primarily the human lung epithelial cells and IAV PR/8/34(H1N1) and WSN/1933(H1N1) strains as a model.

We have found that IAV downregulates the HDAC1 expression (both at mRNA and polypeptide level) as well as its deacetylase activity, which, consistent with our hypothesis indicated an anti-IAV role of host HDAC1. Indeed, silencing of HDAC1 expression by RNA interference augmented the IAV infection by more than 6-fold,
and conversely, the ectopic expression of HDAC1 from a plasmid decreased it by more than half. To efficiently replicate, IAV has evolved multiple strategies to circumvent the host innate antiviral response. The dysregulation of host HDACs could be part of that strategy as HDACs have been shown to be an important component of host innate response in a heterologous system. Consistent with this hypothesis, treatment of infected cells with trichostatin A (TSA), a widely-used HDAC inhibitor resulted in the downregulation of the phosphorylation of STAT1, a critical component of host innate antiviral response and expression of interferon-stimulated genes, IFITM3, ISG15, and viperin, which have been previously reported to have the anti-IAV function. Consequently, TSA treatment also resulted in the enhancement in IAV infection by more than 5-fold. Further, knockdown of HDAC1 expression resulted in decreased level of phosphorylated interferon regulatory factor 3, a key molecule in interferon signalling and subsequently the reduced expression of interferon α. Furthermore, the expression of viperin was also reduced or enhanced by about 58% or 55% in HDAC1-depleted or HDAC1-overexpressing cells, respectively.

Similarly, HDAC2 mRNA and polypeptide expression was also downregulated in IAV infected cells, albeit by a mechanism distinct to HDAC1 downregulation. Nevertheless, the knockdown of HDAC2 expression resulted in about 4 fold increase in IAV infection. In addition, there was a modest, but consistent decrease in the level of phosphorylated STAT1 in HDAC2-depleted cells and consequently, a decrease in viperin expression.

In summary, this PhD study has demonstrated an anti-IAV role of host HDAC1 and 2 and provided a significant insight into their antiviral mechanism. Evolutionary, HDAC1 and 2 are similar proteins and both were found to have the anti-IAV properties. However, HDAC1 and 2 seem to have a slightly distinct and independent interaction with IAV. Based upon the experimental evidence presented here, further mechanistic roles of these HDACs in IAV infection has been discussed and relevant future research directions have been outlined. In conclusion, both HDAC1 and HDAC2 provide a cellular refractory state to IAV infection by regulating the host innate immune response. The data presented here will contribute to further molecular understanding of the IAV-HDACs interactions.
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CHAPTER 1: INTRODUCTION
1.1 The burden of influenza virus on global public health

Influenza A virus (IAV) is a common cause of respiratory illness in humans. Influenza virus infects the upper respiratory tract and causes an acute febrile respiratory disease, commonly referred to as ‘flu’ (Taubenberger and Morens 2008). Aerosol droplets and respiratory secretions are the primary modes of influenza virus transmission in humans (Killingley and Nguyen-Van-Tam 2013). Influenza virus usually causes a mild and self-limiting infection, which can be severe in young children (6-60 months), elderly, and people living with chronic health conditions like asthma, chronic obstructive pulmonary disease (COPD), diabetes and, cardiovascular diseases (Mallia and Johnston 2007). Infected individuals start shedding the virus before presenting with clinical symptoms (Killingley and Nguyen-Van-Tam 2013). About 3,000 infectious influenza virus particles are required for establishing a successful infection (Brooke 2014). The incubation period for influenza virus infection is about two days, but can last up to four days (Lessler, Reich et al. 2009) and is characterized by high fever (38-40°C), cough, muscle pain, and headache. According to the World Health Organization (WHO) estimates, influenza virus causes approximately one billion cases of flu, 3 – 5 million cases of severe illness, and 300,000 – 500,000 deaths worldwide annually (Lessler, Reich et al. 2009, Palache, Oriol-Mathieu et al. 2015). These numbers increase significantly in the event of an influenza virus pandemic. In New Zealand, annually, an average of 401 deaths was documented as a result of influenza infection (Kessaram, Stanley et al. 2015). The socio-economic impact of influenza has often been underestimated as it results in substantial financial burdens due to increased hospitalization, doctor visits, work and school absenteeism and loss of trade (Principi, Esposito et al. 2003). In some cases, the disease caused by influenza is exacerbated by influenza-related
complications which include pneumonia, secondary bacterial infections, chronic bronchitis, myocarditis, and chronic pulmonary diseases. HIV patients are immunocompromised and are therefore at a higher risk of developing long lasting influenza illness (Kunisaki and Janoff 2009).

So far, influenza virus has been reported to have caused four pandemics: 1918, 1957, 1968, and the latest one in 2009. ‘Spanish flu’ was the first and worst ever recorded pandemic which lasted from 1918-1919 during the First World War. It was first observed in the United States of America and then gradually spread across Europe, North America, Asia and remote Pacific islands (Mamun and Huda 2011). Over 500 million people were infected over this period, resulting in about 40 million deaths (Mamun and Huda 2011). During 1957-58, the second pandemic outbreak was recorded in China and was referred to as ‘Asian flu’ (influenza A/Japan/H2N2), causing an estimated 1.5-2 million deaths worldwide (Martin, Kunin et al. 1959). The third pandemic, which lasted from 1968-1969, referred to as ‘Hong Kong flu’ (influenza A/Hong Kong/H3N2), was first isolated in Hong Kong in July 1968. It spread across America and Europe and was considered, a relatively mild as compared to the 1918 pandemic, resulting in an estimated one million to four million deaths worldwide. A secondary outbreak of Hong Kong flu pandemic was reported between 1970-1972 (Viboud, Grais et al. 2005). The most recent influenza pandemic outbreak referred to as ‘Swine flu’ (H1N1), occurred between 2009-2010, and originated in Mexico, which resulted in about 2.8 million deaths worldwide (Mamun and Huda 2011, Dawood, Iuliano et al. 2012).

In addition to seasonal epidemics and pandemics, which are caused by human-adapted viruses, animal (mostly avian) influenza viruses also cause zoonotic outbreaks resulting in high morbidity. The first reported zoonotic outbreak caused by
a pure avian-origin influenza virus occurred in Hong Kong in 1997 when avian influenza H5N1 virus was found to infect 18, of which 6 died. So far, more than 700 cases of H5N1 infection have been reported (Huai, Xiang et al. 2008). Additionally, several zoonotic avian influenza strains (e.g. H6N1, H7N3, H7N7, H7N9 and, H10N8) have been reported to infect humans and cause severe respiratory disease (Maurer-Stroh, Lee et al. 2013, Zhang, Hale et al. 2013, Scotch, Suchard et al. 2015, Sun, Belser et al. 2016, Xiao, Ma et al. 2016, Yang, Chen et al. 2016).

Influenza virus belongs to the family Orthomyxoviridae and exists in three types: A, B, and C (Matsuoka, Matsumae et al. 2013). Influenza virus type A is the most significant one and infects multiple hosts ranging from humans, birds, pigs, dogs, cats, horses, and seals. Recently, influenza A virus has also been isolated from bats (Dlugolenski, Jones et al. 2013). Aquatic birds including shorebirds and, waterfowls are the main reservoir of influenza A virus (Parrish, Murcia et al. 2015). On the other hand, type B and C influenza viruses only infect humans (Kanetsu, Hidekazu et al. 1986, Adachi, Kitame et al. 1989, Matsuoka, Matsumae et al. 2013, Subbarao and Matsuoka 2013). Recently, a close variant of type C influenza virus has been discovered to infect bovines (Hause, Collin et al. 2014). Although this virus has about 50% nucleotide sequence similarity to type C, it has been classified as type D influenza virus (Hause, Collin et al. 2014). The inherent ability of IAV to infect different hosts results in broad host tropism, which makes influenza a global burden causing seasonal epidemics and occasional pandemics (Zhang 2009).

1.2 Influenza A virus genome

Influenza A virus (IAV) particle size varies from 80-120nm. IAV is pleomorphic, but it predominantly exists in a spherical form (Mubareka and Palese 2011, Zheng and
IAV is an enveloped virus with a segmented, negative-sense RNA genome (see Figure 1). The genome of IAV is comprised of eight segments that encode up to 17 proteins (Shtyrya, Mochalova et al. 2009). At least 10 proteins are well characterized viz., hemagglutinin (HA), neuraminidase (NA), polymerase basic 1 (PB1), polymerase basic 2 (PB2), polymerase acidic (PA), nucleoprotein (NP), matrix protein (M1), ion channel protein (M2), and non-structural protein (NS1 and NS2/NEP). Additionally, IAV possess a variant of PB1 known as PB1-F2 which plays a crucial role as a pro-apoptotic protein (Chen, Calvo et al. 2001). Recently, additional IAV proteins namely, PA-X (frameshift product of PA), M42, PA-N155 (N-terminal truncation of PA), PA-N182 (N-terminal truncation of PA) and NS3 (point mutation in NS1) have been discovered (Vasin, Temkina et al. 2014). The function of recently-described proteins is not fully understood, however, PA-X has been characterised to be a major virulence factor of IAV (Gao, Sun et al. 2015).

IAV envelope is composed of a lipid bilayer, which is derived from the host cell. The envelope contains two viral surface proteins, HA and NA, which are major viral antigens (see Figure 1). HA binds to the host cell receptors and enables virus entry, whereas NA enables the release of newly formed virions from the host cell (Shi, Wu et al. 2014). IAV is further subtyped based upon the antigenic nature of HA and NA. So far, 18 HA and 11 NA subtypes have been identified (Memorandum 1980, Tong, Li et al. 2012, Tong, Zhu et al. 2013).
Figure 1. Schematic diagram of Influenza A virus and its surface protein hemagglutinin. (A) Influenza A virus is an enveloped virus with eight negative-stranded RNA genome segments. The envelope contains three membrane proteins (HA, NA, and M2), M1 proteins are located underneath the envelope, and the viral ribonucleoprotein (vRNP) complex is comprised of genomic segments, NP, and polymerase complex (PA, PB1 and PB2). (B) Major surface antigen, HA is a cylindrical heterotrimeric protein with a variable globular head and conserved stem domain. (Lofano, Kumar et al. 2015).
1.3 Influenza A virus life cycle

IAV life cycle is a complex process and utilises the whole host cell (Figure 2)

1.3.1 Entry

IAV enters the host cell via apical surface, wherein, viral HA binds to the sialic acid receptors on the host cell in a process known as ‘virus adsorption’ (Bouvier and Palese 2008). Human IAV preferentially binds to α-2,6 sialic acid, whereas avian IAV preferentially binds to α-2,3 sialic acid (Matrosovich, Tuzikov et al. 2000). The majority of cells in upper human respiratory tract express α-2,6 sialic acid, whereas ciliated epithelial cells in the lower respiratory tract express α-2,3 sialic acid. Though sialic acid receptors were thought to play a crucial role in virus adsorption (Gamblin and Skehel 2010), a study by Stray et al., revealed that IAV can successfully infect cells which are devoid of sialic acid receptors (Stray, Cummings et al. 2000), suggesting multiple strategies adapted by IAV to enter a host cell.

1.3.2 Internalization

After receptor binding, IAV is internalized into the cell via receptor-mediated endocytosis. Clathrin-mediated endocytosis has been the most studied pathway and is also considered an important pathway for IAV entry (Patterson, Oxford et al. 1979, Matlin, Reggio et al. 1981). However, recent studies show IAV also enters through non-clathrin mediated pathways such as macropinocytosis, which is the major pathway for uptake of extracellular fluids by the cells (De Vries, Tscherne et al. 2011). After endocytosis, Rab5 and Rab7 GTPase-mediated trafficking transports the virus-containing endosomes to the cytoplasm (Sieczkarski and Whittaker 2003, Costello, Whittaker et al. 2015).
**Figure 2.** The life cycle of influenza A virus. The life cycle of influenza A virus begins by binding of the viral surface protein HA to sialic acid receptors on the host cells. The virus is then internalized into the endosome. The low pH of the endosome results in fusion of the viral and endosomal membranes and the viral ribonucleoprotein (vRNP) is released into the cytoplasm. This vRNP is transported into the nucleus for replication and transcription of viral RNA. The negative sense strand is first converted into positive sense strand or complementary RNA (cRNA) which is used as a template for viral RNA synthesis. The viral RNA is exported from the nucleus and is assembled along with the viral proteins into the newly budding virion near the plasma membrane. Finally, NA cleaves the sialic acid receptors and releases the newly formed virions on the host cell surface. (Adapted from Squires, R Burke. (2009) Influenza Life Cycle).
In addition, lysosomal-associated membrane proteins (LAMPs) and Cullin-3 (Cul3), a member of Cullin-RING-ligase family (Hubner and Peter 2012), play an essential role in endosomal maturation and trafficking (Zhou, Xue et al. 2011, Hubner and Peter 2012, Huotari, Meyer-Schaller et al. 2012).

1.3.3 Envelope Fusion

Once in endosomes, host proteases then cleave the viral HA (also known as HA0) into HA1 and HA2 (Skehel, Cross et al. 2001). The low pH of endosomes then triggers an reversible conformational change in the HA, exposing the HA2 fusion peptide, which inserts into the endosomal membrane, resulting in the fusion of viral and endosomal membranes (Skehel, Cross et al. 2001). Following membrane fusion, the viral M2 ion channel allows an influx of protons into the virion from the endosome which acidifies the virus particle, dissociating M1 from vRNP complex. Host vacuolar-ATPase (vATPase) contributes to both acidification and fusion of viral and endosomal membranes, resulting in the release of the vRNP complex into the cytoplasm (Marjuki, Gornitzky et al. 2011).

1.3.4 Nuclear import

Once in the cytoplasm, the vRNP complex is imported into the nucleus through the nuclear pore complex guided by importin α, (Martin and Helenius 1991, Deng, Engelhardt et al. 2006, Satterly, Tsai et al. 2007, Tafforeau, Chantier et al. 2011). The nuclear pore complex proteins (Nup98 and Nup153) and importin molecules were shown to be involved in the nuclear import of vRNP complex (Martin and Helenius 1991, Satterly, Tsai et al. 2007, Resa-Infante, Thieme et al. 2014).
1.3.5 Viral replication and transcription

The viral replication and transcription takes place in the nucleus and is catalysed by the ternary viral polymerase complex PB1, PB2, and PA (Engelhardt and Fodor 2006). Viral RNA (vRNA) replication begins with the synthesis of positive-strand RNA, referred to as ‘complementary RNA’ (cRNA) from the negative-stranded genomic viral RNA (Resa-Infante, Jorba et al. 2008). Subsequently, multiple copies of vRNA are made from this cRNA (Resa-Infante, Jorba et al. 2008). The vRNA is then transcribed into viral mRNAs (Engelhardt and Fodor 2006). A key step in transcription of IAV mRNAs is the pre-mRNA cap snatching of host mRNA (Dias, Bouvier et al. 2009). The PB2 protein binds to the 5’-cap region of host pre-mRNAs, which is then cleaved by PA. Finally, PB1 catalyses the addition of a poly(A) tail to (Dias, Bouvier et al. 2009) form mature mRNAs. Further, M and NS mRNAs are spliced to yield M1, M2, and NS1, NS2 mRNAs, respectively. Tat-SF1 (Naito, Kiyasu et al. 2007) and BAT1 (or UPA56), are among the host mRNA splicing factors which facilitate the splicing (Momose, Basler et al. 2001). Additionally, host heat shock protein 90 (Hsp90), interacts with PB2 and stimulates the vRNA replication (Momose, Basler et al. 2001, Momose, Naito et al. 2002).

1.3.6 Translation

The viral mRNAs are translated by host cell machinery, thus it is not surprising that several host translational factors such as eukaryotic translational initiation factor 4A (eIF) 4A, eIF4B, eIF4E, eIF4G interact with viral polymerase complex (Burgui, Aragon et al. 2003, Yángüez, Rodriguez et al. 2012). Upon virus infection, host protein synthesis is limited, and viral mRNAs are favourably translated over host mRNAs. In particular, virus-mediated cap snatching of host mRNAs results in their
rapid degradation (Katze, Chen et al. 1984, Katze, DeCorato et al. 1986). Additionally, NS1 interacts with host poly(A)-binding protein II and CPSF (cleavage and polyadenylation specificity factor) protein, and PB1, PB2, and PA interact with host DNA-dependent RNA polymerase II, which potentially contributes to the shutdown of host mRNA synthesis (Katze, Detjen et al. 1986, Mukaigawa and Nayak 1991). Following viral protein synthesis in the cytoplasm, viral membrane proteins, HA, NA, and M2 follow the host secretory pathway and are transported to the plasma membrane. Whereas, the viral polymerase subunits (PA, PB1, PB2) and NP are imported into the nucleus to catalyse the replication and transcription of vRNA (Greenspan, Palese et al. 1988, Weber, Kochs et al. 1998). In addition, NS1, NS2, and M1 proteins are also imported into the nucleus. NS2, also known as Nuclear Export Protein (NEP) and M1 guide the nuclear export of assembled vRNP, whereas, NS1 guides the processing and export viral mRNAs (Ye, Robinson et al. 1995, van Wielink, Harmsen et al. 2012).

### 1.3.7 Post-translational processing

After translation, viral proteins undergo post-translational modifications, which facilitate their normal function in IAV life cycle. For instance, HA undergoes glycosylation in the endoplasmic reticulum and Golgi complex that results in the masking of antigenic sites recognized by the neutralizing antibodies (Tate, Job et al. 2014). The phosphorylation of NP regulates the vRNP assembly (Mondal, Potts et al. 2015). Phosphorylation of NS1 and PB1-F2 is essential for antagonizing the host immune response, thereby playing a significant role in the virulence of IAV (Hale, Knebel et al. 2009). N-terminal acetylation of PB1, PA, NP, M1, M2, NS1 and NEP was reported but the functional role of these modifications still remains to be investigated. Further, PB1 and M1 protein of IAV were ubiquitinated by the host
proteins to attenuate IAV replication (Liu, Zhao et al. 2012). Furthermore, the M2 ion channel protein of IAV is subjected to various post-translational modifications, including disulphide bond formation, sumoylation, palmitoylation, fatty acid acylation and phosphorylation (Veit, Klenk et al. 1991, Holsinger, Shaughnessy et al. 1995, Liu, Li et al. 2015).

### 1.3.8 The vRNP assembly and nuclear export

The newly formed vRNA is coated with NP, PA, PB1 and PB2 to form vRNP complex. The newly formed vRNPs are then exported out of the nucleus guided by two major host pathways mediated by nuclear RNA export factor 1 (NXF-1) and chromosome region maintenance-1 (CRM-1) (Ma, Roy et al. 2001, Hutten and Kehlenbach 2007). NXF-1 is employed by IAV for nuclear export of vRNPs (Read and Digard 2010, Watanabe, Watanabe et al. 2010). In addition, Satterley et al., demonstrated that binding of NS1 to NXF-1 results in the nuclear retention of host mRNAs, which are used for cap snatching (Satterly, Tsai et al. 2007).

### 1.3.9 Virus release by budding

After synthesis and processing, all IAV components are transported to the apical surface of plasma membrane domains known as lipid rafts for assembly and release by budding (Zhang, Pekosz et al. 2000, Bruce, Digard et al. 2010). Historically, two models have been proposed for viral segments’ packaging; (a) random packaging and (b) specific packaging models. The random packaging model suggests that the viral segments are incorporated in a random manner ensuring that there are eight segments incorporated into the genome (Brunotte, Flies et al. 2014). Contrastingly, the specific package model suggests that specific packaging signals are used to
incorporate each copy of vRNP appropriately into a budding virion (Goto, Muramoto et al. 2013).

The assembly of vRNP complex is still controversial. One school of thought suggests that M1 protein associated with vRNP in the nucleus and guides the nuclear export of M1-vRNP complex to the apical plasma membrane for budding (Brunotte, Flies et al. 2014). The other school of thought suggests that the NP in vRNP complex contains a nuclear export signal that guides the nuclear export of vRNP complex to the apical plasma membrane for budding (Mayer, Molawi et al. 2007). During the process of budding, M2 and NA play a crucial role in directing virus release from the polarized cells. The M2 protein tends to form a positive curvature pushing the viral particles towards the distal end, and aids in packing them into their envelopes. Finally, when the virions are ready to bud out, the sialidase activity of NA cleaves the HA bound to sialic acid receptors (Air and Laver 1989, Taylor 1996). As the progeny virus buds out, they infect the neighbouring cell, continuing the infection cycle. Interestingly, the recently discovered NA had no detectable sialidase activity, suggesting a possibly unique role for these NA molecules (Tong, Zhu et al. 2013).

1.4 Evolution of IAV and emergence of new IAV strains

IAV has been under constant selection pressure including host environment, antiviral drugs and host immunity, (Smith, Lapedes et al. 2004, Landolt and Olsen 2007) which leads to antigenic shift or drift, resulting in generation of genetically and antigenically novel IAV strains (Figure 3).
Figure 3. The ecological cycle of influenza A virus. The ecological cycle of IAV can be divided into avian, animal and human cycles. Swine express both α-2,3 and α-2,6 sialic acid IAV receptors, which make them an intermediate host that serves as a mixing vessel for generation of novel IAV strains. Adapted from (Shi, Wu et al. 2014).
Antigenic drift is a mechanism of accumulation of mutations by viruses within the antibody-binding sites of a protein. Antigenic drift occurs in the HA and NA of both influenza A and B viruses (Hope-Simpson and Golubev 1987, Zambon 1999). The IAV RNA genome exhibits a high mutation rate, ranging from approximately $10^{-3}$ to $8\times10^{-3}$ nucleotide substitutions per site per year resulting in antigenic drift (Chen and Holmes 2006). The RNA polymerase of IAV lacks exonuclease proof-reading activity, hence it is inherently error prone resulting in a high gene mutation rate (Chen and Holmes 2006). Mutations in the surface proteins, HA and NA, offer an antigenic advantage to the virus as they can evade the pre-existing host immunity. Due to this mutation-selection process, IAV and influenza B virus (IBV) exist as quasi-species resulting in seasonal epidemics (Domingo, Baranowski et al. 1998).

Antigenic shift is contributed by the exchange of genomic segments between two different strains of IAV, co-infecting a host. The resultant virus has novel antigenic properties towards which the humans have no significant immunity, and thus causes pandemics (Greenbaum and Ghedin 2015). Theoretically, 256 ($2^8$) different combinations of the eight IAV segments are possible when two parental viruses co-infect a host (Dugan, Chen et al. 2008). This high mutation rate of IAV enables it to evolve rapidly and thereby overcome host barriers. Therefore, control of IAV infection using novel antiviral strategies would not only result in reduced occurrence of epidemics but would also develop an effective way to combat the threat of unforeseen pandemics.

1.5 The ecological cycle of influenza A virus

IAV exhibits broad host tropism which enables it to infect multiple host types. Based on this, the ecological cycle of IAV can be divided into avian, animal and human
cycles. Aquatic birds including shorebirds and waterfowls are the natural hosts, referred to as the ‘reservoir hosts’, of IAV (Figure 3). Most of the natural avian IAV circulates in birds, providing the genetic diversity for the emergence of epidemic and pandemic IAV with tropism for humans and animals. From these reservoir hosts, IAV is transmitted to domestic birds including chickens and ducks. Subsequently, avian IAV is transmitted to swine which act as intermediate hosts. The upper respiratory tract of humans expresses α-2,6 sialic acid receptors whereas the lower respiratory tract expresses a small population of α-2,3 sialic acid receptors. Human IAV strains preferably bind to α-2,6 sialic acid receptors, whereas, avian strains bind to α-2,3 sialic acid receptors. Intriguingly, swine express both α-2,3 and α-2,6 receptors, and are therefore susceptible to infection by both human and avian IAV, serving as a ‘mixing vessel’ for genetic reassortment of IAV (Ito, Couceiro et al. 1998) (Figure 3). The result of genetic reassortment is the evolution of antigenically novel strains of IAV which result in pandemics.

1.6 Need for new anti-IAV strategies

The host switching ability of IAV has always placed the researchers and medical professionals in a quandary. Currently, vaccination is the first line of defence against IAV. However, there is no universal vaccine against IAV, and the currently available vaccines are strain specific and have to be administered on an annual basis (Ohmit, Petrie et al. 2013). Further, to keep the disease under check, WHO surveys circulating IAV strains globally and administers vaccine formulations accordingly twice a year in both northern and southern hemispheres. The constantly evolving IAV is being monitored globally by WHO through the Global Influenza Surveillance and Response System (GISRS) which partners national influenza centres across the world. WHO recommends a trivalent vaccine composition (two IAV and one IBV),
considering the circulating strains in the season. Since 2013-14, during the northern hemisphere influenza season, WHO has updated the vaccine formulation from trivalent to quadrivalent (two IAV and two IBV strains), providing a wider protection against influenza infection (Kolber, Lau et al. 2014).

Antivirals are the second line of defence against IAV. There are two classes of anti-IAV drugs available (Figure 4): (i) Adamantanes such as amantadine and rimantadine, and (ii) Neuraminidase inhibitors such as Oseltamivir, Zanamivir Paramivir, and lanamamivir. Currently, circulating strains of IAV are rapidly gaining drug resistance which renders the available antivirals almost ineffective. Almost all circulating IAV strains have developed resistance to adamantanes. Therefore, WHO recommends neuraminidase inhibitors as the first-line treatment for IAV infection, especially with novel strains, as developing vaccine against them takes at least six months (Kolber, Lau et al. 2014). In 2008, European Early Warning and Response system (EWRS) reported a high rate of resistance to Oseltamivir (Tamiflu) to the WHO. This resistance was developed by a single amino acid mutation H275Y (histidine substituted by tyrosine at amino acid 275) (Yusuf, Mohamed et al. 2016). In addition, point mutations in PB1 protein are increasing the burden of drug resistance leading to genetic drift (Zaraket, Saito et al. 2010). Moreover, anti-IAV drugs like Tamiflu reduce the risk and complications of the disease, only if they are administered within 48 hours of the onset of symptoms (Yusuf, Mohamed et al. 2016). The lack of a universal vaccine and the emergence of drug-resistant IAV strains emphasize a constant need to develop novel anti-IAV strategies. One such strategy is to target specific virus-host interactions during IAV infection, as the virus is dependent on the host cell machinery for its replication.
1.7 Role of host factors in influenza A virus infection

As the virus infects the cell, it has to evade the host defense to establish successful infection (Josset, Frobert et al. 2008). During this process, the virus hijacks the host cell machinery and manipulates it to favour the synthesis of viral proteins. In response to virus infection, one set of host factors (e.g. receptors) promotes virus replication whereas the other set of host factors (e.g. Interferons, antibodies) restrict virus infection (Coulombe and Divangahi 2014). Understanding the role of these host factors and the mechanisms adapted by virus to compromise the host protein synthesis would potentially reveal novel insights into the molecular interplay between virus and the host. Henceforth, a comprehensive understanding of the role of these host factors would broaden the understanding of host-virus interaction and may uncover potentially novel anti-IAV targets (Duggal and Emerman 2012).
Figure 4. Classification of anti-IAV drugs. There are two broad classes of anti-IAV drugs currently available: (i) Adamantanes, inhibit the M2 ion channel and subsequently the release of vRNP into the cytoplasm, and (ii) Neuraminidase inhibitors which inhibit the release of newly formed virions.
1.7.1 Proviral host factors

Hao et al., made a breakthrough discovery using RNA interference (RNAi) technology and identified all host factors involved in IAV virus infection. About 90% of the host genes targeted by RNAi resulted in either inhibition or stimulation of over 100 host genes in IAV infection. Notably, vacuolar ATPase subunit D (ATP6V0D1), cytochrome c oxidase subunit 6A 1 (COX6A1) and nuclear RNA export factor 1 (NXF1) were found to regulate IAV infection and silencing of these genes decreased the virus replication (Hao, Sakurai et al. 2008). Similarly, König et al., identified 295 host factors involved in IAV virus replication. Of these 295 factors, 219 were found to be essential for virus replication. Further analysis revealed a subset of 23 proteins, crucial for virus entry including vacuolar ATPase family, coat protein I (COPI) family, fibroblast growth receptor and glycogen synthase kinase 3 beta. Furthermore, 10 proteins were found to be essential for post-entry events including nuclear import components, proteases, and calcium-calmodulin dependent kinase II beta (König, Stertz et al. 2010). In a similar study conducted by Shapira et al., using yeast two hybrid system, 135 host proteins were regulated during H1N1 infection, whereas, 81 host proteins were regulated during H3N2 infection, indicating differential host response to IAV subtypes (Shapira, Gat-Viks et al. 2009). During H1N1 infection, 56 human proteins interacted with the virus. Out of the 135 host factors identified, 30 (e.g. (PML, MAPK, NF-kB, Wnt and, p38)) were involved in signaling and about 19 transcription factors directly interacted with H1N1. On the other hand, 28 signaling proteins and 16 transcription factors interacted with H3N2. Additionally, 1,056 genes were regulated by the virus, of which 666 were interferon stimulated genes (Shapira, Gat-Viks et al. 2009). The detection of the viral genome by host TLRs and RIG-I influenced the expression of about 721 genes. Importantly, 68 virus-regulated genes
that were identified are involved in influencing the apoptosis and NF-κB pathway thereby dampening the host response (Shapira, Gat-Viks et al. 2009). Christopher et al., used computational modelling to characterize and predict the potential gene signatures associated with IAV infection. This modelling revealed a crosstalk between NFκB and the interferon regulatory factor (IRF) signalling pathway during infection further confirming the involvement of host factors in IAV infection (Zaslavsky, Nudelman et al. 2013).

Karlas et al., employed genome-wide RNAi screening and identified 287 host factors influencing IAV replication. Further, using both epidemic and pandemic IAV strains, 68% of these identified host factors were found to inhibit IAV infection. Out of 287 host factors identified, 119 inhibited the infection of influenza strain A/WSN/33 and 121 inhibited the infection of influenza strain A/Hamburg/04/2009, whereas 72 factors were common between the two strains, confirming their strong inhibitory potential of IAV infection (Karlas, Machuy et al. 2010).

Su et al., conducted genome-wide RNAi screening using shRNA and discovered 110 host factors involved in IAV infection. Notably, this study discovered the role of Itch, an E3 ubiquitin ligase, in the release and transport of vRNP from the endosome into the nucleus during IAV infection. The knockdown of Itch results in the decreased endosomal trafficking of viral RNA, consequently dropping the virus titres by about 10 fold (Su, Chen et al. 2013). Similarly, silencing of Itch also reduced budding of human T-cell leukemia virus type 1, showing a role for this gene in the infection of other viruses (Dorjbal, Derse et al. 2011). York et al., discovered 171 host factors including chaperones, cytoskeletal proteins, kinases, phosphatases and ubiquitin ligases involved in IAV infection. Among the subset of proteins identified, specifically serine/threonine protein phosphatase 6 (PP6) directly interacted with the PB1 and
PB2 complex and promoted viral RNA synthesis. Further, the knockdown of PP6 resulted in decreased IAV titre \cite{York, Hutchinson et al. 2014}. Yet another study by Tran et al., identified the role of TNFSF13, TNFSF12-TNFSF13 (TWE-PRIL) and USP47 in IAV infection. Silencing of USP47 inhibited IAV entry, whereas TNFSF13/TNFSF12-13 silencing inhibited a late stage of IAV replication \cite{Tran, Rahim et al. 2013}.

From the microarray study performed by Su et al. \cite{Su, Chen et al. 2013}, DR1 was identified as a host factor involved in viral RNA/protein synthesis and inhibiting the host innate immune response \cite{Hsu, Su et al. 2015}. DR1 promoted IAV replication by directly associating with viral RNA-dependent RNA polymerase, besides inhibiting the interferon β synthesis \cite{Hsu, Su et al. 2015}. Additionally, from the microarray data generated by Su et al. \cite{Su, Chen et al. 2013}, two more host factors viz., prolidase \cite{Pohl, Edinger et al. 2014} and cathepsin \cite{Edinger, Pohl et al. 2015} were discovered involved during initial stages of IAV replication. The dipeptidase activity of prolidase and the proteolytic activity of cathepsin W were reported to be crucial for infection, indicating their pro-viral function. Intriguingly, the PI3K pathway has been reported to play a bivalent role during IAV infection \cite{Hrincius, Dierkes et al. 2011, Yeon, Song et al. 2015}. During the early stages of infection, this pathway is exploited by the virus to gain entry into cells \cite{Hrincius, Dierkes et al. 2011}. However, during later stages of infection, PI3K promotes the expression of interferons and interferon stimulated genes to combat the virus \cite{Hrincius, Dierkes et al. 2011}. Interestingly, silencing of the squamous antigen recognized by T-cell 1 (SART1) decreased viral HA, NP and M2 expression, having an overall impact on viral protein synthesis. Similarly, vesicular transport complex and COPI are crucial for secretion of host cell receptors and trafficking of viral HA to the plasma membrane via ER-
Golgi transport (Cai, Reinisch et al. 2007, Morriswood, Ryzhakov et al. 2007). Most recently, ANP32, a nuclear protein was found to be essential for viral RNA polymerase activity (Long, Giotis et al. 2016), mediating suboptimal function of avian polymerases in human cells as siRNA-mediated depletion of ANP32 resulted in a profound (about 70%) decrease in IAV infection.

In summary, significant progress has been made on the discovery of proviral host factors (summarized in Table 1) promoting IAV replication and rendering the host predisposed for infection.
<table>
<thead>
<tr>
<th>Host factor</th>
<th>Stage of IAV infection</th>
<th>Proposed mechanistic role</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR1</td>
<td>RNA/protein synthesis</td>
<td>Inhibits interferon β synthesis and ISG expression</td>
<td>(Hsu, Su et al. 2015)</td>
</tr>
<tr>
<td>Prolidase</td>
<td>Entry</td>
<td>Early endosomal routing during entry</td>
<td>(Pohl, Edinger et al. 2014)</td>
</tr>
<tr>
<td>Cathepsin W</td>
<td>Entry</td>
<td>Release of viral particles from late endosome</td>
<td>(Edinger, Pohl et al. 2015)</td>
</tr>
<tr>
<td>E3 ubiquitin ligase, Itch</td>
<td>Endosomal release of viral particles</td>
<td>Release of viral particles from endosomes for nuclear import</td>
<td>(Dorjbal, Derse et al. 2011)</td>
</tr>
<tr>
<td>HDAC8</td>
<td>Endosomal trafficking</td>
<td>Loss of centrosome-associated microtubules and lysosomal trafficking</td>
<td>(Yamauchi, Boukari et al. 2011)</td>
</tr>
<tr>
<td>Nuclear factor kB</td>
<td>Entry/RNA replication</td>
<td>Cytokine burst, cellular damage</td>
<td>Nimmerjahn, Dudziak et al. 2004</td>
</tr>
<tr>
<td>Annexin V</td>
<td>Budding</td>
<td>Antagonize immune response</td>
<td>(Berri, Haffar et al. 2014)</td>
</tr>
<tr>
<td>Epidermal growth factor receptor</td>
<td>Entry</td>
<td>Virus uptake/internalization</td>
<td>(Eierhoff, Hrincius et al. 2010)</td>
</tr>
<tr>
<td>COPI</td>
<td>Entry/late endosome</td>
<td>Protein synthesis</td>
<td>(Sun, He et al. 2013)</td>
</tr>
<tr>
<td>Protein phosphatase 6</td>
<td>RNA polymerase activity</td>
<td>Interacts with viral RdRp</td>
<td>(York, Hutchinson et al. 2014)</td>
</tr>
<tr>
<td>Focal adhesion kinase</td>
<td>RNA replication</td>
<td>PI3K activation/actin reorganization</td>
<td>(Ver, Marcos-Villar et al. 2015)</td>
</tr>
<tr>
<td>NXP2/MORC3</td>
<td>RNA replication</td>
<td>Interacts with RdRp complex</td>
<td>(Ver, Marcos-Villar et al. 2015)</td>
</tr>
<tr>
<td>Formyl peptide receptor 2</td>
<td>Viral replication</td>
<td>Acts through ERK signaling</td>
<td>(Ver, Marcos-Villar et al. 2015)</td>
</tr>
<tr>
<td>ANP32A</td>
<td>Viral replication</td>
<td>PB2</td>
<td>(Long, Giotis et al. 2016)</td>
</tr>
</tbody>
</table>
1.7.2 Antiviral host factors

As IAV enters the host cell, different host sensors including retinoic acid-inducible gene 1 (RIG-I), melanoma differentiation-associated protein 5 (MDA5), toll-like receptors (TLRs), and laboratory of genetics and physiology 2 (LGP2) detect an incoming virion or viral nucleic acid and prepare the host cell to fight virus infection. In this process, a plethora of host factors combat IAV and barricade the invading virion at various stages of infection (Table 2). Brass et al., employed siRNA-mediated gene screening and identified 120 host factors involved in IAV infection. Of these host factors, interferon-inducible transmembrane (IFITM) family members were discovered to be anti-IAV host factors. The IFITM family comprising of IFITM1, IFITM2 and IFITM3 restrict IAV infection at a very early stage by preventing the acidification of endosomes. Besides IAV infection, IFITM proteins conferred cross protection from West Nile virus and Dengue virus but had no effect on Moloney Leukaemia virus, Hepatitis C virus and HIV infection (Brass, Huang et al. 2009). Recently, Wang et al., (Wang, Chi et al. 2014) demonstrated eukaryotic translation initiation factor 4B (eIF4B) as another anti-IAV host factor as IAV infection was enhanced in the absence of eIF4b and contrastingly, the overexpression of eIF4B resulted in decreased virus replication (Wang, Chi et al. 2014). Further investigation revealed that eIF4B positively regulates the expression of IFITM3 (Wang, Chi et al. 2014) (Brass, Huang et al. 2009). In conjunction with this, Wang et al., described the role of another interferon-inducible host protein, ‘viperin’ (also known as rsad2, cig5) as an anti-IAV factor (Wang, Hinson et al. 2007). IAV assembles and buds at the host apical surface plasma membrane micro-domains known as lipid rafts (Wang, Hinson et al. 2007). Viperin exerts its antiviral function by disrupting the lipid rafts, hence perturbing the virus release and subsequent rounds of infection (Wang,
Hinson et al. 2007). A similar role for viperin was reported in human cytomegalovirus infection (Chin and Cresswell 2001).

Another interferon stimulated gene, ISG15, an ubiquitin-like protein, which conjugates to both cellular and viral proteins was also found to inhibit IAV replication (Haas, Ahrens et al. 1987, Hsiang, Zhao et al. 2009). ISG15 exhibited a similar role in HIV-1 (Lenschow, Lai et al. 2007), herpes virus, Sindbis virus (Lenschow, Lai et al. 2007) and Ebola virus infections (Malakhova and Zhang 2008) but not in vesicular stomatitis virus and lymphocytic choriomeningitis virus infections (Lenschow, Lai et al. 2007). Furthermore, the role of another interferon stimulated gene, Myxovirus resistance-1 (Mx-1) (Horisberger and Gunst 1991), inducible by type I and II interferon was delineated by Verhelst and colleges (Verhelst, Parthoens et al. 2012). Mx-1 inhibits IAV replication through its GTPase activity by disrupting the association between IAV proteins, PB2 and NP (Verhelst, Parthoens et al. 2012).
Table 2. Host factors restricting the IAV replication

<table>
<thead>
<tr>
<th>Host factor</th>
<th>Stage of IAV infection</th>
<th>Proposed mechanistic role</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC1</td>
<td>undetermined</td>
<td>Promotes host innate immune response</td>
<td>(Nagesh and Husain 2016)</td>
</tr>
<tr>
<td>HDAC6</td>
<td>Virus assembly</td>
<td>Regulates trafficking of IAV components to plasma membrane</td>
<td>(Husain and Harrod 2009, Husain and Cheung 2014, Nagesh and Husain 2016)</td>
</tr>
<tr>
<td>IFITM3</td>
<td>Entry</td>
<td>Inhibits endosome acidification</td>
<td>(Wang, Chi et al. 2014)</td>
</tr>
<tr>
<td>ISG15</td>
<td>Early stage/viral replication</td>
<td>Cellular protein conjugation and anti-viral defence</td>
<td>(Malakhova and Zhang 2008, Hsiang, Zhao et al. 2009)</td>
</tr>
<tr>
<td>MX1</td>
<td>Virus replication</td>
<td>Disrupts PB2-NP interaction</td>
<td>(Verhelst, Parthoens et al. 2012)</td>
</tr>
<tr>
<td>eIF4b</td>
<td>Endosomal release of vRNA</td>
<td>Promotes host defence by enhancing IFITM3 expression</td>
<td>(Wang, Chi et al. 2014)</td>
</tr>
<tr>
<td>TRIM22</td>
<td>Early stages</td>
<td>Promotes interferon α synthesis</td>
<td>(Di Pietro, Kajaste-Rudnitski et al. 2013)</td>
</tr>
<tr>
<td>Ubiquitin ligase</td>
<td>undetermined</td>
<td>Inhibits apoptosis by destabilizing p53</td>
<td>(Nailwal, Sharma et al. 2015)</td>
</tr>
<tr>
<td>RNF43</td>
<td></td>
<td>Promotes type I interferon synthesis via RIG-I</td>
<td>(Tawaratsumida, Phan et al. 2014)</td>
</tr>
<tr>
<td>PACT</td>
<td>undetermined</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lectins</td>
<td>Early stages</td>
<td>Glycosylation of HA</td>
<td>(Ng, Tate et al. 2012)</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Early stages</td>
<td>Host innate immune response</td>
<td>(Guo, Chen et al. 2007)</td>
</tr>
<tr>
<td>Clusterin</td>
<td>Late stage</td>
<td>Inhibit apoptosis</td>
<td>(Tripathi, Batra et al. 2013)</td>
</tr>
</tbody>
</table>
As mentioned above, in a genome-wide high throughput study conducted by Shapira et al., Tripartite motif 22 (TRIM22) was identified as a candidate host restriction factor. TRIM22 belongs to the RING (Really Interesting New Gene) family of proteins comprising of over 70 members which are involved in innate and adaptive immune pathways (Nisole, Stoye et al. 2005). TRIM22 enhances type I interferon production which further transcribe a plethora of over 300 genes to combat and restrict IAV infection at different stages of its life cycle (Der, Zhou et al. 1998). Evidently, silencing of TRIM22 resulted in a significant decrease in type I interferon production and a subsequent ~10-fold increase in IAV replication. Contrastingly, the overexpression of TRIM22 reduced the IAV replication by about 100 fold (Di Pietro, Kajaste-Rudnitski et al. 2013). Similarly, TRIM19 has anti-viral activity against IAV as well as vesicular stomatitis virus, human cytomegalovirus, herpes simplex virus type 1, Ebola virus, Lassa fever virus, lymphocytic choriomeningitis virus, human foamy virus, and HIV-1 (Nisole, Stoye et al. 2005). A recent report by Tripathi et al., (Tripathi, Batra et al. 2013) uncovered the role of a host anti-apoptotic protein clusterin (CLU) in IAV infection. CLU binds to pro-apoptotic protein Bax and prevents initiation of the intrinsic apoptotic pathway. However, upon infection, NP dysregulates the expression of CLU, disrupting the interaction between CLU and Bax. Thus, unbound or active Bax triggers apoptosis via the intrinsic pathway, which is exploited by IAV for efficient replication (Tripathi, Batra et al. 2013). In addition to other events, apoptosis increases the nuclear pore size and facilitate the increased transport of viral components in and out of the nucleus, hence replication (Muhlbauer, Dzieciolowski et al. 2015). A similar observation for viral NP-induced apoptosis was reported by Nailwal et al.,(Nailwal, Sharma et al. 2015). RING finger protein 43 (RNP43), a host ubiquitin ligase, offers cytoprotective effects by inhibiting
apoptosis. As IAV infection triggers apoptosis, RNP43 expression was dysregulated by IAV to enhance virus infection (Giaccia and Kastan 1998, Benchimol 2001).

Protein activator of interferon-induced protein kinase (PACT) is an essential component of the host anti-viral system which triggers RIG-I signaling and subsequently the interferon production. NS1 binds to PACT and antagonizes its anti-viral activity (Tawaratsumida, Phan et al. 2014).

Although RNAi technique contributed to increased understanding of IAV-host interactions (Chin and Brass 2013), there was not much overlap between the host factors identified in various RNAi screenings, which brings into question the reliability of this method. Hence, there is a constant need to study IAV-host interaction by exploring and understanding the molecular pathways involved in infection.

Recently, a novel class of host enzymes referred to as histone deacetylases (HDACs) have gained significant interest in viral infection, cancer, neurogenerative disease, cardiovascular complications and various other diseases (Das Gupta, Shakespear et al. 2016, Lopez, Sullivan et al. 2016, Yoon and Eom 2016). Specifically, there is growing evidence demonstrating the role of HDACs during virus infection (Guise, Budayeva et al. 2013). Recently, our lab discovered the role of histone deacetylase 6 (HDAC6) as an anti-IAV host factor (Husain and Cheung 2014). Notably, the activity of HDAC6, a tubulin deacetylase, and a member of class II HDACs was downregulated in IAV-infected cells, and HDAC6 polypeptide was cleaved by IAV-induced caspase 3 (Husain and Harrod 2009). Furthermore, endogenous HDAC6 has been shown to have anti-IAV properties as the overexpression of HDAC6 resulted in the reduction of IAV replication, whereas the knockdown of HDAC6 expression or inhibition of its activity resulted in an increase in
IAV release (Husain and Cheung 2014). Furthermore, HDAC6 was shown to exert its antiviral function by negatively regulating the trafficking of IAV components to the plasma membrane, the site of virus assembly, via its substrate acetylated microtubules (Husain and Cheung 2014). This discovery prompted me to investigate the role of class I HDACs, HDAC1 and HDAC2 in IAV infection.

1.8 Chromatin remodelling: histones dancing to the tunes of histone deacetylases (HDACs)

In the mid-1960s, Allfrey and colleagues were the first to observe histone acetylation and proposed that acetylation of core histones regulates transcription (Allfrey 1966, Thiagalingam, Cheng et al. 2003). Chromatin is a highly condensed and packed structure comprised of a dynamic-protein DNA complex. The fundamental subunit of chromatin consists of nucleosomes which are comprised of DNA and the core histones (H2A, H2B, H3, and H4). The local organization of chromatin determines the state of gene expression. Histone acetyltransferases (HATs) are enzymes which add acetyl groups to histones (Ito, Barnes et al. 2000). This reaction is processed by generating acetyl groups from Acetyl-CoA, a molecule generated during cellular metabolism. The addition of negatively charged acetyl groups to the chromatin creates a repulsive force between the histones and DNA (Strahl and Allis 2000). This renders the DNA accessible to the transcription factors and marks an active state of gene transcription (Strahl and Allis 2000, Forsberg and Bresnick 2001). Conversely, HDACs are the enzymes which remove acetyl groups from histones. Histone acetylation has often been correlated with an active genome. In contrast, deacetylation events have been associated with gene silencing. However, in the light of advanced genomics, the deacetylase activity of HDACs was also shown to be involved during active genomic events (Strahl and Allis 2000, Forsberg and Bresnick

1.8.1 Classification of HDACs

HDACs are evolutionarily conserved cellular enzymes, present in plants, animals, fungi, and bacteria (archaebacteria and eubacteria) (Leipe and Landsman 1997). The mammalian HDACs are comprised of 18 different members, which are classified into four classes based on their structure, enzymatic activity, intracellular localization, and expression pattern (Spiegel, Milstien et al. 2012, Moser, Hagelkuys et al. 2014). The class I, II, and IV HDACs are considered classical HDACs and their activity is zinc dependent (Spiegel, Milstien et al. 2012, Moser, Hagelkuys et al. 2014). The class I HDACs (HDAC1, 2, 3, and 8) are expressed in all tissues and mainly localized in the nucleus. The class II HDACs are further sub-divided into class IIa (HDACs 4, 5, 7, and 9) and class IIb (HDACs 6 and 10) and shuttle between the nucleus and the cytoplasm (Haberland, Montgomery et al. 2009, Spiegel, Milstien et al. 2012, Moser, Hagelkuys et al. 2014). The class III HDACs, commonly known as sirtuins, are comprised of seven members (SIRT1-7). Each sirtuin has a unique subcellular localization and distinct function (Choi and Mostoslavsky 2014). The sirtuins are different to classical HDACs and require NAD\(^+\) for enzymatic activity.

Lastly, the stand-alone member of class IV, HDAC11 shares similarities with class I and II HDACs, and is reported to be involved with immune tolerance (Gao, Cueto et al. 2002, Villagra, Cheng et al. 2009). The localization of HDACs and their knockout phenotype is described in table 3.
Table 3. HDACs: intracellular localization and knockout phenotype.

<table>
<thead>
<tr>
<th>Class</th>
<th>HDAC</th>
<th>Localization</th>
<th>Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>HDAC1</td>
<td>Nucleus</td>
<td>Lethal due to general growth and retardation</td>
<td>(Lagger, O’Carroll et al. 2002)</td>
</tr>
<tr>
<td>I</td>
<td>HDAC2</td>
<td>Nucleus</td>
<td>Not viable after 24 hours. Die due to severe cardiac defects</td>
<td>(Montgomery, Davis et al. 2007)</td>
</tr>
<tr>
<td>I</td>
<td>HDAC3</td>
<td>Nucleus/cytoplasm</td>
<td>Lethal due to imperfect gastrulation/cardio myocyte maturation</td>
<td>(Montgomery, Potthoff et al. 2008)</td>
</tr>
<tr>
<td>II</td>
<td>HDAC4</td>
<td>Nucleus</td>
<td>Lethal within 7 days due to ossification</td>
<td>(Vega, Matsuda et al. 2004)</td>
</tr>
<tr>
<td>II</td>
<td>HDAC5</td>
<td>Nucleus/cytoplasm</td>
<td>Viable but observed cardiac abnormalities</td>
<td>(Chang, Mckinsey et al. 2004)</td>
</tr>
<tr>
<td>II</td>
<td>HDAC6</td>
<td>Nucleus/cytoplasm</td>
<td>No phenotype except for increased tubulin acetylation</td>
<td>(Zhang, Kwon et al. 2008)</td>
</tr>
<tr>
<td>II</td>
<td>HDAC7</td>
<td>Nucleus/cytoplasm</td>
<td>Lethal due to impaired vasculature</td>
<td>(Chang, Young et al. 2006)</td>
</tr>
<tr>
<td>II</td>
<td>HDAC8</td>
<td>Nucleus/cytoplasm</td>
<td>underdetermined</td>
<td>(Haberland, Mokalled et al. 2009)</td>
</tr>
<tr>
<td>II</td>
<td>HDAC9</td>
<td>Nucleus/cytoplasm</td>
<td>Viable but observed cardiac abnormalities</td>
<td>(Chang, Mckinsey et al. 2004)</td>
</tr>
<tr>
<td>II</td>
<td>HDAC10</td>
<td>Nucleus/cytoplasm</td>
<td>undetermined</td>
<td>(Tong, Liu et al. 2002)</td>
</tr>
<tr>
<td>IV</td>
<td>HDAC11</td>
<td>Nucleus</td>
<td>undetermined</td>
<td>(Gao, Cueto et al. 2002)</td>
</tr>
</tbody>
</table>
1.9 Physiological role of HDACs

A functional balance between HATs and HDACs serve as key regulators of gene expression and governs numerous developmental processes and disease states (Allfrey, Faulkner et al. 1964, Galvani and Thiriet 2015). Historically, HDACs are known to deacetylate histones (Gregoretti, Lee et al. 2004). However, cytoplasmic proteins are also the substrates of HDACs (Kim, Sprung et al. 2006). A proteomic study has identified at least 3,600 acetylation sites in 1,750 histone and non-histone proteins, indicating a broader role of HDACs in the cell. The functional characterization of class I HDACs, HDAC1, and HDAC2, revealed that these enzymes perform their activity either independently or together as a complex to influence gene expression (Kao, Downes et al. 2000). Further, Sin3, NuRD, and CoREST complexes were found to be necessary for HDAC1 and HDAC2 activity. In addition, HDAC1 and HDAC2 have been reported to form a complex with DNA binding proteins like YY-1 (Ying Yang 1), Rb binding protein-1, and SP-1 (Wu, Li et al. 2001, Yao, Yang et al. 2001). Although, hyperphosphorylated forms of HDAC1 and HDAC2 are enzymatically active, their activity is regulated and co-repressor complex formation is promoted when they are hypophosphorylated, suggesting a dynamic role of these HDACs (Pflum, Tong et al. 2001, Galasinski, Resing et al. 2002). HDAC1 and HDAC2 are expressed ubiquitously in all cell types and are involved in diverse cellular pathways (Yao, Yang et al. 2001, Yamaguchi, Tonou-Fujimori et al. 2005). HDAC1 and HDAC2 play a redundant role in cardiac development (Nusinzon and Horvath 2005, Montgomery, Davis et al. 2007). Another class I member, HDAC3 exhibits about 68% sequence similarity with HDAC1 and HDAC2 (Chang, McKinsey et al. 2004). HDAC3 forms a complex with SMRT (silencing mediator for retinoic acid and thyroid hormone receptors) and N-CoR
(nuclear receptor co-repressor) to exhibit its activity. HDAC3 has been reported to co-immunoprecipitate with HDAC4, HDAC5, and HDAC7 \cite{Fischle2001, Yang2002}. Unlike other class I members, HDAC3 polypeptide has a nuclear export signal (NES) \cite{Yang2002}. HDAC3 knockout mice exhibit a lethal phenotype resulting from increased lipid storage in the heart and causing cardiac hypertrophy \cite{Chang2004, Bhaskara2008, Montgomery2008}. Moreover, conditional deletion of HDAC3 in liver, results in impaired lipid metabolism \cite{Kyle2016}. The last class I member, HDAC8 has about 34% amino acid sequence identity to HDAC3 \cite{Van_den_Wyngaert2000}. The co-repressor complex for HDAC8 has not yet been identified \cite{Van_den_Wyngaert2000}. HDAC8 knockouts are viable but exhibit a craniofacial abnormality \cite{Haberland2009}.

The class IIa members, HDAC4 and 5 have about 70% amino acid sequence similarity, whereas HDAC4 and HDAC7 have 58% and HDAC5 and HDAC7 have about 57% amino acid sequence similarity. Interestingly, HDAC 4 and HDAC5 interact with HDAC3 and regulate gluconeogenesis \cite{Kao2001, Miska2001, Wu2001, Mihaylova2011, Oiso2011}. HDAC4 is a key regulator of calcium signalling and regulates chondrocyte development \cite{Vega2004}. Additionally, HDAC4 is implicated in the pathogenesis of Huntington’s disease and several other neurogenerative diseases \cite{Li2012, Mielcarek2013}. HADC5 controls memory function and its dysregulation is reported in Alzheimer’s disease \cite{Taniguchi2012, Agis-Balboa2013}. Likewise, HDAC9 is also reported in the normal functioning of mature neuron and is implicated in the pathophysiology of Schizophrenia \cite{Lang2012}. Class IIb
HDAC, HDAC6 is a unique enzyme containing two catalytic domains and is more closely related to HDAC9 (Salian-Mehta, Xu et al. 2015). HDAC6 shuttles between the nucleus and cytoplasm (Li, Zhuang et al. 2016). HDAC6 knockouts have no lethal phenotype, however, increased acetylation of α-tubulin was observed (Zhang, Kwon et al. 2008). HDAC7 knockouts are embryonically lethal as they result in loss of vasculature (Chang, Young et al. 2006). HDAC7 is also a lymphoid-specific transcriptional repressor involved in regulation of B-cell and T-cell biology (Kasler, Lim et al. 2012). Interestingly, HDAC9 has three splice variants, HDAC9a, b, and c. HDAC9c has no identified catalytic domain and its N-terminus has about 50% sequence similarity to HDAC4 and HDAC5 (Zhou, Richon et al. 2000). The different isoforms of HDAC9 might be attributed to their tissue-specific expression (Bertos, Wang et al. 2001, Zhou, Marks et al. 2001). Mice lacking either HDAC5 or HDAC9 are viable but mice lacking both genes result in embryonic lethality due to immature cardiomyocyte development (Chang, McKinsey et al. 2004). HDAC9 controls the adaptive immune system by regulating the function of T regulatory cells (Tao, de Zoeten et al. 2007). The absence of HDAC9 improves glucose tolerance and insulin resistance under high-fat diet condition (Chatterjee, Basford et al. 2014), suggesting a promising role in treating obesity-related metabolic complications. HDAC10 is the most recently identified member of class IIb (Guardiola and Yao 2002). Phylogenetic analysis reveals its close identity to HDAC6 (about 36% sequence similarity) (Guardiola and Yao 2002) with a catalytic domain at the N-terminus and another putative catalytic domain at the C-terminus (Guardiola and Yao 2002). HDAC10 is implicated in B-cell malignancies, autophagy and renal carcinoma (Fan, Huang et al. 2015, Pinto, Shtaif et al. 2016, Powers, Lienlaf et al. 2016).
The only member of class IV HDACs, HDAC11 shares similarities between both class I and class II HDACs (Gao, Cueto et al. 2002) and is therefore placed separately under class IV (Gregoretti, Lee et al. 2004). HDAC11 is localized to the nucleus and is reported to interact with HDAC6 (Gao, Cueto et al. 2002). HDAC11 is reported to play a role in Hodgkin’s lymphoma (Buglio, Khaskhely et al. 2011), immune tolerance and various other physiological and pathological conditions (Villagra, Cheng et al. 2009).

1.10 Role of HDACs in virus infection and innate antiviral response

HDACs are also involved in viral infections, primarily through regulation of host innate antiviral responses, as they can act as both co-activators and co-repressors of gene transcription. The nuclear factor κB (NFκB) pathway is primarily involved in host antiviral response and apoptosis signalling (Akira and Kishimoto 1992). NFκB has also been shown to be regulated by deacetylation/acetylation and this process is modulated by different viruses in multiple ways to enable efficient infection (Kwon, Brent et al. 2008). Acetylation is also reported to play a key role in regulating the innate immune pathway. For instance, acetylation of signal transducer and activator of transcription (STAT) 1/2/3 or IRF9 at their DNA binding domain results in the enhanced expression of innate immune genes (Yuan, Guan et al. 2005). Further, acetylation of interferon alpha receptor (IFNAR) results in its decreased association with interferon alpha (IFNA). However, upon deacetylation by HDAC3, IFNAR show higher binding capacity (Yuan, Guan et al. 2005). Mounce et al., demonstrated that HDACs 1 and 2 are involved in the attenuation of mouse gammaherpesvirus 68 (MHV68) replication. They have showed that MHV68 orf36 protein, a protein kinase, interacts with host HDACs 1 and 2 and prevents their association with the viral promoter and consequently attenuates viral replication (Mounce, Mboko et al. 2013).
To further study the role of HDAC 1 and 2 during herpes virus infection, the same group showed that HDAC 1 and 2 are essential for the type I Interferon signaling by activating the IRF3 pathway and subsequently expressing ISGs. This activity accounts for conferring host defence against herpes viruses (Guise, Budayeva et al. 2013).

Recently, sirtuins, class III HDACs, have been discovered as evolutionarily conserved host anti-viral factors. The knockdown of sirtuins resulted in an increase in the infection of cytomegalovirus and IAV as well as bacteriophage (Koyuncu, Budayeva et al. 2014). Strikingly, HIV infection has been reported to downregulate the activity of SIRT1, a member of the class III HDACs (Mohr and Sonenberg 2012). In addition, SIRT1 is also reported to be regulated during vesicular stomatitis virus and Kaposi’s sarcoma-associated herpes virus infection (Mohr and Sonenberg 2012) (Campagna, Herranz et al. 2011, Li, He et al. 2014). A recently identified ISG, IFI16 is localized to the nucleus when acetylated and is involved in immunosurveillance. By doing so, it regulates the expression of viral DNA in the nucleus and also induces interferon production (Unterholzner, Keating et al. 2010, Li, Diner et al. 2012). A classic example of the role of co-repressors in inhibiting virus replication was reported in herpes virus infection (Gu and Roizman 2007). The HDAC1-CoREST co-repressor complex formation is disrupted by ICP10, a viral protein (Gu and Roizman 2007).

Host HDACs have paradoxical roles in the infection of DNA and RNA viruses. In the acute phase of infection, they restrict the virus, whereas in HIV and herpes virus infection they promote latency. The major barrier which stands in the way of discovering a cure for HIV is the latent virus residing in resting T cells. To reboot the
latency, HDAC inhibitors (HDACi) have been proving as potential molecules in latent viral infections (Wightman, Lu et al. 2013, Banga, Procopio et al. 2016). HDAC4, HDAC5, SIRT1 and SIRT2 were found to be upregulated during human Borna disease virus infection. The expression of these proteins was essential for transportation of the viral components outside the nucleus (Liu, Zhao et al. 2014). Likewise, HDACi treatment (SAHA and sodium butyrate) stimulated the expression of Simian virus 40 transcription at appropriate times during infection (Balakrishnan and Milavetz 2008). Treatment with the class I and II HDACi, TSA also resulted in the hyperacetylation of the viral promoter region in the fatal viral disease, leukoencephalopathy caused by human polyoma JC virus. Similarly, sirtuins have been reported to be involved in antiviral defence against varicella zoster virus, herpes simplex virus and vesicular stomatitis virus (Kwon, Brent et al. 2008, Thakur, Chandra et al. 2012) (Allison, Jiang et al. 2009). Recently, Li et al., reported that the inhibition of SIRT2a activity using nicotinamide resulted in a decrease in promoter activity and a subsequent reduction in Hepatitis B virus (HBV) replication both in vitro and in vivo (Li, Ren et al. 2016). Similarly, SIRT-1 was found to inhibit the host factor AP-1 which supported HBV replication by enhancing their promoter binding activity. Knockdown of SIRT-1 resulted in impaired HBV replication whereas its overexpression augmented the promoter activity (Ren, Tao et al. 2014).

The acetylation/deacetylation status altering the promoter activity resulting in virus latency has been reported in herpes simplex virus 1 (HSV-1), Kaposi’s sarcoma-associated herpesvirus (KSHV), Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV) (Lu, Zhou et al. 2003, Danaher, Jacob et al. 2005, Sinclair and Sissons 2006). Inhibition of class I and class II HDACs resulted in reactivation of Kaposi’s sarcoma induced herpes virus.
Besides their role in virus infection, HDACs have also been attractive targets for inducing antitumor activity in oncolytic viruses. Nakashima et al., (Nakashima, Kaufmann et al. 2015) accelerated the immune response against glioma cells by delivering oncolytic herpes simplex virus type I. Inhibition of HDAC6 activity further enhanced the virus replication, eventually boosting the immune response against the tumour (Nakashima, Kaufmann et al. 2015).

1.11 Focus of my PhD research

It is not surprising that viruses have evolved multiple strategies to breach the host defence and establish a successful infection. There is growing evidence pertaining to the role of HDACs in mediating the host defence. However, the exact mechanism by which they exhibit their antiviral activity still remains elusive. Thus, understanding the molecular basis of IAV-host interaction in the light of HDACs could potentially result in unravelling novel mechanisms adapted by the host which has not been discovered hitherto.

My PhD thesis focuses on understanding the role of class I HDACs, HDAC1 and HDAC2 in IAV infection using a cell culture model system by using mainly influenza virus IAV/PR/8/34 (H1N1) (hereafter referred to as PR8) and influenza virus strain A/WSN/1933 (H1N1) strains (hereafter referred to as WSN). PR8 is a lab adapted strain (Thangavel and Bouvier 2014) whereas WSN is reported as both lab and mice adapted strain (Forbes, Selman et al. 2013, Thangavel and Bouvier 2014). For studying IAV-host interaction, these two strains are widely used especially in A549 cells (Kroeker, Ezzati et al. 2012, Thangavel and Bouvier 2014). Hence these two strains were employed for this study.
1.11.1 Hypothesis

Based on the findings in our lab demonstrating the anti-viral role of HDAC6, it was hypothesized that class I HDACs, HDAC1 and HDAC2 regulate IAV infection, and in turn, they are regulated by IAV. In order to test my hypothesis, the following objectives were proposed.

1.11.2 Main objectives

1. Investigate the aberrant expression of HDAC1 and HDAC2 in response to IAV infection.
2. Identify if the aberrant expression of HDAC1 and HDAC2 has a pathological role in IAV infection.
3. Understand the molecular mechanism of HDAC1 and HDAC2 and IAV interaction.
CHAPTER 2: RESULTS
2.1 Role of HDAC1 in IAV infection

2.1.1 Introduction

HDAC1 is a mammalian homolog of yeast pleiotropic transcriptional regulator Rpd3 (reduced potassium deficiency 3). HDAC1 is reported to be predominantly present in the nucleus; however, its cytosolic localization is reported during herpes simplex virus type 1 infection (Gu and Roizman 2009) as well as in damaged axons of human brain (Kim, Shen et al. 2010). HDAC1 was the first protein identified to exhibit deacetylase activity and is believed to be the main driver of overall cellular deacetylase activity (Taunton, Hassig et al. 1996, Lagger, O’Carroll et al. 2002). The catalytic activity of HDAC1 is mainly attributed to its association with multiprotein co-repressor complexes comprising of HDAC2, Sin3, nucleosome remodelling and deacetylase (NuRD), corepressor of element-1-silencing transcriptional factor (CoREST) and Nanog and Oct4-associated deacetylase (NODE) complexes (Alland, Muhle et al. 1997) (Heinzel, Lavinsky et al. 1997, Zhang, Iratni et al. 1997, Alland, David et al. 2002, Grozinger and Schreiber 2002, Moser, Hagelkruys et al. 2014). Interestingly, as a part of NuRD co-repressor complex, metastasis-associated protein (MTA1), recruits HDAC1 to the catalytic core in inositol phosphate-dependent manner to alter the target gene expression (Millard, Varma et al. 2016).

2.1.1a Dual role of HDAC1 in transcription regulation

HDAC1 has been gaining significant interest as it exhibits a prominent role in the regulation of various infectious diseases, inflammatory diseases, cancer, metabolic pathways, cardiovascular complications, neurogenerative disorders and maintains the overall cellular homeostasis. Initially, HDAC1 was believed to be a repressor of gene transcription (Doetzlhofer, Rotheneder et al. 1999). However, there is growing
evidence demonstrating the role of HDAC1 as well as other HDACs as the activators of gene transcription \textit{(Nusimzon and Horvath 2003, Zupkovitz, Tischler et al. 2006)}. Genome-wide studies revealed that deletion of yeast Rpd3, a human HDAC1 homologue, resulted in a two-fold decrease in the expression of 264 genes and upregulation of about 170 genes \textit{(Bernstein, Tong et al. 2000)}. Likewise, about 5% of mouse genes were dysregulated in HDAC1-deficient embryonic stem cells \textit{(Zupkovitz, Tischler et al. 2006)}, indicating their key role in gene expression. HDAC1 plays an essential role in cell cycle progression, proliferation and differentiation, adipogenesis, muscle development and autophagy \textit{(Woods and Rogina 2016)}. HDAC1 is an essential gene and HDAC1-knockout mice exhibit severe cardiac abnormalities resulting in a lethal phenotype, indicating their crucial role in cardiac growth and morphogenesis \textit{(Montgomery, Davis et al. 2007)}. HDAC1 activity is also known to play a key role in maintaining intestinal metabolism by regulating the acetylation status of inflammatory genes, which would otherwise result in metabolic stress and impaired response to oxidative stress. Moreover, HDAC1 depletion in intestinal cells resulted in altered activation of the adenosine monophosphate Activated kinase (AMPK), which plays a key role in nutrient sensing and metabolism \textit{(Lu, Borthwick et al. 2011, Gonneaud, Turgeon et al. 2015)}.

2.1.1b Role of HDAC1 in viral infections

Consistent with its dual role as a transcription repressor as well as an activator, HDAC1 paradoxically influences virus replication too. During persistent viral infection, HDAC1 promotes latency, whereas, during acute viral infection, it inhibits virus replication. Latent virus infections in the case of HIV-1 has been recently been overcome by using HDAC inhibitors. Breaking this latency is very crucial as the host immune response in combination with anti-retroviral therapy could abolish HIV-1
from its reservoir cells. HDAC1 induces epigenetic silencing and represses the expression of vitamin D receptor, causing podocyte injury and thereby promoting HIV latency (Chandel, Ayasolla et al. 2015). An interesting study by Hye et al. demonstrated the role of class I and II HDACs in Kaposi’s sarcoma-associated herpesvirus (KSHV) infection. In this case, inhibition of HDAC activity using sodium butyrate breaks the latency of virus by switching to a lytic cycle, thereby inducing the expression of Rta, a viral protein (Shin, DeCotiis et al. 2014).

In hepatitis B virus-induced hepatocellular carcinoma, there is an increase in the expression of HDAC1 and HDAC1-co-repressor complex, which promotes carcinogenesis by prolonged activation of the interleukin-6/STAT3 pathway (Zhang, Diab et al. 2015, Yuan, Lei et al. 2016). In addition, HDAC1 has been reported to reduce airway inflammation and, intriguingly, support fungal and bacterial infection (Lagger, O’Carroll et al. 2002) by altering the cytokine expression. Notably, in lung macrophages, HDAC1 depletion resulted in reduced rate of *Mycobacterium tuberculosis* (Chandran, Antony et al. 2015).

Contrastingly, during acute infection, HDAC1 represses hepatitis B replication by exhibiting its deacetylase activity at the viral DNA promoters (Koumbi, Pollicino et al. 2016). In HIV-1 infection, a viral accessory protein, Vpr induced depletion of HDAC1 and HDAC3 result in hyperacetylation of histones on HIV-1 long terminal repeat (LTR), which enhances acute infection in macrophages (Romani, Baygloo et al. 2016). In lymphocytic choriomeningitis virus (LCMV) infection, T cell-specific loss of HDAC1 resulted in an impaired antiviral response, suggesting its critical role in cell-mediated immunity (Tschismarov, Firmer et al. 2014).
Inhibition of HDAC1/HDAC3 activity during human papillomavirus infection resulted in impaired host antiviral response and cytokine signalling, indicating a crucial role of these HDACs in regulating the infection (Tummers, Goedemans et al. 2015). During human cytomegalovirus (HCMV) infection, pUL97, a viral protein kinase phosphorylates HDAC1 and regulates its repressor activity, which would otherwise interfere with viral gene expression (Bigley, Reitsma et al. 2013). Similarly, the role of HDAC1 was demonstrated during mouse gammaherpesvirus (MHV68) and Epstein-Barr virus (EBV) infection. HDAC1 and HDAC2 are phosphorylated by viral kinases, orf36 or BGLF4 during the de novo lytic infection of MHV68 and EBV, respectively. The inactivation of HDAC1/HDAC2 by phosphorylation enhances early viral replication events, suggesting a role of HDAC1 and HDAC2 in repressing viral replication (Mounce, Mboko et al. 2013). Likewise, ORF66p, a varicella-zoster viral kinase has been reported to phosphorylate HDAC1 and 2 (Walters, Erazo et al. 2009). Thus, evidence so far suggests a critical role of HDAC1 in regulating virus infection. However, in the context of IAV infection, the molecular role of HDAC1 has not been studied.

Previously, Husain lab demonstrated that HDAC6, a class IIb HDAC, inhibits IAV infection (Husain and Harrod 2009, Husain and Harrod 2011, Husain and Cheung 2014). Based on this finding, the role of class I HDAC, HDAC1 was examined in IAV infection. This chapter describes my findings showing that HDAC1 also possesses anti-IAV properties and is an important component of host innate antiviral response against IAV. Major parts of this chapter have been published in Journal of Virology (see appendix II).
2.1.1c Main aim of this chapter

To determine if HDAC1 has a role during IAV infection.

2.1.1d Objectives achieved

- Determined the mRNA and protein expression as well as the deacetylase activity of HDAC1 in response to IAV infection.
- Studied the IAV growth kinetics in HDAC1-depleted and -overexpressing cells.
- Investigated the underlying molecular mechanism of HDAC1 as an anti-IAV host factor.
2.1.2 Results

2.1.2a IAV downregulates the expression of HDAC1 in epithelial cells

A cell culture model system comprising of human lung epithelial cells, A549 and influenza virus strain A/PR/8/34 (H1N1) (hereafter referred to as PR8) were used for this study. In some experiments, Madin-Darby canine kidney (MDCK) cells and influenza virus strain A/WSN/1933 (H1N1) and influenza A/New Caledonia/20/1999 (H1N1) strains (hereafter referred to as WSN and NC, respectively) were also used. Unless otherwise stated, a multiplicity of infection (MOI) of 0.5 plaque forming unit (PFU) per cell was used in all the experiments to allow the virus to undergo multiple rounds of replication cycle. One replication cycle of IAV is reported to take around 8 hours (Abdoli, Soleimanjahi et al. 2013). Unless otherwise stated, 24 h infection time point was used throughout the thesis, which allows around three virus replication cycles. Cells were constantly monitored under the microscope; the infection did not have any obvious effect on the cell monolayer as it was still intact at 24 h after infection. As discussed in Chapter 1, viruses subvert host cell transcription and translational machinery to express their own polypeptides, and decrease or shut down the expression of host proteins involved in antiviral response (Walsh and Mohr 2011, Walsh, Mathews et al. 2013). Hence, we first analysed the expression of HDAC1, both at the mRNA and polypeptide level, in response to the IAV infection.

2.1.2b HDAC1 mRNA level is downregulated in response to IAV infection

A549 cells were infected with PR8 at an MOI of 0.5, and the culture media and the infected cells were harvested separately. The cells were then processed to isolate total RNA. Initially, the integrity of isolated RNA was analysed using
Agilent RNA 6000 Nano Kit (Agilent Technologies). Further, the quality and quantity of RNA were analysed by measuring the 260/280 ratio using a Nanodrop reader spectrophotometer (ThermoScientific). Subsequently, cDNA was synthesized and used as a template to measure the level of HDAC1 mRNA by quantitative real-time PCR (qPCR) using SYBR green chemistry. The data obtained was then analysed using the relative quantitation (2-\(\Delta\Delta ct\)) method (Livak and Schmittgen 2001) using the housekeeping gene actin as a reference. The analysis revealed that IAV infection caused a moderate 23%, but statistically significant (p-value – 0.005) reduction in HDAC1 mRNA level in A549 cells when infected with a low MOI of 0.5 (Figure 5.A). Similar results were obtained when A549 cells were infected with a high MOI (5) of PR8 (Figure 5.B). The infection was confirmed by detecting the presence of IAV progeny in the culture media by plaque assay (Figure 5.D). As mentioned in Chapter 1, host cells produce interferon-stimulated genes (ISGs) in response to IAV infection, and viperin is one such ISG. The expression of viperin is exponentially increased in response to IAV infection (Wang, Hinson et al. 2007). Therefore, viperin mRNA expression level was also detected as an infection marker for 0.5 MOI infection (Figure 5.D).
Figure 5. IAV downregulates HDAC1 transcript level. A549 cells were infected with either 0.5 MOI (A) or 5 MOI* (B) of PR8. After 24 hours of infection, qPCR was performed to determine HDAC1 mRNA level using β-actin as internal control. Plaque assay for 0.5 MOI (D) was performed to confirm the infection. Viperin expression (C) was used as an infection marker for 0.5 MOI using relative quantitation. Each bar represents means ± standard errors of the means of three independent experiments. P value was calculated using Students t test. * Experiment was performed in technical duplicates. UNI-Uninfected, INF-infected.
2.1.2c HDAC1 polypeptide level is downregulated in response to IAV infection

After detecting a moderate, but significant reduction in HDAC1 mRNA level in response to IAV infection, the next step was to determine the level of HDAC1 polypeptide in A549 cells in response to IAV infection. A549 cells were infected with PR8 at an MOI of 0.5 and harvested after 2, 6, 12 and 24h of infection. The total cell lysates were prepared and resolved on SDS-PAGE. The HDAC1 polypeptide was then detected by WB (WB). Protein disulphide isomerase (PDI) was detected as a loading control and viral nucleoprotein (NP) was detected as an infection marker. Protein bands were visualized by Odyssey Fc imaging system (Li-COR).

The HDAC1 and PDI protein bands were quantified using Image Studio Lite V4.0 software (Li-COR) and HDAC1 was normalized to PDI. The normalized amount of HDAC1 in uninfected sample or 2h sample was considered 100% for comparisons to 6h, 12h, and 24h samples. IAV infection was found to reduce the level of HDAC1 polypeptide (62 kDa) in A549 cells in a time-dependent manner (Figure 6.A). There was a significant 44% ($p$-value – 0.015), 63% ($p$-value – 0.001), and 67% ($p$-value – 0.001) decrease in HDAC1 polypeptide level after 6, 12, and 24h of infection, respectively, compared to 2h post-infection time point (Figure 6.B). Furthermore, there was a significant 57% ($p$-value – 0.002) decrease in HDAC1 polypeptide level after 24h of infection when compared to the uninfected cells harvested at the same time.

In addition, the HDAC1 polypeptide level was also analysed in MDCK cells in response to IAV infection. Although not physiologically relevant, MDCK cells are conducive to IAV infection and have been extensively used to study IAV biology. MDCK cells were infected with 0.5 MOI of PR8. Samples were harvested 2, 6, 12 and 24 hours post-infection, and were processed for WB. About 92% reduction in
HDAC1 polypeptide level was observed in MDCK cells after 24h of infection, which was more profound than in A549 cells at corresponding time (see Figure 12.A).

2.1.2d Downregulation of HDAC1 polypeptide was H1N1 strain-independent and required replication competent IAV

To assess whether downregulation of HDAC1 polypeptide is H1N1 strain-dependent and required a replication competent IAV, A549 cells were infected with UV-treated or untreated IAV WSN or NC strains at an MOI of 0.5. For infection with WSN, an MOI of 0.5, 3 and 5 were used to analyse a dose-dependent response. After 24h of infection, cells were harvested and processed for to detect the expression of HDAC1, Actin, and NP by WB. The reduction in HDAC1 polypeptide level was even more profound and dose-dependent when A549 cells were infected with WSN for 24h (Figure 7.A). WSN causes more profound infection in cell culture as compared to PR8. Compared to the uninfected cells, there was about 80% and 95% decrease in the level of HDAC1 polypeptide in the cells infected with WSN strain at an MOI of 3.0 and 5.0, respectively (Figure 7.A). Infection with higher MOI overwhelmed the system resulting in disruption of the monolayer and reduced cell viability (as observed visually under microscope). However, infection with higher MOI gave a very comparable result.

To confirm that the downregulation of HDAC1 polypeptide was a result of IAV pathogenicity and not its antigenic nature, A549 cells were infected with the UV-treated virus. UV treatment results in the inactivation of the genetic material of the virus; hence the virus loses its replication potential. However, the viruses retain their antigenic nature, capable of eliciting an immune response (He, Xu et al. 2010). WB analysis revealed that HDAC1 polypeptide expression in cells infected with the UV-
treated virus was comparable to mock-infected cells (Figure 7.B). Further, almost identical results were obtained when the experiment was repeated using IAV NC strain, a more clinically relevant strain of IAV (Figure 7.C).
Figure 6. HDAC1 polypeptide expression is downregulated upon IAV infection. (A) A549 cells were infected with PR8 (0.5 MOI) and cells were harvested at indicated time points followed by WB to detect indicated proteins. (B) Quantification of HDAC1 protein expression using Image studio version 4.0, LI-COR. PDI was used as a loading control. The signal from HDAC1 protein band was normalized with PDI signal and was considered as 100% in uninfected cells (UNI) for comparison with infected cells (INF). Data are presented as means ± standard errors of the means from three independent experiments: P value was calculated using one-way ANOVA followed by Dunnett’s multiple comparison test.
Figure 7. Downregulation of HDAC1 polypeptide was H1N1 strain-independent and required replication competent IAV. A549 cells were infected with (A) WSN (0.5, 3 and 5 MOI as indicated) or (B) PR8 (0.5 MOI) or UV-pretreated or (C) NC (0.5 MOI) or UV-pretreated virus as indicated. Cells were harvested at 24 hours post infection followed by WB.
2.1.2e HDAC1 polypeptide undergoes proteasome-mediated degradation in IAV-infected cells

The lack of a linear correlation between the reduction of HDAC1 mRNA and polypeptide (Figure 5 and 6) levels in PR8-infected cells prompted me to investigate whether IAV promotes the degradation of HDAC1 polypeptide in infected cells. Previously, IAV has been shown to induce the expression of caspase 3 in infected cells (Zhirnov, Konakova et al. 1999, Wurzer, Planz et al. 2003). Further, our lab has demonstrated caspase 3 mediated cleavage of HDAC6 in IAV infected cells (Husain and Harrod 2009). Therefore, the first step was to determine if HDAC1 polypeptide downregulation is mediated by caspase 3 degradation.

CaspDB software identified 22 putative caspase cleavage sites in HDAC1 polypeptide (Kumar, van Raam et al. 2014) (Table 4). Based on this information and previous findings on HDAC6 (Husain and Cheung 2014), we investigated if HDAC1 is also being cleaved in a similar fashion during IAV infection. A549 cells were infected with 0.5 MOI of PR8 and the media was supplemented with either mock inhibitor, caspase 3 inhibitors (40 µM) or left untreated. WB analysis revealed the HDAC1 downregulation, regardless of the treatment (Figure 8.A), indicating a pathway independent of caspase-mediated cleavage downregulating the HDAC1 polypeptide levels in infected cells. A follow up experiment with pan-caspase inhibitor was not performed because a role of caspase 3, an executor caspase (Walsh, Cullen et al. 2008) in HDAC1 (or HDAC2 in subsequent results section) was not observed. This prompted further investigation into the alternative pathways of protein turnover. Mainly two pathways, one mediated by the proteasome and other mediated by lysosome govern the degradation of proteins in eukaryotic cells. Of these, the lysosomal pathway is the major pathway involved in the turnover of cytosolic...
proteins. Lysosomes are membrane-bound organelles which contain digestive enzymes to break down the proteins. During starvation, lysosomes degrade cytosolic proteins in a selective manner and supply amino acids and energy to the cell for continuing basal metabolic processes (Ciechanover 2013). In contrast, the proteasomal pathway is involved in the turnover of both cellular and nuclear proteins. This pathway utilizes a small protein referred to as ubiquitin which tags their substrates and directs it to the proteasome machinery for degradation. This process is energy-driven and utilizes ATP. There are three ubiquitin conjugating enzymes, E1, E2, and E3. Of these, E3 is the effector enzyme which tags the substrate ubiquitin.

Several E3 enzymes promote IAV infection by targeting degradation of host antiviral proteins (Su, Chen et al. 2013, Chesarino, McMichael et al. 2015) Since HDAC1 polypeptide was downregulated during IAV infection, the next step was to identify which of these two pathways guide HDAC1 degradation. To do this, PR8-infected cells were treated with proteasome inhibitor MG132 (10 µM) or lysosome inhibitor NH₄Cl (20 mM). At these concentrations, MG132 and NH₄Cl have been shown to inhibit proteasomal and lysosomal activity, respectively (Shen, Zhang et al. 2009, Wang, Chi et al. 2014). The levels of HDAC1 polypeptide were then analysed and quantified as above. MG132 treatment reversed the effect of IAV infection on HDAC1 polypeptide level and rescued its level in infected cells almost to that of uninfected cells, whereas, NH₄Cl treatment did not reverse the effect of IAV infection on HDAC1 polypeptide level (Figure 8.A). Consistent with above data, there was a significant 54% (p-value – 0.001) and 60% (p-value – 0.0004) decrease in HDAC1 polypeptide level in mock-treated and NH₄Cl-treated infected cells, respectively, compared to their uninfected controls (Figure 8.C). In contrast, there was a non-
significant 3% decrease in HDAC1 polypeptide level in MG132-treated infected cells compared to the uninfected control (Figure 8.C), indicating that IAV promoting the degradation of HDAC1 polypeptide by the proteasome pathway. Inhibition of proteasomal pathway using MG132 results in increased accumulation of ubiquitinated protein as the proteasome machinery is blocked. Therefore, an increase in ubiquitinated proteins can be used as a marker to confirm the potency of MG132, which inhibits proteasomal degradation. Hence, an increase in the amount of ubiquitinated proteins in MG132-treated cells confirmed the potency of MG132 (Figure 9.D).
Table 4. Potential caspase cleavage sites in HDAC1 polypeptide. Using Caspodb online programme (http://caspdb.sanfordburnham.org/browse.php), 22 putative caspase cleavage sites for human HDAC1 polypeptide were identified. Dotted lines (----) represents the site of caspase cleavage.

<table>
<thead>
<tr>
<th>Amino acid no</th>
<th>Potential Caspase cleavage site</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>213</td>
<td>GDLRD---- IAGDK</td>
<td>0.945</td>
</tr>
<tr>
<td>181</td>
<td>IHKGID---- GVEEA</td>
<td>0.937</td>
</tr>
<tr>
<td>82</td>
<td>SIRPD---- NMSEY</td>
<td>0.896</td>
</tr>
<tr>
<td>264</td>
<td>QCGSD---- SLSGD</td>
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<tr>
<td>104</td>
<td>CPVFD---- GLFEF</td>
<td>0.874</td>
</tr>
<tr>
<td>230</td>
<td>YPLRD---- GIDDE</td>
<td>0.861</td>
</tr>
<tr>
<td>233</td>
<td>RDGID---- DESYE</td>
<td>0.839</td>
</tr>
<tr>
<td>387</td>
<td>AIPEP---- AIPEE</td>
<td>0.811</td>
</tr>
<tr>
<td>332</td>
<td>LPYNDD---- YFEYF</td>
<td>0.781</td>
</tr>
<tr>
<td>176</td>
<td>YIODD---- IHHGD</td>
<td>0.779</td>
</tr>
<tr>
<td>321</td>
<td>AVALD---- TEIPN</td>
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<tr>
<td>422</td>
<td>EEFSD---- SEEEG</td>
<td>0.741</td>
</tr>
<tr>
<td>340</td>
<td>YFGPD---- FKLHI</td>
<td>0.735</td>
</tr>
<tr>
<td>402</td>
<td>EDDPD---- KRISI</td>
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</tr>
<tr>
<td>99</td>
<td>NVGED---- CPVFD</td>
<td>0.677</td>
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<tr>
<td>191</td>
<td>FYTTD---- RVMTNV</td>
<td>0.642</td>
</tr>
<tr>
<td>16</td>
<td>CYYYD---- GDVGNN</td>
<td>0.603</td>
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<tr>
<td>269</td>
<td>SLSGD---- RLGCF</td>
<td>0.581</td>
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<tr>
<td>130</td>
<td>KQOQD---- IAVNW</td>
<td>0.544</td>
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<td>447</td>
<td>VKTED---- EKEKD</td>
<td>0.54</td>
</tr>
<tr>
<td>18</td>
<td>YYDGD---- VGNYY</td>
<td>0.524</td>
</tr>
<tr>
<td>70</td>
<td>KYHSD---- DYIKF</td>
<td>0.511</td>
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</table>
Figure 8. HDAC1 polypeptide undergoes proteasome-mediated degradation in IAV-infected cells. (A) A549 cells were infected with 0.5 MOI of PR8 and subsequently treated with 40μM of inhibitor control or caspase 3 specific inhibitor. Total cell lysate was used to detect HDAC1, PDI and NP by WB. (B) A549 cells were infected with PR8 as above and subsequently treated with NH4Cl (20 mM) or MG132 (10 μM) for 24 h. Total cell lysates were prepared, and HDAC1, Actin, and NP were detected in UNI and INF cell lysates by WB. (C) HDAC1 and actin protein bands were quantified as above and the amount of HDAC1 was normalized to actin. The normalized amount of HDAC1 in respective UNI samples was considered 100% for comparisons to INF samples. Data presented are means ± standard errors of the means of three independent experiments; P value was calculated using one-way ANOVA followed by Dunnett’s multiple comparison test. (D) The Ubiquitin (Ubiq) and Actin were detected in the above cell lysates by WB; U, uninfected; I, infected. MW, molecular weight markers.
2.1.2f Endogenous HDAC1 possesses an anti-IAV property

The downregulation of HDAC1 expression in infected cells indicated a potential anti-IAV function for HDAC1. To test this, the growth kinetics of IAV was determined in A549 cells depleted of HDAC1 expression by RNA interference. To this end, a concentration of HDAC1 siRNA was first optimized to obtain an efficient knockdown. Various concentrations (10, 25, 50 and 100nM) of a human HDAC1-targeting siRNA and a non-targeting control siRNA (50nM) were delivered to A549 cells. After 72h, the level of HDAC1 polypeptide was analysed by WB. All siRNA concentrations used resulted in an efficient knockdown of HDAC1 polypeptide (Figure 9.A). A concentration of 10nM siRNA was sufficient to reduce the HDAC1 polypeptide level by 97% reduction. Further, 10nM HDAC1 siRNA exhibited negligible cytotoxicity as compared to the control siRNA (Figure 9.B). Therefore, in the next and all subsequent experiments, a 10nM siRNA concentration was used for depleting HDAC1 expression. Further to confirm that the effect of knockdown had no considerable effect on the infection, immunofluorescent staining of NP followed by confocal microscopy was used to ascertain that a similar percentage of HDAC1-targeting siRNA (92.4 ± 1.2, n = 5) and control siRNA (93.2 ± 2.2, n = 5) transfected cells were infected with PR8 after 12h (see Figure 10.A-B).

In order to determine the IAV growth kinetics in HDAC1 depleted cells, A549 cells were transfected with CT or HD1 siRNA for 72h and subsequently infected with PR8 at an MOI of 0.5. The culture medium and the infected cells were harvested separately after 2, 6, 12, and 24h of infection. The culture media was divided into two parts; one part was analysed by WB and the other part was titrated by plaque assay to measure the amount of total and infectious IAV progeny released, respectively. The lysates of infected cells were subjected to WB to confirm the depletion of
HDAC1 expression. Notably, IAV exhibited a higher growth characteristic in HDAC1-depleted cells (Figure 9.C). After 12h and 24h of infection, the cells transfected with HDAC1-targeting siRNA released more total virions (live and dead virus release in the culture medium) than the cells transfected with control siRNA (Figure 9.D). Similarly, cells transfected with HDAC1-targeting siRNA released 2.4-fold and 3.2-fold ($p$-value – 0.002) more infectious virions after 12 h and 24 h of infection, respectively, than the cells transfected with control siRNA at the corresponding times (Figure 9.E). The WB analysis of infected cell lysates confirmed the depletion of HDAC1 expression at each time points (Figure 9.A).
Figure 9. Endogenous HDAC1 possess the anti-IAV property. (A) A549 cells were transfected with indicated concentrations of non-targeting control (CT) siRNA or HDAC1-targeting (HD1) siRNA for 72 h. Total cell lysates were prepared and HDAC1 and PDI were detected by WB. (B) The cell viability in HDAC1 depleted cells (10nM) was then determined using MTT assay. (C) A549 cells were transfected with 10 nM of CT siRNA or HD1 siRNA for 72 h. Cells were then infected with PR8 at an MOI of 0.5 and the culture medium and the cells were harvested separately after 2h, 6h, 12h, and 24h of infection. Total lysates of the cells were prepared, and HDAC1, PDI, and NP were detected by WB. Total virion yield in the culture medium was measured by (D) WB of NP and infectious virion was quantified using (E) microplaque assay. (F-G) A549 cells transfected with CT or HD1 siRNAs as above, were infected with PR8 at an MOI of 0.1 (F) or 0.01 (G) in the presence of 0.1 µg/ml trypsin, and the virion yield in the culture medium was measured by microplaque assay after 24 h and 48 h. The virion yield from respective CT siRNA samples was considered 1-fold for comparisons to HD1 siRNA samples. Data presented are means ± standard errors of the means of three independent experiments; P values were calculated using one-way ANOVA followed by Dunnett’s multiple comparison test (F-G) or two-way ANOVA followed by Sidak’s multiple comparison test (E). MW, molecular weight markers; ns, not significant.
Figure 10. Equivalent numbers of cells were infected both in CT and HD1 siRNA treated cells. A549 cells were transfected with indicated concentrations of non-targeting control (CT) siRNA or HDAC1-targeting (HD1) siRNA for 72 h. Subsequently, cells were infected with PR8 at an MOI of 0.5. After 12 h, cells were fixed, permeabilized, and then stained with mouse anti-viral NP followed by Alexa 594-conjugated rabbit anti-mouse IgG antibody. DNA-binding dye Hoechst was used for nuclear staining and then analysed by confocal microscopy. Scale bar, 10 µm.
To further substantiate the higher IAV growth kinetics in the absence of endogenous HDAC1, HDAC1-depleted cells were infected with PR8 at a lower MOI (0.1 and 0.01) in the presence of trypsin (0.1µg/ml) to enable multiple rounds of IAV replication cycle. During infection, surface protein of IAV, HA i.e., HA0 is cleaved into two subunits, HA1 and HA2. HA cleavage enables the virus to gain entry into the host cell and release its RNA into the host nucleus (Lazarowitz and Choppin 1975). Therefore, HA cleavage is essential for multiple cycles of replication at lower MOI. Trypsin is involved in the cleavage of HA and is often supplemented in cell culture under low MOI condition to support multiple rounds of infection (Seitz, Isken et al. 2012). To this end, A549 cells were transfected and infected with 0.1 MOI of PR8, and the release of infectious IAV progeny was measured after 24h and 48h as above. Consistent with above data, cells transfected with HDAC1-targeting siRNA released a 2.15-fold ($p$-value – 0.0001) more infectious virions than the cells transfected with control siRNA after 24 h of infection (Figure 9.F). After the 48 h of infection, the release of infectious IAV progeny from HDAC1-depleted cells was further increased to 6.4-fold ($p$-value – 0.005) compared to control cells (Figure 9.F). Similarly, when infected with PR8 at an MOI of 0.01, cells transfected with HDAC1-targeting siRNA released 1.6-fold and 6.8-fold more infectious virions than the cells transfected with control siRNA after 24 h and 48 h of infection, respectively (Figure 9.G).

2.1.2g Ectopically expressed HDAC1 inhibits IAV infection

The knockdown data demonstrated that endogenously expressed HDAC1 has an anti-IAV property and IAV grows to a higher titre in HDAC1-depleted A549 cells. The next goal was to identify whether ectopically expressed HDAC1 would have the opposite effect as siRNA mediated knockdown of HDAC1 on IAV replication. First,
the optimal concentration of plasmid DNA for efficient HDAC1 overexpression was determined. To achieve this, A549 cells were transfected with three different concentrations (1, 2, and 3 µg) of HDAC1-expressing plasmid or the empty plasmid using a constant volume (3 µl) of Lipofectamine 2000 reagent. After 48 h, cells were harvested and HDAC1 overexpression was detected by WB. All three concentrations resulted in the overexpression of HDAC1, but there was slightly more overexpression with 3:1 and 3:2 combinations. Henceforth, 3:1 combination was used for all subsequent experiments (Figure 11.A). At the same time, a GFP-expressing plasmid using above combinations was used to assess the transfection efficiency of A549 cells (Figure 11.B).
Figure 11. Ectopically expressed HDAC1 inhibits IAV infection. (A and C) A549 cells were transfected with empty pcDNA3 (pc) or pcDNA3 containing HDAC1 (HD1) using indicated Lipofectamine 2000: DNA combination for 48 hours. Total cell lysates were prepared and indicated proteins were detected by WB. The top bands in PDI blot under HD1 in (A) are the leftover HDAC1 bands as HDAC1 blot was stripped to probe for PDI. (B) Cells transfected with a GFP plasmid were viewed and imaged under an inverted fluorescence microscope (Olympus), magnification of X 100 to assess the transfection efficiency. (E-F) A549 Cells were transfected with CT or HD1 and then infected with PR8 at an MOI of 0.5 (E) or 5 (F) and the culture medium and the cells were harvested separately after 24 h. The virion yield in the culture medium was measured by (D) WB of NP. (G-H) A549 cells were transfected with empty plasmid pcDNA3 (pc) or pcDNA3 containing HDAC1 (HD1) for 48 hours. Subsequently, they were infected with PR8 at an MOI of 0.1 (G) or 0.01 (H) supplemented with 0.1 µg/ml trypsin, and the virion yield in the culture medium was measured by microplaque assay after 24h, 48 h and 72h of infection. The virion yield from respective pc samples was considered 1-fold for comparisons to HD1 transfected samples. Data presented are means ± standard errors of the means of three independent experiments; P values were calculated using t test (E-F) or one-way ANOVA followed by Dunnett’s multiple comparison test (G-H).
To determine if ectopically-expressed HDAC1 inhibits IAV infection, A549 cells were transfected with the HDAC1-expressing plasmid or empty plasmid as above and subsequently infected with PR8 at an MOI of 0.5 for 24h. The culture medium and the infected cells were harvested separately. The culture medium was analysed by WB and plaque assay was performed to determine the virus release. The cell lysates were subjected to WB to analyse the HDAC1 overexpression and subsequent effect of overexpressing HDAC1 on its substrate acetylated-histone H3 (Lys9) (Lagger, O’Carroll et al. 2002, Dovey, Foster et al. 2010). The overexpression of HDAC1 in cells was confirmed (Figure 11.C). Further, a visually noticeable reduction in acetylated histone H3 (Lys9) level and a negligible change in total histone H3 level in HDAC1-overexpressing cells compared to empty plasmid control confirmed that the ectopically-expressed HDAC1 was enzymatically active (Figure 11.C). Indeed, in contrast to the results obtained with HDAC1 depletion, the overexpression of HDAC1 caused a reduction in IAV infection (Figure 11.E). The HDAC1-overexpressing cells released less viral progeny (Figure 11.D) and a significant 2.3-fold (p-value – 0.01) fewer infectious virions (Figure 11.E) in the culture medium than the cells transfected with empty plasmid. Similar results were obtained when cells were infected with PR8 at an MOI of 5.0 for 24h (Figure 11.F).

When infected with PR8 at an MOI of 0.1 in the presence of trypsin, the HDAC1-overexpressing cells released 42% (p-value – <0.01), 47% (p-value – <0.01), and 57% (p-value – <0.01) less infectious viral progeny after 24, 48, and 72 h of infection, respectively, than the cells transfected with empty plasmid (Figure 11.G). Similar results were obtained when 0.01 MOI of PR8 was used for infection (Figure 11.H).
2.1.2h IAV downregulates HDAC1 activity

After discovering that HDAC1 polypeptide has an anti-IAV property and IAV downregulates its expression to potentially undermine its antiviral function, the next step was to examine whether HDAC1 enzymatic activity is also downregulated in infected cells. To assess this, the levels of HDAC1 substrate, acetylated histone H3, were compared in the infected and uninfected cell lysates by WB. An increase in acetylated histone H3 level would mean that HDAC1 activity is downregulated and vice versa. To detect acetylated-histone H3, an antibody (C5B11, Cell Signaling) that detects acetylated-histone H3 (Lys9) – histone H3 acetylated on lysine residue at position 9 was used. Previously, acetylated-histone H3 (Lys9) has been shown to be a specific substrate for HDAC1, and the HDAC1 overexpression data presented above (Figure 11.C) has confirmed it as an HDAC1 substrate. MDCK cells were infected with PR8 at an MOI of 0.5 and the cells were harvested at 2h, 6h, 12h and 24h of infection. Total cell lysates were prepared and processed for WB. The data from this experiment revealed that IAV downregulates the HDAC1 activity and it does this mainly between 6h and 12h of infection (Figure 12.A), which is the growth period of IAV in cultured cells (Figure 9.E). There was a significant 3.0-fold ($p$-value – 0.02) and 3.38-fold ($p$-value – 0.009) increase in the levels of acetylated histone H3 (Lys9) in infected cells after 6h and 12h of infection, respectively, compared to the uninfected cells (Figure 12.B). Interestingly, after 24h of infection HDAC1 activity in infected cells (1.74-fold) came back up almost to the level of uninfected cells (Figure 12.A-B).
Figure 12. IAV downregulates the activity of HDAC1. (A) MDCK cells were infected with PR8 at an MOI of 0.5 and harvested after 2h, 6h, 12h, and 24h of infection. Total cell lysates were prepared, and acetylated-histone H3 (Lys9) (Acet H3), total histone H3 (Total H3), PDI, HDAC1, Actin and NP were detected in uninfected (UNI) and infected (2h, 6h, 12h, 24h) cell lysates by WB. Acet H3, an HDAC1 substrate was detected as a marker of HDAC1 activity, and Total H3, PDI, and Actin as loading controls. (B) Acet H3 and Total H3 protein bands were quantified and the amount of Acet H3 was normalized to Total H3. The normalized amount of Acet H3 in UNI sample was considered 1-fold for comparisons to 2h, 6h, 12h, and 24h samples. Data presented are means ± standard errors of the means of three independent experiments; $P$ value was calculated using one-way ANOVA followed by Dunnett’s multiple comparison test. MW, molecular weight markers.
2.1.2i Deacetylase activity of class I and class II HDACs exhibit anti-IAV properties

The downregulation of HDAC1 activity by IAV suggested an anti-IAV role for HDAC1 activity. One way to further investigate this is by using a selective inhibitor of HDAC1 enzymatic activity. However, a selective HDAC1 inhibitor is not commercially available; most of the available HDAC inhibitors cross-inhibit other class I and class II HDACs (Seitz, Isken et al. 2012). Therefore, we determined the combined activity of class I and II HDACs in response to IAV infection (Spiegel, Milstien et al. 2012). To accomplish this, an in situ fluorometric HDAC activity assay kit was used, which essentially measures the activity of class I and class II HDACs. This kit utilises a cell-permeable HDAC substrate containing an acetylated lysine side chain, which is deacetylated by intracellular HDACs. A developer then cleaves the deacetylated substrate and releases a fluorophore, which can be quantified. In order to accomplish this, MDCK cells were seeded in a black well 96-well plate and infected with 0.5 MOI of PR8. After 20h of infection, deacetylase activity was determined using the manufacturer’s protocol. Briefly, deacetylated standard was serially diluted and the fluorescence readings were measured for plotting a standard graph (Figure 13.A). The deacetylase activity of unknown samples was calculated using the standard graph. Fluorescence value of the unknown samples was measured and normalized with the inhibitor controls. These normalized values were then used for quantifying the activity of respective samples. HDAC activity in infected cells was about 35% less as compared to uninfected cells (Figure 13.B), indicating the dysregulation of class I and II HDAC activity.
Figure 13. Deacetylase activity of class I and II HDACs is downregulated in IAV-infected cells. MDCK cells were infected with PR8 at an MOI of 0.5 in a black 96-well plate. After 20h of infection, (B) HDAC activity was measured using manufacturer’s protocol. (A) A standard deacetylase activity curve to calculate the activity of UNI and INF samples. Data presented are means ± standard errors of the means of three independent experiments; $P$ value was calculated using students’ $t$ test.
Having observed that class I and II HDAC activity was being downregulated upon IAV infection, the next goal was to determine the effect of the inhibition of class I and II HDAC activity on IAV infection. To accomplish this, we employed trichostatin A (TSA), an antifungal antibiotic that is widely used as an inhibitor of class I and class II HDACs including HDAC1 (Balasubramanian, Verner et al. 2009). To achieve this, A549 cells were infected with PR8 at an MOI of 0.5. One hour after infection, fresh medium, was added to the cells supplemented with TSA in increasing concentrations of 0, 1, 5, 10, and 20 (µM). Like HDAC1 depletion, TSA treatment increased the release of infectious IAV progeny in a dose-dependent manner (Figure 14.B). Inhibition of HDAC activity was confirmed by detecting increased acetyl histone H3 levels in infected cells (Figure 14.A). However, there was a decrease in IAV release with 20µM which could be attributed to the severe cytotoxic effect of TSA (as observed under the microscope). Below the 1 µM concentration, TSA did not inhibit the deacetylase activity (Figure 14.C). Based on this, 1µM and 5µM TSA were used for subsequent experiments. Further, to estimate the effect of TSA treatment on cell viability, an MTT assay was performed as described earlier. The TSA treatment had a negligible effect on the viability of infected cells at 1 and 5 µM (Figure 14.G). Compared to mock-treated infected cells, treatment of infected cells with 1µM and 5µM of TSA resulted in a significant 3.1-fold ($p$-value – 0.03) and 5.3-fold ($p$-value – 0.001) increase in the release of infectious IAV progeny, respectively (Figure 14.F). Correspondingly, there was an increase in total virion release which was detected using WB (Figure 14.E). A corresponding increase in the levels of acetylated histone H3 (Lys9), but not total histone H3 in TSA-treated cells indicated the inhibition of class I (including HDAC1) and class II HDAC activity (Figure 14.F).
Figure 14. Inhibition of HDAC activity promotes IAV infection. (A – C) A549 cells were infected with PR8 at an MOI of 0.5 and subsequently treated with dimethyl sulfoxide (DMSO) or the indicated concentrations of TSA (in DMSO) for 24 h. The culture medium and the cells were harvested separately and the virion yield in the culture medium was measured by microplaque assay. The virion yield in the culture medium was measured by (E) WB of NP and (B and F) microplaque assay. (G) A549 cells were infected and treated with DMSO and TSA for 24 h and the cell viability was determined by MTT assay. The viability of DMSO-treated infected cells was considered 100% for comparison to TSA-treated infected cells. (A, C-D) Total cell lysates were prepared, and Acet H3, Total H3, and NP were detected by WB as the markers of TSA potency, loading control, and infection, respectively. MW, molecular weights. Data presented are means ± standard errors of the means of three independent experiments; P value was calculated using one-way ANOVA followed by Dunnett’s multiple comparison test.
2.1.2j Deacetylase activity of class I and class II HDACs is important for IAV-induced host type I interferon-mediated response

An increase in the release of infectious IAV progeny from TSA-treated cells confirmed that enzymatic activity of class I and class II HDACs has anti-IAV properties. Besides its role in host gene transcription, deacetylase activity of class I and class II HDACs has been shown to be a co-activator of type I interferon (IFN)-mediated host innate antiviral response and subsequent expression of interferon-stimulated genes (ISGs) (Nusinzon and Horvath 2003, Chang, Paulson et al. 2004, Sakamoto, Potla et al. 2004, Xu, Holko et al. 2009). However, a precise role of class I and class II HDACs in IAV-induced host innate antiviral response is yet to be understood. It has been well established that host cells produce type I IFNs upon IAV infection (Garcia-Sastre 2011). Type I IFNs then engage host cell type I Interferon receptor in an autocrine and paracrine manner and activate the cytoplasmic Janus kinases (JAKs) that in turn, phosphorylate the cytoplasmic Signal Transducer and Activator of Transcription I (STAT1). The phosphorylated STAT1 (pSTAT1) then translocate to the nucleus and forms a transcription complex called interferon-stimulated gene factor 3 (ISGF3). ISGF3 then binds to interferon-stimulated response element (ISRE) of ISGs in a sequence-specific manner and induces the expression of over 300 ISGs, including IFITM3, ISG15, and viperin that inhibit IAV infection by targeting various steps of virus life cycle (Brass, Huang et al. 2009, Garcia-Sastre 2011). To induce the expression of ISGs, ISGF3 specifically interacts with several coactivators, which include class I and class II HDACs, particularly HDAC1 (Nusinzon and Horvath 2003, Nusinzon and Horvath 2005). Therefore, to determine the role of class I and class II HDAC activity in type I IFN-mediated host response against IAV, PR8-infected cells were treated with TSA (1
µM and 5 µM) and the levels of pSTAT1 and ISGs - IFITM3, ISG15, and viperin were analysed by WB. A decrease in the levels of these proteins in TSA-treated infected cells compared to untreated infected cells would indicate the involvement of class I and class II HDAC activity in type I IFN-mediated host antiviral response against IAV. As expected, IAV infection induced the phosphorylation of STAT1 and expression of IFITM3, ISG15, and viperin in A549 cells (Figure 15.A-D). And, consistent with the above hypothesis, TSA treatment reduced the level of pSTAT1 (Figure 15.A) as well as IFITM3 (Figure 15.B), ISG15 (Figure 15.C), and viperin (Figure 15.D) in infected cells. TSA seems to have a dose-dependent effect on the phosphorylation of STAT1 (Figure 15.A) and the expression of ISG15 (Figure 15.C); whereas, it has more profound effect on the expression of IFITM3 (Figure 15.B) and viperin (Figure 15.D) as they were barely detectable in infected cells treated with TSA. The potency of TSA was confirmed by an increase in acetyl histone H3 in TSA-treated cells (Figure 15.E).
Figure 15. Deacetylase activity of class I and class II HDACs is important for IAV-induced host type I interferon-mediated response. (A – E) A549 cells were infected with PR8 at an MOI of 0.5 and subsequently treated with DMSO or the indicated concentrations of TSA (in DMSO) for 24 h. Total cell lysates were prepared, and (A) phosphorylated STAT1 (pSTAT1, 91/84 kDa) along with total STAT1 (tSTAT1) as loading control, and (B) IFITM3 (15 kDa), (C) ISG15 (15 kDa), and (D) viperin (42 kDa) along with PDI as loading control were detected in uninfected (UNI) and infected (INF) cell lysates by WB. (E) In same lysates, Acet H3, Total H3, and NP were detected by WB as the markers of TSA potency, loading control, and infection, respectively. MW, molecular weight markers.
2.1.2k HDAC1 regulates the host innate antiviral signaling in IAV infected cells

In the event of virus infection, viral or RNA sensor molecules of the host including RIG-I, MDA5, TLRs and LGP2, detect the incoming virions and prepare the host cell to fight the virus infection. RIG-I recruits mitochondrial antiviral signaling proteins (MAVS) which further activates downstream kinases like IκB kinase (IκK) and tank binding kinase 1 (TBK1) (Patel and Garcia-Sastre 2014). Consequently, these kinases phosphorylate interferon regulatory factor 3 (IRF3), which translocate into the nucleus, resulting in interferon production (Yan and Chen 2012). These interferons act both in an autocrine and a paracrine manner, alerting the producer cell or neighbouring cells to produce more interferons or ISGs (Figure 16.A) (O’Shea, Gadina et al. 2002, Xu, Holko et al. 2009). Interferon exerts its function by modulating the expression of ISGs through activation of JAK-STAT pathway (Figure 16.A) (Yan and Chen 2012). During paracrine signaling, interferons bind to the neighbouring host cell receptors and signal via Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, creating a cellular refractory state to virus infection. Canonically, upon interferon stimulation, JAK phosphorylates cytoplasmic STAT at serine or tyrosine residues. Phosphorylation activates STATs resulting into homo- or heterodimerization and subsequent translocation to the nucleus and binding to the DNA binding region to alter target gene expression. This signalling also activates the expression of promyelocytic leukemia zinc finger (PLZF) which acts as a co-stimulator of ISG expression. Earlier, it has been demonstrated that HDAC1 specifically co-activates the PLZF and promyelocytic leukemia (PML) protein that are involved in type I IFN-mediated innate immune response and subsequent expression of ISGs like viperin (Xu, Holko et al. 2009).
Figure 16. Host innate immune response to virus infection. Binding of a cytokine (interferon, growth factors, etc) dimerizes the receptors and activates JAK which further phosphorylates STAT which subsequently localize into the nucleus, influencing the production of interferons and ISGs. (Figure adapted from Shuai & B. Liu, 2003). 

(B-C) Investigate the role of HDAC1 in IRF3 and STAT1 signaling and subsequently, interferon and ISG production.
In a similar context, HDAC1 has been reported to regulate IRF3 signaling during gamma herpes virus infection and induce type I interferon production to fight virus infection. Therefore, it was plausible to study if HDAC1 regulates IRF3 signaling and type I interferon production (Mounce, Mboko et al. 2014) during IAV infection (Figure 16.B-C). As a representative member of type-I interferons, the expression of interferon-α was determined for this study. In order to investigate the role of HDAC1 in interferon-α production, A549 cells were depleted of HDAC1 expression by RNA interference and WB was performed to analyse the expression of phosphorylated IRF3 and type I interferon-α production in HDAC1-depleted infected cells. Remarkably, the level of phosphorylated IRF3 (pIRF3) was downregulated in HDAC1-depleted infected cells (Figure 17.A). Further, the interferon-α production was also decreased in the absence of HDAC1 (Figure 17.A). These observations suggested that HDAC1 is involved in promoting the host innate antiviral response against IAV by regulating IRF3 signaling and interferon-α production.

Next, we sought to identify if HDAC1 regulates the paracrine signaling by regulating the STAT1 pathway. To this end, A549 cells were depleted of HDAC 1 expression or overexpressed using HDAC1 expressing plasmid as described earlier and subsequently infected with 0.5 or 5 MOI of PR8. WB analysis revealed that the expression of the activated STAT1 i.e., phosphorylated STAT1 (pY701) was not altered in the absence of HDAC1 (Figure 17.B). However, it was interesting to note that there was a marginal increase in the levels of total STAT1 in HDAC1 depleted cells (Figure 17.B).
Figure 17. HDAC1 regulates host innate antiviral response. A549 cells were transfected with non-targeting control (CT) siRNA or HDAC1-targeting (HDAC1) siRNA for 72 h. Cells were then infected with PR8 at an MOI of 0.5 and samples were harvested at indicated time points (A and B). Total cell lysates were prepared, and HDAC1, pIRF3, pSTAT1, STAT1, interferon-α, Actin, and NP were detected in uninfected (UNI) and infected (INF) cell lysates by WB.
2.1.2 HDAC1 has a differential role in the expression of ISGs

The data above suggested the role of HDAC1 regulating the IRF3 signaling and type I Interferon IFN mediated antiviral response during IAV infection. Therefore, we next sought to determine whether HDAC1 plays a specific role in the expression of ISGs. To accomplish this, HDAC1 expression was either depleted by RNA interference (Figure 18.A) or increased from a plasmid (Figure 18.B) in A549 cells before infecting them with PR8 and subsequently measuring the ISG (IFITM3, ISG15 and viperin) levels by WB (Figure 18.A). Consistent with the data presented in Figure 15.D, viperin expression was decreased in HDAC1-depleted infected cells (Figure 18.A-B) and increased in HDAC1-overexpressing infected cells (Figure 18.C-D). About 58% less ($p$-value – 0.03) viperin was detected in infected cells transfected with HDAC1-targeting siRNA than the infected cells transfected with non-targeting control siRNA (Figure 18.B). Conversely, about 55% more viperin was detected in infected cells transfected with HDAC1-expressing plasmid than the infected cells transfected with empty plasmid (Figure 18.D). Interestingly, a noticeable increase in the basal level of viperin was also observed in uninfected cells transfected with an HDAC1-expressing plasmid (Figure 18.C). However, HDAC1 has a differential role in regulating the expression of other ISGs as, unlike viperin, there was an increase in the expression of IFITM3 and ISG15 in HDAC1-depleted cells (Figure 19.A-C). About 2.5-fold increase in the IFITM3 expression was observed in HDAC1-depleted cells compared to the control (Figure 19.B). The expression of IFITM3 and ISG15 were also analysed in HDAC1-overexpressing cells which showed no detectable change in the expression of these two ISGs in HDAC1-expressing cells as compared to their control (data not shown).
Figure 18. HDAC1 is involved in the expression of viperin in IAV-infected cells. (A and B) Knockdown of HDAC1 expression decreased viperin expression in infected cells. A549 cells were transfected with non-targeting control (CT) siRNA or HDAC1-targeting (HD1) siRNA for 72 h. Cells were then infected with PR8 at an MOI of 0.5 for 24 h. (A) Total cell lysates were prepared, and HDAC1, viperin, PDI, and NP were detected in uninfected (UNI) and infected (INF) cell lysates by WB. (B) The viperin and PDI protein bands were quantified and the amount of viperin was normalized to PDI. The normalized amount of viperin in CT siRNA-transfected INF cells was considered 100% for comparisons to HD1 siRNA-transfected INF cells. (C-D) Overexpression of HDAC1 increased viperin expression in infected cells. A549 cells were transfected with empty plasmid pcDNA3 or pcDNA3 containing HDAC1 for 48 hours. Cells were then infected with PR8 at an MOI of 0.5 for 24 h. (C) Total cell lysates were prepared, and HDAC1, viperin, PDI, and NP were detected in uninfected (UNI) and infected (INF) cell lysates by WB (D) The percent change in viperin level was calculated as above. Data presented are means ± standard errors of the means of three independent experiments; P value was calculated using t test. MW, molecular weights.
Figure.19. HDAC1 has differential role in the expression of IFITM3 and ISG15. A549 cells were transfected with non-targeting control (CT) siRNA or HDAC1-targeting (HD1) siRNA for 72 h. Cells were then infected with PR8 at an MOI of 0.5 or 5 for 24 h. (A and C) Total cell lysates were prepared, and HDAC1, IFITM3, ISG15, PDI, and NP were detected in uninfected (UNI) and infected (INF) cell lysates by WB. (B) The IFITM3 and PDI protein bands were quantified and the amount of IFITM3 was normalized to PDI. The normalized amount of IFITM3 in CT siRNA-transfected INF cells was considered 1 fold for comparisons to HD1 siRNA-transfected INF cells. Data presented are means ± standard errors of the means of three independent experiments; P value was calculated using two way ANOVA followed by Sidak’s multiple comparison test.
2.1.3 Discussion

In order to efficiently replicate in host cells, IAV modulates and exploits the host cell machinery. Thus, the effect of host factors on IAV replication and in turn, the effect of IAV on these host factors remains indisputable. The data presented in this chapter demonstrate that HDAC1 is a component of host antiviral response against IAV and in turn, IAV dysregulates HDAC1 to undermine its role in host antiviral response. IAV downregulates HDAC1 expression at both mRNA and polypeptide level. The reduction in HDAC1 mRNA level could be attributed to the global mRNA level downregulation caused by the host mRNA synthesis shut off induced by IAV (Inglis 1982). Nevertheless, the reduction in HDAC1 mRNA level in IAV-infected cells observed here is consistent with a recent microarray-based gene expression profiling study (Buggele, Krause et al. 2013). By using mainly, a luciferase reporter assay and quantitative real-time PCR, Buggele et al. (Buggele, Krause et al. 2013) demonstrated that IAV induces host microRNA (miR), miR-449b that targets the 3’-UTR of HDAC1 mRNA, and when added exogenously, miR-449b further reduced HDAC1 mRNA level in infected cells. However, they did not determine HDAC1 polypeptide level in response to the direct IAV infection and could not establish a direct anti-IAV role of miR-449b or HDAC1 as depletion or overexpression of miR-449b did not have an effect on IAV infection. Here, we have demonstrated that IAV also reduces HDAC1 polypeptide level in infected cells by promoting its degradation by the proteasomal pathway. A proteasome-mediated degradation of HDAC1 polypeptide is plausible because HDAC1 has been reported to be ubiquitinated by Mdm2 E3 ubiquitin ligase (Kwon, Eom et al. 2016). In cardiovascular and metabolic diseases, vascular calcification is regulated by HDAC1. MDM2 E3 ligase targets HDAC1 to proteasomal degradation which increased vascular calcification (Kwon,
Similarly, in IAV infection, the E3 ubiquitin ligase, Itch, is upregulated and plays an important role in the endosomal release of viral RNA and their nuclear transport (Su, Chen et al. 2013). This upregulation of E3 ligase would potentially ubiquitinate HDAC1. In addition, the nuclear localization of HDAC1 supports its proteasomal degradation, as observed in this study. Therefore, one of the future directions of this study is to determine whether HDAC1 is also ubiquitinated in IAV-infected cells and identify the potential role of E3 ligase.

A previous study reported an increase in IAV entry in HDAC1-depleted cells using a microscopy-based assay, but it did not monitor productive IAV replication cycles measuring the release of IAV progeny from HDAC1-depleted cells and did not envisage an anti-IAV role for HDAC1 (Yamauchi, Boukari et al. 2011). Using a productive IAV replication cycle, we envisaged and confirmed the anti-IAV role of host HDAC1 and have demonstrated that knockdown and overexpression of HDAC1 increases and decreases IAV infection, respectively. However, such increase or decrease did not result in a noticeable increase or decrease in the intracellular NP levels in HDAC1-depleted or HDAC1-overexpressing cells, respectively, though we confirmed by immunofluorescence microscopy that almost an equal number of HDAC1 knockdown cells were being infected with IAV (HDAC1-targeting siRNA (92.4%)- and control siRNA (93.2%)-transfected cells. A plausible explanation for this is that NP is one of the most abundant IAV proteins in infected cells, and a significant, but modest 2-3-fold difference in IAV infection may only slightly alter the intracellular abundance of NP in HDAC1-depleted or HDAC1-overexpressing cells, which may not be discernible by WB. Further, a significant, but modest 3-fold increase in the growth characteristics of IAV in HDAC1-depleted cells after 24h could be partly attributed to the compensatory role of other class I HDACs in the absence
of HDAC1. As mentioned above, HDAC1 shares at least 86% similarity in nucleotide sequence with HDAC2 and about 63% similarity with HDAC3 (Moser, Hagelkruys et al. 2014). It is believed that HDAC1 and HDAC2 genes are a duplicated copy of one gene derived from a common ancestor (Moser, Hagelkruys et al. 2014). Evidently, depletion of HDAC1 expression results in the enhanced expression of HDAC2, which has been presented in Section 2.5.

The data showed that deacetylase activity also has an anti-IAV function. Therefore, in addition to expression, IAV also downregulated the deacetylase activity of HDAC1 as determined by detecting the increase in the levels of specific HDAC1 substrate, acetylated-histone H3 (Lys9) in infected cells. One could argue that the increase in the levels of acetylated histone H3 (Lys9) (Zupkovitz, Tischler et al. 2006) in IAV-infected cells is due to the decrease in the levels of HDAC1 polypeptide. However, time-course experiments revealed slightly different kinetics for the decrease in the HDAC1 polypeptide levels and increase in the acetylated histone H3 (Lys9) levels. For instance, HDAC1 polypeptide level continued to decrease after 12 h of infection, whereas the HDAC1 deacetylase activity started to come back up to the basal level after 12 h of infection. Interestingly, both the expression and activity of HDAC1 was upregulated in Newcastle disease virus infection (Ng, Chang et al. 2011). This observation further explains the functional disparity of HDAC1 specifically in IAV infection and for virus infection in general. In another report, HDAC1 activity was downregulated by Gam1, an early gene product of adenovirus (Chiocca, Kurtev et al. 2002). Gam1 directly binds to HDAC1 and undermines its deacetylase activity, resulting in enhanced viral transcription (Chiocca, Kurtev et al. 2002). Similarly, HDAC activity is also dysregulated in bovine herpes virus infection at an early stage of virus infection (Zhang and Jones 2001). Contrastingly, herpes viruses and
retroviruses which induce latency require HDAC activity for epigenetic silencing (Hatama, Otake et al. 2001). Interestingly, different viruses adopt unique and independent strategies to circumvent the host defence. Therefore, one of the future directions is to study IAV-HDAC1 interaction and identify the IAV protein which regulates HDAC1 expression and activity. Another future direction of this study is to delineate the independent roles of HDAC1 catalytic domain and rest of the HDAC1 polypeptide in its anti-IAV function.

HDAC1 is believed to be the main driver of overall cellular deacetylase activity (Lagger, O’Carroll et al. 2002). The catalytic activity of HDAC1 (and HDAC2) is largely dependent on its association with multiprotein complexes, which include Sin3, NuRD, CoREST, and NODE complexes (Moser, Hagelkruys et al. 2014). Additionally, various post-translational modifications also regulate HDAC1 activity. For instance, casein kinase CK2 mediated phosphorylation of HDAC1, result in regulation of enzyme activity (Pflum, Tong et al. 2001, Khan, He et al. 2013). Phosphorylation of HDAC1 at Serine 421 and 423 residues enhanced its enzymatic activity and complex formation (Pflum, Tong et al. 2001). Moreover, HDAC1 and HDAC2 are phosphorylated by CK2 during the cell cycle, which reduces the complex formation between HDAC1 and HDAC2 but enhances complex formation with their corresponding repressors (Khan, He et al. 2013). Additionally, HDAC1 is acetylated by CBP/p300 which negatively regulates their activity (Qiu, Zhao et al. 2006). This is the first report describing the global HDAC activity in IAV infected cells. There is a paucity of information describing the role of HDAC1 in IAV infection. It remains to be investigated whether IAV destabilizes or prevents the formation of the above complexes to downregulate the HDAC1 activity.
Historically, HDAC1 has been described as the regulator of chromatin structure and repressor of gene transcription (Mounce, Mboko et al. 2014). However, lately, HDAC1 has also been implicated in non-transcriptional functions such as DNA repair, splicing and cell division, and described as both positive and negative regulator of gene expression (Mounce, Mboko et al. 2014). Some of the genes that are positively regulated by HDAC1 include type I IFNs and ISGs such as ISG15, ISG54, IFITM1, IFITM2, and viperin (Nusinzon and Horvath 2003, Nusinzon and Horvath 2006, Xu, Holko et al. 2009). The data presented here indicate that HDAC1 and deacetylase activity are involved in innate antiviral signaling pathway induced in IAV-infected cells (Figure 15). Further, this data also demonstrates that HDAC1 works upstream and regulates the phosphorylation of IRF3 and subsequently type I IFNs production. (Figure 18 B-C). The complex IFN-signalling pathways represent the first-line of host antiviral defence against invading viruses including IAV. And, like other viruses, IAV has evolved various strategies to subvert IFN-signalling to multiply and cause respiratory disease. Thus, the data presented here suggest the downregulation of HDAC1 in IAV infection to dampen the host innate immune response. However, it would be interesting to investigate the interaction of HDAC1 with STAT1 and pIRF3 in IAV infected cells by co-immunoprecipitation to describe the direct role of HDAC1 in the innate immune pathway. In addition, PML and PLZF nuclear bodies confer antiviral resistance either by physically interacting with viral proteins or by inducing host antiviral proteins. Intriguingly, PML and PLZF have been shown to restrict IAV infection and in conjunction with this, upon interferon stimulation, HDAC1 along with PLZF and PML acts as a co-activator and induces ISG production (Ozato 2009, Xu, Holko et al. 2009). Our observations are in agreement, with these studies reinforcing the point that HDAC1 regulates viperin
expression (Figure 20) (Ozato 2009, Xu, Holko et al. 2009). These observations build a hypothesis that IAV potentially downregulates PML-PLZF-HDAC1 interaction to counteract the host antiviral response. However, the direct role of PML-PLZF-HDAC1 interaction and their fate during IAV infection need further investigation. Indeed, a follow-up study should determine whether HDAC1 plays more of a canonical role or a unique role in the IAV-induced host antiviral response. Supporting this, a noticeable decrease in IFITM3, ISG15, and viperin levels in TSA-treated IAV-infected cells shown here indicated that class I and class II HDAC activity is an important component of canonical type I IFN-mediated host antiviral response against IAV. Specifically, the decrease or increase in the IAV-induced expression of viperin in HDAC1-depleted or HDAC1-overexpressing cells, respectively, indicated that HDAC1 is a cofactor for the expression of at least viperin in IAV-infected cells. However, it is unlikely that viperin is the only ISG through which HDAC1 is exerting its anti-IAV function. Direct role of HDAC1 in the expression of other ISGs (IFITM3 and ISG15) that inhibit IAV infection were also studied. In contrast to the observations with viperin, expression of IFITM3 and ISG15 in HDAC1 depleted-IAV infected cells was upregulated. A plausible explanation for this disparity could be that in the absence of HDAC1, potentially HDAC2 or other HDACs try to compensate for their loss. HDACs are a large family of proteins which are known to play a compensatory role. Loss of HDAC1 is partially compensated by an increase in HDAC2 expression and their recruitment at HDAC1 binding sites (Xu, Holko et al. 2009) IFITM3 restricts the virus during the virus uncoating in the cytoplasm whereas ISG15 exhibits its prominent role in the later half of IAV life cycle. The change in the expression of these ISGs with higher MOI could be potentially attributed to their role in mechanism of IAV inhibition. A future follow up study would potentially reveal
some interesting answers to this question. Additionally, loss of both HDAC1 and HDAC2 in embryonic stem cells resulted in about seven-fold increase in HDAC3 expression potentially explaining their compensatory role (Jamaladdin, Kelly et al. 2014). In this study, we observed an increase in HDAC2 in HDAC1 depleted cells and vice versa. (Figure 26). There is the likelihood that either HDAC2 or class I HDAC play a compensatory role in their absence. However, a more plausible explanation is that the expression of these ISGs is reported to be controlled by interferon β and HDAC1 is known to repress interferon β expression (Nusinzon and Horvath 2006, Buggele, Krause et al. 2013).

Therefore, it cannot be excluded that dysregulation of viperin expression in HDAC1 depleted cells could have potentially resulted in a compensatory increase in the expression of other ISGs like IFIMT3 and ISG15. Therefore, in the absence of HDAC1 and its antiviral effector viperin, there could be an increase in interferon β expression, consequently resulting in the increased expression of other ISGs to combat IAV infection (Buggele, Krause et al. 2013). However, a more clear understanding of the regulation of host innate immune response could be revealed by infecting the cell with NS1 deficient (delat-NS1) IAV, as NS1 is mainly reported to antagonize host innate immune response during IAV infection (Fernandez-Sesma, Marukian et al. 2006).

In summary, the data provided in this chapter, demonstrate a prominent role of HDAC1 in exhibiting its anti-IAV function by enhancing the expression of ISGs, notably viperin.
Figure 20. Role of HDAC1 in host antiviral signaling. (A) IRF3 and ISGF3 are recruited along with STAT forming a complex with HDAC1 and bind to the DNA and exert their antiviral response. (B) IAV downregulates the expression of HDAC1 which subsequently is reflected on impaired antiviral response.
2.2 Role of HDAC2 in IAV infection

2.2.1 Introduction

Histone deacetylase 2 (HDAC2) another class I HDAC, was the second member of the HDAC family to be discovered. HDAC2 is ubiquitously expressed in all tissue types, playing a major role in cell differentiation and proliferation (Gonneaud, Turgeon et al. 2016) (Eom and Kook 2015). HDAC2 knockout mice are only partially viable, depending on the allele used for knockout (Eom and Kook 2015). The knockout phenotype is usually associated with severe cardiac defects, leading to hypertrophy (Montgomery, Davis et al. 2007, Haberland, Montgomery et al. 2009). HDAC2 lacks direct DNA binding activity and was first identified in a DNA-binding complex with Ying Yang 1 and polycomb protein (Fesik 2005, Segre and Chiocca 2011). However, all three were later identified in a major co-repressive complex including HDAC1, Sin3, NuRD, CoREST, DNA methylase 1 (DNMT1), and DMAP1 (DNMT1 associated protein) that regulates transcription (Rountree, Bachman et al. 2000, Liang, Wan et al. 2008).

2.2.1a Role of HDAC2 in cellular physiology

Aberrant overexpression of HDAC2 was reported in various cancers including gastric, colon and prostate cancer and is often linked with tumour progression with their distinct role in regulating apoptosis (Ree, Folkvord et al. 2008, Kramer 2009, Jung, Noh et al. 2012, Wagner, Brand et al. 2014). Notably, the non-redundant function of HDAC2 activity was reported in brain development and architecture, regulating perinatal lethality (Hagelkruys, Mattes et al. 2016). HDAC2 associates with the promoter region of spinal motor neuron 2 and negatively regulates spinal motor atrophy (Peart, Smyth et al. 2005, Song, Noh et al. 2005). Interestingly, the

2.2.1b Role of HDAC2 in virus infection

The IRF1 promoter region is exploited by HIV-1 for its replication. Silencing of IRF1 promoter restricted the infection and intriguingly, HDAC2 is specifically involved in IRF1 silencing (Su, Sivro et al. 2011). Similarly, HDAC2 activity had a repressive effect on viral promoters in HCMV infection (Park, Kim et al. 2007). Additionally, a functional interaction of HDAC2 with HDAC1, PML, STAT1 and STAT2 was essential for the expression of ISGs in HCMV infection (Kim and Ahn 2015). Paradoxically, hyper-phosphorylation of HDAC2 by U3 kinase, a highly conserved viral kinase, was reported during alpha herpes virus, pseudorabies virus, and varicella-zoster virus infection, resulting in the regulation of HDAC2 activity, which would otherwise silence the viral genome (Walters, Kinchington et al. 2010).

HDAC2 also exhibits a paradoxical role during acute and latent virus infection. Epigenetic reprogramming of host chromatin mediated by HDAC2 was observed in African swine fever virus infection, resulting in heterochromatin formation and subsequent host gene silencing (Simoes, Rino et al. 2015). In HIV-1 infection, viral protein Tat upregulates the expression of HDAC2 resulting in neurocognitive disorders (Saiyed, Gandhi et al. 2011, Wightman, Lu et al. 2013). In addition, HDAC2 is recruited by COUP-TF interacting protein 2 (CTIP2) at the HIV-1 promoter region to induce local histone H3 deacetylation, inducing HIV-1 latency (Barton,
Archin et al. 2014). Notably, silencing or pharmacological inhibition of HDAC2 resulted in breaking the HIV-1 latency, suggesting their potential role in anti-latency therapy (Barton, Archin et al. 2014). Contrastingly, HDAC2 activity was upregulated and exploited by coxsackievirus B3, which is the leading cause of viral myocarditis (Shim, Park et al. 2013). Intriguingly, HDAC2 has been employed by Blimp-1 (a transcription factor) to regulate the virus-specific accumulation of CD8+T cells during acute lymphocytic choriomeningitis virus infection ((Shin, Kapoor et al. 2013)). In herpes simplex virus 1 (HSV-1) infection, ICP8, a single-stranded DNA binding viral protein, interacted with HDAC2 inducing epigenetic silencing and subsequently promoting the latency (Taylor and Knipe 2004). In a similar fashion, HDAC2 is reported to interact with K-bZIP, a Kaposi’s sarcoma-associated herpes virus protein and exhibit a repressive effect on lytic gene promoters (ORF50 and OriLyt), therefore inducing latency (Martinez and Tang 2012).

Like HDAC1, HDAC2 has shown to be a key regulator of various physiological and pathological diseases, yet its role in IAV infection remains elusive. Therefore, the aim was to determine the role of HDAC2 in IAV infection by following the similar experimental plan and design as that was used for HDAC1.
2.2.1c Aim of this study

To determine if HDAC2 has a role during IAV infection.

2.2.1d Objectives achieved

- Determined HDAC2 mRNA and protein expression in response to IAV infection.
- Studied the IAV growth kinetics in HDAC2 depleted cells.
- Investigated the underlying molecular mechanism of HDAC2 as an anti-IAV host factor.
2.2.2 RESULTS

2.2.2.a HDAC2 mRNA level is downregulated in response to IAV infection

As shown in Chapter 2, compared to PR8 or NC strain, infection with WSN strain showed a more profound effect on the downregulation of HDAC1 polypeptide infected cells (Figure 7.A). Therefore, infection with WSN at 0.5 MOI was chosen for HDAC2 study, however, 5.0 MOI was also used. To determine whether HDAC2 is involved in IAV infection, A549 cells were infected with 0.5 MOI of WSN. At 24h post-infection, the expression of HDAC2 mRNA was determined in uninfected and infected cells by quantitative real-time PCR. Unlike HDAC1, HDAC2 mRNA was considerably downregulated in infected cells. Compared to the uninfected cells, there was a significant 54% \((P < 0.001)\) reduction in HDAC2 mRNA level in WSN-infected cells (Figure 21.A). Furthermore, HDAC2 mRNA downregulation by IAV was dose dependent and was more profound in the cells infected with 5 MOI of WSN (Figure 21.C). The increase in viperin expression in corresponding experiments served as an infection marker (Figure 21.B and D).

2.2.2.b IAV induces downregulation of HDAC2 polypeptide in a strain-independent manner and requires a replication competent virus

After detecting a significant reduction in HDAC2 mRNA level in infected cells, we next determined whether IAV also downregulates the HDAC2 polypeptide level. To accomplish this, A549 cells were infected with WSN at an MOI of 0.5 and cells were harvested after 0h, 6h, 12h and 24h of infection. As compared to 0h, there was no change in HDAC2 polypeptide level at 6h, and a non-significant 35% decrease at 12h. However, there was a significant 48% \((p < 0.0081)\) decrease in HDAC2 polypeptide level at 24h of infection when compared with 0h (Figure 22.A). Similar
results were obtained when the cells were infected with a higher MOI (5MOI) (Figure 22.B). To assess if the downregulation of HDAC2 was IAV strain dependent or independent, A549 cells were infected with either PR8 or NC strain for 24h. Similar to WSN, infection with PR8 and NC infection also resulted in HDAC2 polypeptide downregulation. Notably, at 5 MOI, there was a profound HDAC2 polypeptide downregulation for both strains (Figure 22.C-D). This observation confirmed that IAV dysregulates HDAC2 polypeptide expression in a H1N1 strain-independent manner (Figure 22.A-D). Finally, by using the live and UV-inactivated WSN strain, it was confirmed that a replication-competent IAV was required to downregulate HDAC2 expression (Figure 23.A).

Unlike HDAC1, there was almost a linear relationship between the downregulation of HDAC2 mRNA (about 54%) and polypeptide (about 48%). Nevertheless, we wanted to assess if HDAC2 polypeptide undergoes caspase 3 mediated or proteasomal or lysosomal degradation in IAV infected cells. To identify the HDAC2 degradation pathway in IAV infected cells, A549 cells were infected with 0.5 MOI of WSN and subsequently treated with either caspase 3 inhibitor (40 µM) or proteasome inhibitor MG132 (10 µM) or lysosome inhibitor NH₄Cl (20 mM). After 24h of infection, cells were lysed and processed for WB to detect HDAC2 expression. Neither caspase 3 inhibitor (data not shown) or MG132 or NH₄Cl treatment restored the HDAC2 polypeptide levels in infected cells, suggesting that IAV dysregulate HDAC2 at mRNA level. (Figure 23.B). The potency of the proteasomal inhibitor was confirmed by increased expression of ubiquitin in MG132 treated cells (Figure 23.C).
Figure 21. IAV dysregulates HDAC2 transcript level. A549 cells were infected with either (A) 0.5 MOI or (C)* 1, 3 and 5 MOI of WSN. After 24 hours of infection, qPCR was performed to determine either the relative HDAC2 mRNA level using β-actin as internal control. (B and D*) Viperin expression was used as an infection marker. Each bar represents means ± standard errors of the means of three independent experiments. *Experiment was performed in technical duplicates.
Figure 22. IAV induces downregulation of HDAC2 polypeptide. (A) A549 cells were infected with either (A) WSN (0.5 MOI) or (C) NC or (D) PR8 (0.5 or 5 MOI) and cells were harvested at indicated time points followed by WB. (B) Quantification of HDAC2 protein expression using Image studio version 4.0, LI-COR. Actin was used as a loading control. The signal from HDAC1 protein band was normalized with actin signal and was considered as 100% increase in cells at 0h of infection for comparison with infected cells at different time points. Data are presented as means ± standard errors of the means from three independent experiments. P value was calculated using one-way ANOVA followed by Dunnett’s multiple comparison test.
Figure 23. A replication competent virus is required for HDAC2 downregulation. A549 cells were infected with (A) UV-treated or untreated WSN (0.5 MOI) and cells were after 24 hours of infection followed by WB. (B) A549 cells were infected with WSN and subsequently treated with NH₄Cl (20mM) or MG132 (10µM) for 24 h. Total cell lysates were prepared, and HDAC2, Actin, and NP were detected in UNI and INF cell lysates by WB. (C) Ubiquitin bands were detected to confirm the potency of MG132 treatment on cells.
2.2.2.c Endogenous HDAC2 possess anti-IAV property

The above data indicated that, as for HDAC1, IAV also downregulated the expression of HDAC2, albeit, by a different mechanism. Nevertheless, this indicated that HDAC2 has a potential anti-IAV function. Therefore, the IAV infection kinetics in A549 cells were analysed by depleting the expression of HDAC2 using RNA interference. Firstly, siRNA concentration was optimized to obtain a significant knockdown of HDAC2 expression. Different concentrations of siRNA (10, 25, 50, and 100nM) targeting HDAC2 and a non-targeting siRNA control (50nM) were delivered to A549 cells. After 72h, the expression of HDAC2 was determined by WB. As low as 10nM siRNA efficiently depleted (about 90%) the HDAC2 expression (Figure 24.A), with negligible effect on cell viability (about 10% cell death as compared to control) as estimated by MTT assay (Figure 24.B). Henceforth, 10nM siRNA was used for HDAC2 knockdown.

To determine IAV growth kinetics in the absence of HDAC2, A549 cells were transfected with control or HDAC2 siRNA for 72h and subsequently infected with WSN at an MOI of 0.5. After 2h, 6h, 12h, and 24h of infection, culture medium, and cells were harvested separately. The culture media was used to determine the virus titre by plaque assay, whereas the cells were processed for WB. Knockdown of HDAC2 expression was confirmed by WB (Figure 24.C). Consistent with HDAC2 downregulation, plaque assay revealed significantly higher virus titre in HDAC2 depleted cells as compared to the cells transfected with control siRNA. Cells transfected with HDAC2 targeting siRNA released an insignificant 2.6 fold more ($p$-value – 0.905) and a significant 4.1 fold ($p$-value < 0.0001) more infectious virions after 12h and 24h of infection, respectively, as compared to the cells transfected with non-targeting control siRNA (Figure 24.D). Taken together, this data indicate that
endogenous HDAC2 possesses anti-IAV properties and IAV growth is enhanced in the absence of HDAC2.
Figure 24. Endogenous HDAC2 possess anti-IAV property. (A) A549 cells were transfected with indicated concentrations of non-targeting control (CT) siRNA or HDAC2-targeting (HD2) siRNA for 72 h. Total cell lysates were prepared and HDAC2 and Actin were detected by WB. (B) Cell viability was then determined using MTT assay. (C) A549 cells were transfected with 1nM of CT siRNA or HD2 siRNA for 72 h. Cells were then infected with WSN at an MOI of 0.5 and the culture medium and the cells were harvested separately after 2h, 6h, 12h, and 24h of infection. Total lysates of the cells were prepared, and HDAC2, Actin, and NP were detected by WB. Infectious virion yield in the culture medium was determined by (D) microplaque assay. Data presented are means ± standard errors of the means of three independent experiments; P value was calculated using two-way ANOVA test. Data presented are means ± standard errors of the means of three independent experiments; P values were calculated using one-way ANOVA test. MW, molecular weights.
2.2.2.d HDAC2 positively regulates STAT1 signaling and exhibit a differential role in the expression of ISGs

After discovering that HDAC2 is an anti-IAV host factor, the next step was to understand the molecular mechanism of anti-IAV function of HDAC2. As discussed in the HDAC1 Chapter, the host innate immune system responds by regulating STAT1 and ISGs (Uetani, Hiroi et al. 2008, Mauffre, Grimard et al. 2016). Further, STAT1 is phosphorylated and translocated to the nucleus where it is involved in transcription of ISGs and interferons (Uetani, Hiroi et al. 2008). However, IAV antagonizes the host response by inhibiting STAT1 phosphorylation and subsequent downstream events, which involve interferon and ISG production (Uetani, Hiroi et al. 2008). Therefore, to get an insight into the anti-IAV mechanism of HDAC2, the phosphorylation of STAT1 in HDAC2-depleted cells was investigated. To accomplish this, A549 cells were depleted of HDAC2 expression using RNA interference as above. Subsequently, these cells were infected with WSN at an MOI of 0.5 and harvested at 0h, 6h, 12h, and 24h of infection. Intriguingly, unlike HDAC1, STAT1 phosphorylation was dysregulated in HDAC2-depleted-infected cells. Notably, at 6h and 12h post infection, there was a moderate and consistent decrease in pSTAT1 level in HDAC2-depleted infected cells, as compared to the cells transfected with control siRNA, indicating HDAC2’s role in STAT1 signaling (Figure 25.A-B). Two independent replicates are presented in figure 25.A-B to show the moderate effect on HDAC1 downregulation. IAV replication is reported (and as shown in Figure 9.E and 24.D.) to be at its peak between 6h and 12h of infection and then gradually reduces (Pauli, Schmolke et al. 2008, Uetani, Hiroi et al. 2008). This explains the possible reason for no alteration in pSTAT1 expression at 24h of infection. Of note, total STAT1 was cleaved and had a distinct cleavage product at 24h both in control
and HDAC2-depleted cells (Figure 25.A-B). In addition, this data supports the result showing that class I HDAC activity is involved in STAT1 signaling (Klampfer, Huang et al. 2004). This would appear to be the first report showing a role of HDAC2 in STAT1 signaling during IAV infection. Since STAT1 signaling is involved in ISG expression, whether dysregulated STAT1 signaling in HDAC2-depleted cells influences ISG expression was determined. A549 cells were depleted of HDAC2 expression as described earlier, and WB analysis was performed to analyze ISG expression. Consistent with HDAC1 data, viperin expression was decreased, whereas IFITM3 expression was not altered at 12h of infection in HDAC2-depleted cells (Figure 25.C). It is interesting to note that, among the large subset of ISGs that fight against virus infection, the decrease in one ISG is potentially compensated by other ISG as demonstrated in this study. In summary, IAV undermines HDAC2 expression to attenuate host innate antiviral immune response mediated by STAT1 signaling.
Figure 25. HDAC2 is involved in STAT1 phosphorylation and viperin expression. A549 cells were transfected with non-targeting control (CT) siRNA or HDAC2-targeting (HD2) siRNA for 72 h. Cells were then infected with WSN at an MOI of 0.5 for 24 h. (A-B) Total cell lysates were prepared, and pSTAT1 (Y701), STAT1. Inset A' depicts the pSTAT1 expression in CT and HD2 depleted cells at 12h post infection with quantitation from three biological repeats. (C) Viperin, IFITM3, actin, and NP were detected in uninfected (UNI) and infected (INF) cell lysates by WB. Inset C' represents viperin quantitation at 12h post infection from three biological repeats. (A-B) Represents two independent experimental repeats.
2.2.3 Discussion

In this study, we have demonstrated the IAV-mediated downregulation of HDAC2 expression to undermine its antiviral activity. Further, a linear correlation was observed between HDAC2 mRNA and polypeptide downregulation. However, the mechanism of HDAC2 regulation by IAV at the mRNA level still remains to be investigated. A miRNA profiling in IAV infected cells by Buggele et al revealed the upregulation of miR-145 (Buggele, Krause et al. 2013), and interestingly, miR-145 mediated regulation of HDAC2 was observed in hepatocellular carcinoma (Noh, Chang et al. 2013). In addition, miR-233 (Leuenberger, Schuoler et al. 2016), miR-155 (He, Zhu et al. 2016) are reported to silence HDAC2 mRNA under various pathological conditions. Although, miRNA-mediated regulation of HDAC2 mRNA has been reported under various pathophysiological conditions, yet their direct role in IAV infection remains to be addressed. Therefore, miR-145 mediated dysregulation of HDAC2 mRNA could be a plausible strategy adopted by IAV to produce less HDAC2 protein, which can be investigated in future studies.

Further, we also confirmed HDAC2 polypeptide downregulation using different strains of IAV including a clinically-relevant strain. Though ubiquitin-mediated degradation of HDAC2 specifically by E2 ubiquitin conjugase UBCH8 is plausible (Kramer, Zhu et al. 2003), the data presented here suggests that proteasomal pathway has potentially no effect on HDAC2 polypeptide restoration in IAV infection. Nevertheless, it will be worthwhile to investigate if HDAC2 is ubiquitinated in IAV infected cells.

The data on IAV growth kinetics showed a significant 4-fold increase in IAV release in HDAC2 depleted cells. Although there was no profound difference in IAV growth
kinetics between HDAC2 (4-fold increase) and HDAC1 depleted cells (3.2 fold increase), HDAC2 depletion had modestly higher effect on IAV release. This difference could be attributed to a specific and distinct role of HDAC2 in IAV infection. Previously, a specific role for HDAC2 was reported, wherein silencing of HDAC2, but not HDAC1 enhanced acetylation in the hippocampal region of the brain, regulating memory and brain development (Kramer 2009, Graff and Tsai 2013, Hagelkruys, Mattes et al. 2016, He, Zhu et al. 2016). This supports the argument that HDAC2 potentially plays a unique role to HDAC1 in IAV infection. Furthermore, there was no comparable difference in the expression of intracellular NP in the HDAC2 depleted cells as compared to the control. NP is one of the most abundantly expressed IAV proteins, therefore, it is hard to discern the change in NP level by WB. Analysing the level of other viral proteins (like NA1, M1, and others) may reveal better results.

Additionally, as discussed earlier, HDAC1 and HDAC2 are closely related members and are reported to have compensatory roles (Lagger, O’Carroll et al. 2002). Indeed, evidence from previous studies suggests that deletion of either HDAC1 or HDAC2 alone had no effect on infertility in developing mouse oocytes (Ma, Pan et al. 2012). However, a combined depletion of both the genes resulted in infertility, suggesting the complementary role of these two enzymes in the absence of the other. The data presented indicates that both HDAC1 and HDAC2 are downregulated in IAV infection. However, it is interesting to note the increase in expression of HDAC1 in HDAC2 depleted-infected cells and vice versa (Figure 26). In figure 26, HDAC1 and HDAC2 knockdowns were performed as two independent experiments, but the proteins samples were run alongside in the same gel. The inconsistency in the expression of HDAC1 and HDAC2 between these experiments could be attributed to
the technical variation. However, a key observation here is that both HDAC1 and HDAC2 compensate each other’s expression, but IAV still downregulates their compensatory expression. Whether this increase is a direct correlation of the activity of these HDACs remains to be investigated. One could argue that in a knockdown, an increase in the expression of HDAC1 or HDAC2 might have a corresponding increase in their activity which could potentially mask the independent role of HDAC2 depletion in IAV infection. Therefore, in order to rule out the compensatory role of other HDACs and to identify the independent role of HDAC2 in IAV infection, infection of HDAC2-depleted cells in the presence of HDAC inhibitor, TSA could be a potential strategy for future studies.
Figure 26. Expression of HDAC1 and HDAC2 in the absence of the other. A549 cells were transfected with non-targeting control (CT) siRNA or HDAC2-targeting (HD2) siRNA for 72 h. Cells were then infected with WSN at an MOI of 0.5 and the cells were harvested separately after 24h of infection. Total lysates of the cells were prepared, and HDAC1, HDAC2, Actin, and NP were detected by WB.
HDAC2 has been shown to be associated with HDAC3 during breast cancer and melanoma regulating tumour development (Ye, Jin et al. 2013, Ohya, Kanatsuka et al. 2016). Notably, combined ablation of HDAC2 and HDAC3 further aggravated tumour progression (Ohya, Kanatsuka et al. 2016). Therefore, the possible compensatory role of HDAC1 or HDAC3 in the absence of HDAC2 cannot be ruled out. Hence, it will be interesting to examine the expression and activity of HDAC1 and HDAC3 in HDAC2 depleted-IAV infected cells. A further investigation to determine the role of HDAC2 in conjunction with HDAC1 and HDAC1/2/3 triple knockdown would result in a clear understanding of the role played by these HDACs.

Of note, the kinetics of HDAC2 activity in IAV infection still remains to be ascertained in IAV infection. The acetylation status of histone 4 lysine 16 (H4K16) was more prominent in the absence of HDAC2, indicating H4K16 as a specific substrate of HDAC2 (Ma and Schultz 2013). Thus, determining the acetylation status of H4K16 in infected cells would possibly explain the kinetics of HDAC2 activity during IAV infection. Additionally, understanding the role of co-repressor complexes associated with HDAC2 and the fate of their catalytic domains during IAV infection needs further investigation. Furthermore, it would be interesting to investigate the IAV protein, which is involved in HDAC2 downregulation in infected cells. Expressing the individual segments of IAV, and subsequently studying the expression of HDAC2 would potentially help to identify the viral component involved in dysregulating HDAC2.

In the context of host immune response, the STAT1 pathway is a paradigm for cytokine-induced signaling. Canonically, upon cytokine stimulation, Janus-kinase (JAK) activates STAT proteins by phosphorylating at serine/tyrosine residues (Bonni, Sun et al. 1997). However, STAT1 signaling is regulated by various events including
HDACs/HATs activity, sumoylation, and phosphorylation (Kramer, Knauer et al. 2009).

Notably, activation of STAT1 signaling by phosphorylation results in nuclear localization of STAT1 and interacts with their binding partners ISGF3/IRF3/IRF7. This complex further binds to the ISRE sequence of ISGs and transcribes antiviral genes (Cho, Lee et al. 2008). Once translocated to the nucleus, STAT1 is targeted for acetylation by CBP, a histone acetyltransferase, which acetylates STAT1. This acetylation event triggers T-cell protein tyrosine phosphatase 45 mediated dephosphorylation of STAT1 (Figure 27.A) (Kramer, Knauer et al. 2009). In line with this, evidence from previous studies report that HDAC2 activity is essential for STAT1 signaling (Klampfer, Huang et al. 2004). The observations from this study suggested that STAT1 signaling was downregulated in HDAC2-depleted IAV-infected cells (see Figure 25). Based on this evidence, we propose that HDAC2 deacetylates pSTAT1, thereby inhibiting dephosphorylation of pSTAT1, supporting STAT1 mediated antiviral gene transcription (Figure 27.B). Supporting this view, in HCMV infection, HDAC2 associates with PML protein and forms a complex with STAT1/STAT2 and transcribe ISGs. This antiviral activity of PML-STAT-HDAC complex is silenced by IE1, a HCMV protein, (Kim and Ahn 2015), further indicating the role of HDAC2 as a potential cofactor to induce ISG expression. It would be interesting to investigate the IRF3 signaling and type I interferon production in HDAC2 depleted cells and identify if the dysregulated STAT1 signaling is contributed by a decrease in interferon production using qPCR.
Figure 27. HDAC2 regulates acetylation of pSTAT1 and mediate host antiviral response. (A) In the absence of HDAC2 deacetylase activity, acetylation of pSTAT1 mediated by p300 is enhanced which recruits TCP45 and dephosphorylates pSTAT1, resulting in an impaired antiviral response. (B) In contrast, the presence of HDAC2 deacetylates pSTAT1 and mediates host antiviral response. Ac- acetylation, P- phosphorylation.
Tying together, these observations suggest that yet another member of class I HDACs, HDAC2, exhibit an anti-IAV function by regulating STAT1 signaling and viperin expression.

2.2.3.a IAV dissecting the role of HDAC1 and HDAC2 to antagonize the host antiviral innate immune response

Both, HDAC1 and HDAC2 are regulated at the mRNA and polypeptide level. Further, their knockdown resulted in enhanced IAV growth kinetics, and they independently regulated the expression of viperin. Similarly, the knockdown of both HDAC1 and HDAC2 resulted in an increase in the expression of IFITM3 (Figure 8).

While the phenotypes of HDAC1 and HDAC2 in IAV infection are very similar, they also have contrasting and independent roles. The data from this study indicate a non-linear relationship between the downregulation of HDAC1 mRNA and polypeptide level in IAV-infected cells, and IAV targeting HDAC1 for proteasomal degradation (Figure 5.A, 6.A, 8.C-D). Contrastingly, there is a direct linear correlation between the downregulation of HDAC2 mRNA and polypeptide level in IAV-infected cells (Figure 21.A and 22.A). Further, IAV growth kinetics in HDAC2 depleted cells was marginally higher than HDAC1 depleted cells (Figure 8D and 25E). Most significantly, HDAC2 depletion impaired STAT1 signaling (Figure 25.A) whereas HDAC1 depletion had no effect on it (Figure 17A). Taken together, IAV discriminates the role of HDAC1 and HDAC2, and therefore, regulates them independently for enhanced virus infection by suppressing the host immune barricade (Table 5).
Table 5. Dissecting the role of HDAC1 and HDAC2 in the IAV infection. The similarities and differences in the role of HDAC1 and HDAC2 in IAV infection.

<table>
<thead>
<tr>
<th>IAV Infected cells</th>
<th>HDAC1</th>
<th>HDAC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA expression downregulated</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Polypeptide expression downregulated</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Polypeptide degradation</td>
<td>Yes, Proteasomal pathway</td>
<td>No, Linear relation b/w mRNA and Polypeptide</td>
</tr>
<tr>
<td>Enzyme activity downregulated</td>
<td>Not determined</td>
<td></td>
</tr>
<tr>
<td>Knockdown</td>
<td>Increase in IAV growth kinetics</td>
<td>Increase in IAV growth kinetics</td>
</tr>
<tr>
<td>Overexpression</td>
<td>Inhibition in IAV infection</td>
<td>Not determined</td>
</tr>
<tr>
<td>STAT1 signaling</td>
<td>No effect</td>
<td>Downregulated</td>
</tr>
<tr>
<td>ISGs regulated</td>
<td>Viperin, IFITM3</td>
<td>Viperin, IFITM3</td>
</tr>
</tbody>
</table>
CHAPTER 3: CONCLUSION AND FUTURE PERSPECTIVES
3.1 CONCLUSION

This research is the first evidence demonstrating the anti-viral role of host HDAC1 and HDAC2 in IAV infection. This study elucidated the molecular interplay between IAV and HDAC1/HDAC2. The expression and activity of HDAC1 were regulated by IAV. It is noteworthy, that IAV has multiple checkpoints to regulate HDAC1 expression. This is supported by the results showing IAV mediated regulation of HDAC1 expression at the mRNA level, and further targeting HDAC1 for proteasomal degradation at polypeptide level. Contrastingly, HDAC2 was regulated at mRNA level, as evident with the linear relation between the mRNA and protein expression. Knockdown of HDAC1 and HDAC2 and overexpression of HDAC1 revealed an antiviral role of these proteins as there was a significant increase or decrease in the virus release, respectively. Further studies into understanding the mechanistic implication of these proteins revealed their crucial role in regulating the host innate antiviral response. Inhibition of their enzyme activity using TSA, downregulated STAT1 signaling and ISG expression, indicating the role of HDACs in the innate immune pathway. Specifically, knockdown or overexpression of HDAC1 either enhanced or suppressed viperin expression during infection, respectively.

Intriguingly, HDAC2 depleted cells suggested a modest and consistent decrease in pSTAT1 expression and subsequently regulated viperin expression. Though HDAC1 and HDAC2 are very closely related proteins, they seem to work in an independent manner in the same network of the innate immune pathway. Thus, these findings unravel the novel role of host HDAC1 and HDAC2 in IAV infection and contribute to understanding the role of HDAC family proteins.
In summary, this project was initiated to understand the role of HDAC1 and HDAC2 in IAV infection and was successful in discovering their roles as anti-IAV host factors. Constant emergence of drug-resistant strains of IAV, and the lack of a universal vaccine emphasize the need to develop novel anti-IAV strategies. In this regard, a molecular understanding of IAV and HDACs interplay may lead to the development of such a strategy. These findings will contribute to the molecular understanding of the interplay between IAV and HDACs, especially HDAC1 and HDAC2 (Figure 28).

3.2 FUTURE PERSPECTIVES

This study has uncovered the role of HDAC1 and HDAC2 in regulating the antiviral host innate immune response against IAV virus. The virus manipulates these two proteins to circumvent the host innate immune response, thereby establishing a successful infection. The data from this study provide significant information elucidating the antiviral role of HDAC1 and HDAC2 in IAV infection, albeit, a complete understanding of their role is far from being complete.

The proteasomal degradation of HDAC1 polypeptide during IAV infection is a novel observation, however, the ubiquitination status of HDAC1 in IAV infected cells still remains to be investigated. Immunoprecipitation of HDAC1 from IAV infected cells and subsequently staining them with ubiquitin antibody would possibly address this question.
Figure 28. An overview of this project. An inverted pyramid depicting the broader aims at the start of the project and the final conclusion arrived at the end, by deciphering the role of HDAC1 and HDAC2 as anti-IAV host factors.
The expression and activity of HDAC1 seemed to follow different kinetics. HDAC1 polypeptide was downregulated over the period of infection, starting from 6h. Contrastingly, HDAC1 activity reached its peak at 6h and 12h of infection. Unlike the kinetics of polypeptide downregulation, the activity was restored to normal at 24h of infection. Studying the activity between 12h and 24h of infection would give some clue to determine the point where the expression and activity diverge.

Further, the mechanism of dysregulation of HDAC1 during IAV infection activity still remains to be investigated. HDAC1 has the catalytic domain at the C-terminus region of the polypeptide. By constructing deletion mutants in the catalytic domain of HDAC1, the importance of their activity can be understood.

Having demonstrated the antiviral mechanism of HDAC1, it would be tempting to further understand the role of HDAC1 in enhancing viperin expression during IAV infection. It is possible that HDAC1 would act as a component of the innate immune response against IAV. The type I interferons (IFNs) represent the first line of host defense against virus infection (Platanias 2005). The cytoplasmic pattern recognition receptors in host cell sense the incoming virions and trigger the phosphorylation and subsequent nuclear translocation of interferon regulatory factor 3 (IRF3) (Platanias 2005). In the nucleus, IRF3 binds to the IFN-β promoter and mediates the expression of type I IFNs, which are secreted from the host cell (Platanias 2005). The secreted IFNs then engage the type I IFN receptor in an autocrine and paracrine manner and activate the cytoplasmic Janus kinases (JAKs). The JAKs subsequently phosphorylates STAT1, which then translocate to the nucleus, bind to STAT2 and IRF9 and forms the interferon-stimulated gene factor 3 (ISGF3) complex. The ISGF3 complex then induces the expression of hundreds of interferon-stimulated genes (ISGs) that prevent viral replication by targeting various steps of the viral lifecycle.
However, viruses have evolved their own strategies to antagonize the ISG-mediated immune response (Zundler and Neurath 2016). Recently, it has been shown that HDAC1 expression is important for the phosphorylation of IRF3 and induction of type I IFN response following the herpesvirus infection (Mounce, Mboko et al. 2014). Similarly, we believe that HDAC1 is playing an identical role following the IAV infection and IAV dysregulates the HDAC1 to undermine its role in this pathway. Supporting this hypothesis, the data presented here suggest that the phosphorylation of IRF3 and interferon production was downregulated in HDAC1 depleted cells, indicating an antiviral role of HDAC1. Therefore, it would be interesting to determine the type-I interferon response by qPCR or ELISA. Further, chromatin immunoprecipitation assay can be employed to identify if HDAC1 or HDAC2 directly bind to the promoter region of interferon α and support interferon synthesis.

Like HDAC1, the role of HDAC2 as an anti-IAV host factor has been demonstrated here. In this regards, looking into the mechanistic implications of the antiviral role of HDAC2 suggest their potential involvement in regulating NF-kB pathway. Accumulating evidence suggest that the NF-kB pathway is a pre-requisite for IAV infection (Nimmerjahn, Dudziak et al. 2004, Ludwig and Planz 2008). Aberrant activation of this pathway, result in a virus-induced ‘cytokine storm’ which enhances infection by inducing apoptosis (D’Elia, Harrison et al. 2013). Historically, NF-kB was identified in 1986, playing a central role in inflammatory and immune response (Sen and Baltimore 1986, Singh, Sen et al. 1986). NF-kB is predominantly comprised of five members, RelA (p65), RelB, cRel, NF-kB1 (p50 and its precursor p105) and NF-kB2 (p100 and its precursor p52). There are two main pathways of NF-kB activation; (i) canonical pathway induced by TNF-α, IL-1, and (ii) alternative pathway induced
by a variety of cytokines. RelA and p50 subunits are predominantly found in the inflammatory pathway. Upon, influenza infection, NF-kB pathway is activated, resulting in a ‘cytokine storm’ (Liu, Zhou et al. 2016) which results in apoptosis enhancing infection. When this pathway is triggered, the p65 subunit of NF-kB is deacetylated which increases its interaction with IKK. The P65-IKK complex is then exported to the nucleus from the cytoplasm. This deacetylase activity is predominantly contributed by HDACs. However, a direct link between HDAC2 activity and NF-kB deacetylation will provide more insights into the anti-viral role of HDAC2 (Figure 29).

Further in support of this hypothesis, a direct correlation between the downregulation of HDAC2 expression and activity with a corresponding increase in the NF-kB-mediated inflammatory response was observed in COPD patients. This indicates the direct role of HDAC2 in regulating NF-kB activity. The current study demonstrated the prominent role of HDAC1 and HDAC2 in inhibiting IAV infection, therefore it is tempting to investigate IAV growth kinetics in the absence of both HDAC1 and HDAC2. Although the double knockdown might be lethal to the cells, they would at least help to understand the phenotype of the disease with respect to these genes. The absence of HDAC1 is compensated by an increase in HDAC2 and vice versa. However, where this increase is just the observed phenotype or has any functional compensatory role in IAV infection remains to be investigated.

This study revealed the role of host HDAC1 and HDAC2 in regulating the host immune response. NS1 protein of IAV is well known to antagonize host cellular process including, host RNA synthesis, apoptosis, antiviral response and research is still underway to understand the comprehensive regulatory role of NS1 in various cellular pathways (Hale, Randall et al. 2008, Engel 2013). It has been well
established that NS1 of IAV antagonize the host immune response either by targeting the mRNA/protein for degradation or by binding to the antiviral factors and restricting their antiviral function (Kochs, Garcia-Sastre et al. 2007, Haye, Burmakina et al. 2009). Henceforth, it will be interesting to study the role of HDAC1/HDAC2 in regulating the host innate antiviral response in delta-NS1 (NS1 deleted) IAV infected cells.

This study demonstrated the anti-IAV role of host HDAC1 and HDAC2 in regulating the host innate immune using an in vitro model. It would be interesting to further study the role of these two host factors using an animal model. A variety of animal models are employed to study IAV infection, including, mice, ferrets, guinea pigs, Syrian hamsters, Rhesus macaque (Matsuoka, Lamirande et al. 2009, Bouvier and Lowen 2010). Ferrets are considered the ideal model system as they mimic the human infection (Belser, Katz et al. 2011). However, considering the breeding and animal husbandry constraints, mice models are preferred for influenza virus infection. Knockout mice models are generally used to study the role of a particular host factor in regulating virus infection. Unfortunately, HDAC1/HDAC2 knockout mice are not viable, but conditional knockout mice developed using Cre-Lox system specifically targeting the deletion of HDAC1/HDAC2 in the upper respiratory tract could be employed (Bayer and Wirth 2017).
Figure 29. HDAC2 mediated regulation of NF-κB. IAV mediated stimulation of innate immune pathway resulting in the activation of Nk-κB. Viral stimulus targets IkB for proteasomal degradation and results in the nuclear enrichment of p65/p50 and, subsequently acetylation of p65 by p300/CBP is facilitated for transcribing proinflammatory genes. However, in the presence of HDAC2, p65 is deacetylated and is recycled back to the cytoplasm binding to the IkB leading to their inactivation. Ac-acetylation, PKC-Phosphokinase C, IkB- an inhibitor of kB, p300- histone acetyltrasnsferase, p65, and p50-subunits of NF-kB., NLS- nuclear localization signal.
3.3 HDACs: at the crossroads of antiviral therapy

The paradoxical role of HDACs in virus infection has attracted considerable attention as potential antiviral targets. These PhD findings demonstrate the antiviral role of HDAC1 and HDAC2 in IAV infection. These enzymes play a crucial role in regulating innate and adaptive immune pathways by acting as co-factors and promote gene expression and thus have been attractive drug targets against various cancers, autoimmune and neurological diseases. The enzymatic activities of HDACs are essential for driving the innate and adaptive immune response. Notably, Class I, II and IV HDACs are zinc-dependent for their activity. Remarkably, individuals with zinc deficiency or low zinc intake in their diet are more prone to infection with influenza A H1N1 virus (Sandstead and Prasad 2010). These evidence suggest a correlation between the roles of these HDACs in regulating host immune response. Henceforth, a considerable understanding of mechanistic role of HDAC1/HDAC2 in regulating the host innate immune response against IAV will enable us to employ them for fighting IAV infection in the future.
CHAPTER 4: MATERIALS AND METHODS
4.1 Cells and viruses

Human alveolar epithelial cells, A549 and MDCK (Madin Darby Canine Kidney) cells (kindly provided by Kevin Harrod, Lovelace Respiratory Research Institute, Albuquerque, USA). Influenza virus lab adapted strains, PR8 and NC (Infect Drug Resist) (kindly provided by Kevin Harrod, Lovelace Respiratory Research Institute), WSN (kindly provided by Richard Webby at St Jude Children’s Research Hospital) were used for determining the HDAC1 enzyme kinetics.

4.2 Cell culture

For regular maintenance, cells were grown in complete minimum essential medium (Thermo Fisher Scientific) supplemented with 10% foetal bovine serum (Invitrogen), glutamine and antibiotic mixture (penicillin + streptomycin) (Invitrogen) at 37°C under 5% CO₂ atmosphere. For sub-culturing, the medium was removed, cells were washed twice with phosphate buffered saline (PBS) (ThermoFisher Scientific) and then incubated with trypsin-EDTA (ThermoFisher 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.

4.3 Virus propagation

Influenza viruses were propagated in 10-day old embryonated chicken eggs and titrated on MDCK cells by plaque assay (described below). Briefly, 3-4-day old fertilized chicken eggs were incubated at 35°C. The eggs were candled on the first day before incubation and then candled every second day to check the development and viability of the embryo. Ten days later, eggs were candled again to mark the air sac cavity. 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. One hundred microliter of virus inoculum containing 1000 PFU was inoculated into eggs. The
punctured area was then sealed off with tape and the eggs were incubated at 35°C for 48h. The embryos were then sacrificed by placing the eggs overnight in a cold room. The next day, tape was removed and the egg shell was half peeled using a sterile forceps, after which the chorio-allantoic membrane was carefully removed. A broad mouthed spatula was then used to gently push the embryo towards one side in order to harvest the allantoic fluid, which contains the virus. Care was taken not to disrupt the yolk sac or the blood vessels which will contaminate the allantoic fluid. Virus titre of the harvested samples was determined by performing the microplaque assay on MDCK cells (as described below).

4.4 Infection

Cell monolayers were washed twice with serum free (SF) medium. The volume of virus stock required for appropriate MOI was calculated and added to 500µl of the SF media (Thermo Fisher Scientific). About 500µl of this infection inoculum was added to cell monolayers for infection and incubated at 35°C for one hour. After the incubation period, the infection inoculum was removed and 1 ml of SF medium was added. In the case of MDCK cells, the SF media was supplemented with TPCK-trypsin (1µ/ml) (Sigma-Aldrich) for aiding the virus to continue with multiple infection cycles. The medium changed after one hour of virus inoculum incubation was supplemented with the desired concentration of drugs, appropriately. Trichostatin A (TSA) (Sigma-Aldrich) was used for inhibiting HDAC activity, MG132 (Calbiochem) and NH₄Cl (Sigma-Aldrich) were used for inhibiting proteasomal and lysosomal pathways, respectively.
4.5 UV irradiation of virus

In order to make the virus replication deficient, virus inoculum was irradiated with 1-2mJ of UV for 5 minutes prior to infection. UV treatment results in the inactivation of the genetic material of the virus, and hence become replication incompetent (Kim, Latham et al. 2002, He, Xu et al. 2010).

4.6 Microplaque assay

The culture medium from infected cells was harvested, cleared off cell-debris by low-speed centrifugation, and divided into two parts. One part was subjected to protein precipitation by trichloroacetic acid (TCA) (Calbiochem), whereas the other part was mixed with 0.3 % BSA (Sigma-Aldrich) and titrated on MDCK cells followed by microplaque assay. For microplaque assay, confluent monolayers of MDCK cells were infected with 10-fold serial dilutions of the culture medium. The viral inoculum was removed and cells were overlaid with serum-free MEM containing 1 µg/ml TPCK-trypsin and 0.8% Avicel (RC-581; FMC Biopolymer). After 18-20 h incubation, the overlay was removed and the cells were fixed with 4% formalin and, subsequently permeabilized with 0.5% TritonX-100 and 20mM glycine. Cells were then stained with mouse anti-NP antibody (BEI resources, 1:1000) followed by three washes with PBS (Thermo Fisher Scientific). Subsequently, horseradish peroxidase-conjugated anti-mouse IgG antibody (Thermo Fisher Scientific, 1:1000) were added to the cells and incubated for one hour at room temperature followed by three washes with PBS (Thermo Fisher Scientific). Plaques were developed using 0.4 mg/ml 3-Amino-9-Ethylcarbazole (Sigma-Aldrich) in 0.05 M sodium acetate buffer (pH 5.5) and 0.03% hydrogen peroxide (Calbiochem) or commercially available True blue (KPL Biosciences).
4.7 Trichloroacetic acid precipitation of viral protein

For protein precipitation, ice-cold TCA was mixed with culture medium at a final concentration of 20% and incubated on ice for 30 minutes. The mixture was then centrifuged at 20,000xg and 4°C for 30 minutes. The supernatant was removed carefully and the pellet was washed with ice-cold acetone, followed by centrifugation at 20,000xg and 4°C for 15 minutes. The supernatant was discarded and the tube was air dried overnight. White powdery pellet was deposited on the walls of the tubes which were resuspended in 50µl of SDS-PAGE sample buffer per pellet. Proteins were resolved on SDS-PAGE and viral NP was detected by WB.

4.8 RNA interference

Pre-designed siRNA oligonucleotides targeting HDAC1 or HDAC2 gene (Sigma Aldrich) and a universal non-targeting MISSION control (Sigma Aldrich) were used for knockdown (Figure 30). The lyophilized siRNA obtained from the supplier was reconstituted to obtain a master stock of 50µM and was further diluted to obtain a working standard of 10µM. Finally, 10nM siRNA was used for knockdown of HDAC1 or HDAC2 expression. For knockdown of the target gene expression, OptiMEM (Invitrogen) medium was used for preparing the transfection complex. Desired concentration of siRNA was 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 5min, after which, the lipofectamine mix was added to the siRNA mix. This lipofectamine-siRNA complex was incubated for at least 30 min at room temperature to form the transfection complex. Meanwhile during the incubation period, A549 cells were split and cells were counted using a haemocytometer. A cell density of 2×10^5/ml was calculated and added to 200µl of
the transfection complex. The final volume was made to 1ml with complete MEM and the cells were plated in a 12 well plate or 24 well plate over the coverslips (for confocal microscopy).
Figure 30. Experimental design for RNAi to silence the target gene expression. The lipofectamine-siRNA complex was transfected for 72h in A549 cells and incubated at 37°C.
4.9 Plasmid propagation and extraction

Human HDAC1 cloned in plasmid pcDNA3.1 (ADDGENE) (kindly provided by Eric. M. Verdin, University of California) was prepared from *Escherichia coli* DH5α cells using a Qiagen plasmid purification kit. Briefly, 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 manufacturer’s protocol. The DNA was resuspended in 0.5 ml of sterile milliQ water and quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific) and stored at -80°C.
Figure 31. Experimental design for HDAC1-plasmid overexpression. The lipofectamine-DNA complex was transfected for 72h in A549 cells and incubated at 37°C for 48h.
4.10 Plasmid DNA transfection

Cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) by following the manufacturer’s guidelines. Briefly, $4 \times 10^5$ cells were grown to 80–90% confluency in 12-well culture plate (Corning). Routinely, 1µg of plasmid DNA and 3µl of Lipofectamine 2000 were diluted separately in 100µl of OptiMEM (Invitrogen), and incubated for 5min at room temperature. Following this, lipofectamine 2000 (Thermo Fisher Scientific) and DNA were mixed together and incubated at room temperature for 20-30min to form the DNA-Lipofectamine complex which was then added to the cells in a 12 well plate and incubated at 37°C for 48h (Figure 31).

4.11 Quantitative real time PCR

Total RNA was isolated from the uninfected and infected cells using an RNA isolation PureLink® RNA Mini Kit (Thermo Fisher scientific) as per manufacturers protocol. RNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific). The purity of isolated RNA was analysed using the 260/280 ratio obtained (an absorbance in 1.8 - 2.0 range was considered acceptable) using Nano drop (Thermo scientific). For analysing the integrity of RNA, the RNA samples were assayed on a bioanalyser chip. All the samples had an RNA integrity number (RIN) between 8 and 9. The cDNA was then synthesized from 500 ng total RNA using SuperScript® III First-Strand Synthesis System (Cat no; 18080-400, Thermo Fisher scientific).

The quantitative real-time PCR was performed using SYBR select master mix (Invitrogen). Pre-designed Kick start primers (Sigma-Aldrich) for HDAC1, HDAC2, viperin and β-actin was used for amplifying respective genes (Table 6) using quantitative real time (qPCR) (VIIA7, Applied Biosystems). β-actin was used as a
reference gene for normalizing the gene of interest expression, whereas, viperin were used as infection markers. The fold change in the expression of gene of interest was calculated using $2^{-\Delta \Delta CT}$ method and represented as percentage HDAC1 or HDAC2 mRNA expression.

Table 6: List of primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC1</td>
<td>5'-GGATACGGAGATCCCTAATG-3'</td>
<td>5'-CGTGTTCTGGTTAGTCATATTG-3'</td>
</tr>
<tr>
<td>HDAC2</td>
<td>5'-GGTCATGCTAATGTGTAAG -3'</td>
<td>5'-GTCGGTCCAAAATACTCAAG -3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-GACGACATGGAGAAAATCTG-3'</td>
<td>5'-ATGATCTGGGTATCTTTTAC-3'</td>
</tr>
<tr>
<td>Viperin</td>
<td>5'-CTTTTGCTGGGAAGCTCTTG -3'</td>
<td>5'-CAGCTGCTGTTTTTCTCCCTCT -3'</td>
</tr>
</tbody>
</table>

4.12 HDAC activity

MDCK cells ($10^5$) were grown to 80–90% confluency in Nunc 96-well optical bottom plate (165305; Thermo Fisher Scientific). One set was mock infected and other set was infected with 0.5 PFU of IAV/PR/8/34 (H1N1). After 20h post infection, media was removed from the respective wells and an *in situ* fluorometric HDAC activity kit (EPI003; Sigma-Aldrich) was used for determining the global HDAC activity following the manufacturer’s protocol. Briefly, 100µl of the reaction mix containing the substrate was added to the cells. The plate was incubated for 2h at 37°C. Meanwhile, standard deacetylase substrate provided was diluted following the manufacturers protocol to obtain a standard deacetylase activity graph. During the incubation, the HDAC substrate enters the cells and is deacetylated by intracellular HDACs. After 2h of incubation, the developer solution was added to the cells and the standard and samples were incubated for 30min. The developer lysed the cells and cleaved the deacetylated HDAC substrate to release a fluorophore which was
measured at 368/442nm (excitation/emission) on Varioskan Flash fluorimeter (Thermo Fisher Scientific).

4.13 Western blotting

After the desired time points, cells were scraped from the wells, pipetted into a 1.5 ml tube and centrifuged at 14,000 rpm for 30 secs to obtain the cell pellet. The supernatant (medium) was either stored or discarded and 10 µl of PBS was added to the cell pellet. The pellet was then dislodged by vigorous vortexing to get the cells in suspension. Cells were then lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% SDS, 0.5% Sodium deoxycholate, 1% TritonX-100, and protease-inhibitor cocktail). Protease inhibitor cocktail was added just before cell lysis. 20µg of proteins were resolved on 10% or 15% Tris-Glycine SDS-PAGE and transferred onto nitrocellulose membrane (GE Healthcare). Membranes were probed with desired primary antibodies and relevant secondary antibodies (Table 7 and Table 8). Proteins were then visualized 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 Elements 9 or Microsoft PowerPoint 2010. Raw signals for each band were calculated after background removal using the Li-COR software (Image studio 4.0). The signal from the protein of interest was normalized by dividing with PDI or Actin signal. Then each sample was normalized with their corresponding control samples. The control sample was either kept as 1 for fold change or 100% for percentage expression and the corresponding treated samples were calculated for fold change or percentage change, respectively.
4.14 Prediction of potential caspase cleavage sites

The human HDAC1 polypeptide sequence was used to predict the potential caspase cleavage sites by employing CaspDB online tool (http://caspdb.sanfordburnham.org/, (Kumar, van Raam et al. 2014).

4.14 Confocal microscopy

For confocal microscopy, cells were grown on 13 mm cover slips (sterilized with ethanol) in a 24-well plate. After various treatments, cells were carefully washed with PBS and then fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20 minutes at room temperature. Later, the cells were washed with PBS (3X) and then 500 µl of 0.2% Triton-X was added to permeabilize the cells. This was followed by 3 washes of PBS and then cells were incubated with mouse anti-NP primary antibody (1:100) in 10% FBS for one hour at room temperature. Cells were then washed with PBS (3X) followed and then incubated with donkey anti-mouse secondary Alexa flour 594 dye for 30 minutes at room temperature. The cells were then washed with PBS (3X) and then stained with Hoechst dye (Invitrogen) to identify the nucleus (1:5000) for 20 minutes at room temperature. After a final wash with PBS (3X), the coverslip with cells were mounted on to a glass slide with anti-fade mounting agent (Invitrogen).

4.15 Statistical analyses

All statistical analyses were performed using Prism 6 (GraphPad). The p-values were calculated using unpaired t-test, one-way ANOVA or two-way ANOVA for multiple data set comparisons.
**Table 7: List of primary antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Manufacturer</th>
<th>Clone</th>
<th>Catalogue no</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>1:10,000</td>
<td>ABCAM</td>
<td>-</td>
<td>ab 8227</td>
</tr>
<tr>
<td>HDAC1</td>
<td>1:1000</td>
<td>Cell signaling</td>
<td>10E2</td>
<td>5356</td>
</tr>
<tr>
<td>HDAC2</td>
<td>1:1000</td>
<td>Cell signaling</td>
<td>3F3</td>
<td>5113</td>
</tr>
<tr>
<td>HDAC3</td>
<td>1:1000</td>
<td>Cell signaling</td>
<td>7G6C5</td>
<td>3949</td>
</tr>
<tr>
<td>Acetyl histone H3</td>
<td>1:1000</td>
<td>Cell signaling</td>
<td>C5B11</td>
<td>9649</td>
</tr>
<tr>
<td>Total histone H3</td>
<td>1:1000</td>
<td>Cell signaling</td>
<td>D1H2</td>
<td>4499</td>
</tr>
<tr>
<td>pIRF3 (Ser395)</td>
<td>1:1000</td>
<td>Cell signaling</td>
<td>4D4G</td>
<td>4947</td>
</tr>
<tr>
<td>Total IRF3</td>
<td>1:1000</td>
<td>Cell signaling</td>
<td>D6I4C</td>
<td>11904</td>
</tr>
<tr>
<td>Human interferon-2b α (NR-3072)</td>
<td>1:100</td>
<td>BEI bioresources</td>
<td>-</td>
<td>G037-501-572</td>
</tr>
<tr>
<td>ISG15</td>
<td>1:1000</td>
<td>Cell signaling</td>
<td>-</td>
<td>2743</td>
</tr>
<tr>
<td>IFITM3</td>
<td>1:1000</td>
<td>ABCAM</td>
<td>-</td>
<td>ab15592</td>
</tr>
<tr>
<td>Mouse anti-NP</td>
<td>1:1000</td>
<td>BEI bioresources</td>
<td>-</td>
<td>-NR4282</td>
</tr>
<tr>
<td>Goat anti-NP</td>
<td>1:5000</td>
<td>Gift from Richard Webby</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Antibody</td>
<td>Dilution</td>
<td>Supplier</td>
<td>Catalog Number</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>----------</td>
<td>-------------------</td>
<td>----------------</td>
<td></td>
</tr>
<tr>
<td>PDI</td>
<td>1:10,000</td>
<td>SIGMA ALDRICH</td>
<td>-</td>
<td>P7496</td>
</tr>
<tr>
<td>STAT1</td>
<td>1:1000</td>
<td>BD biosciences</td>
<td>42/Stat1</td>
<td>610185</td>
</tr>
<tr>
<td>pSTAT1(pY701)</td>
<td>1:1000</td>
<td>BD biosciences</td>
<td>14/P-STAT1</td>
<td>612132</td>
</tr>
<tr>
<td>Viperin</td>
<td>1:1000</td>
<td>Cell signaling</td>
<td>D5T2X</td>
<td>13996</td>
</tr>
</tbody>
</table>
### Table 8: List of secondary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Manufacturer</th>
<th>Catalogue no</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-mouse HRP</td>
<td>1:2000</td>
<td>Thermo Fisher Scientific</td>
<td>626520</td>
</tr>
<tr>
<td>Donkey anti-rabbit HRP</td>
<td>1:2000</td>
<td>Thermo Fisher Scientific</td>
<td>A16023</td>
</tr>
<tr>
<td>Rabbit anti-goat HRP</td>
<td>1:5000</td>
<td>Sigma-Aldrich</td>
<td>A5420</td>
</tr>
<tr>
<td>IRDye® 680LT Goat anti-Mouse</td>
<td>1:10,000</td>
<td>LI-COR</td>
<td>926-68020</td>
</tr>
<tr>
<td>IRDye® 800CW Goat anti-Rabbit</td>
<td>1:10,000</td>
<td>LI-COR</td>
<td>926-32211</td>
</tr>
<tr>
<td>IRDye® 800CW Donkey anti-Goat</td>
<td>1:10,000</td>
<td>LI-COR</td>
<td>926-32214</td>
</tr>
</tbody>
</table>
Appendix I: Recipes
Table 9: Composition of cell lysis buffer

<table>
<thead>
<tr>
<th>Components</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>
</tbody>
</table>
Table 10: Composition of SDS-Sample buffer

<table>
<thead>
<tr>
<th>Components</th>
<th>Resolving Gels</th>
<th>Stacking Gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 6.8)</td>
<td>50 mM</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>2%</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>30%</td>
<td></td>
</tr>
<tr>
<td>bromophenol blue</td>
<td>0.04%</td>
<td></td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>5%</td>
<td></td>
</tr>
</tbody>
</table>

Table 11: Composition of Resolving and Stacking gels.

<table>
<thead>
<tr>
<th>Components</th>
<th>10%</th>
<th>15%</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ-water (mL)</td>
<td>3.9</td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td>Lower Tris (pH 8.8) (mL)</td>
<td>2</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Upper Tris (pH 6.8) (mL)</td>
<td>-</td>
<td>-</td>
<td>1.75</td>
</tr>
<tr>
<td>10% SDS (µL)</td>
<td>80</td>
<td>80</td>
<td>50</td>
</tr>
<tr>
<td>40% AA:MBA (mL)</td>
<td>2</td>
<td>4</td>
<td>0.7</td>
</tr>
<tr>
<td>10% APS (µL)</td>
<td>80</td>
<td>80</td>
<td>50</td>
</tr>
<tr>
<td>TEMED (µL)</td>
<td>8</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Total (mL)</td>
<td>8</td>
<td>8</td>
<td>5.5</td>
</tr>
</tbody>
</table>
Table 12: Composition of Running Buffer (1x).

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
<th>Amount</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>192mM</td>
<td>14.4g</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Tris base</td>
<td>25mM</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>MilliQ-water (L)</td>
<td></td>
<td></td>
<td>final volume was made up to 1L</td>
</tr>
</tbody>
</table>

Table 13: Composition of Transfer Buffer (1X)

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
<th>Amount</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>192mM</td>
<td>14.4g</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Tris base</td>
<td>25mM</td>
<td>3.2g</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Methanol</td>
<td>20% (vol/vol)</td>
<td>200ml</td>
<td>LabServ</td>
</tr>
<tr>
<td>MilliQ-water (L)</td>
<td></td>
<td></td>
<td>final volume was made up to 1L</td>
</tr>
</tbody>
</table>

Table 14: List of blocking buffers

<table>
<thead>
<tr>
<th>Blocking reagent</th>
<th>Buffer</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% non-fat-milk</td>
<td>Phosphate buffered saline</td>
<td>To detect all proteins</td>
</tr>
<tr>
<td>1% BSA</td>
<td>Tris buffered saline</td>
<td>To detect phosphorylated proteins</td>
</tr>
<tr>
<td>0.1% Casein</td>
<td>Tris buffered saline</td>
<td>To detect phosphorylated proteins</td>
</tr>
</tbody>
</table>
Appendix II: Publications and presentations
Publications


Manuscript under preparation:

1. **Nagesh, P.T. & Husain, M. (2016).** Host histone deacetylase 2 regulates the antiviral response during influenza A virus infection. (Manuscript under preparation.)


Oral presentations

1. **Nagesh, P.T., & Husain, M.** Host histone deacetylase 2 regulates the innate antiviral response in influenza A virus infected epithelial cells, Queenstown Molecular Biology meeting, August 2016, New Zealand. (Abstract submitted)

2. **Nagesh, P.T., & Husain, M.** Influenza A virus dysregulates host histone deacetylase 1 that inhibits viral infection in lung epithelial cells, 35th annual meeting American society for Virology, Blacksburg, Virginia, USA, June 2016.

3. **Nagesh, P.T., & Husain, M.** Histone deacetylase 1: a newly identified antiviral host factor of influenza A virus, One Health Aotearoa - A Symposium on Infectious Diseases, March 2016, Wellington, New Zealand.


5. **Nagesh, P.T., & Husain, M.** Investigating the role of human histone deacetylases in the replication of IAV, 2013, Three minutes’ presentation, Research retreat, Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand.
Poster presentations

1. **Nagesh, P.T, & Husain.M**, Influenza A virus dysregulates host histone deacetylase 1 to efficiently replicate, Queenstown Molecular Biology meeting, August 2015, New Zealand.

2. **Nagesh, P.T, & Husain.M**, Investigating the role of human histone deacetylase 1 in the replication of IAV, Research retreat, 2014, Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand.

3. **Nagesh, P.T, & Husain.M**, Investigating the role of human histone deacetylases in the replication of IAV, 2013, Research retreat, Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand.

Influenza A Virus Dysregulates Host Histone Deacetylase 1 That Inhibits Viral Infection in Lung Epithelial Cells

Prashanth Thevakar Nagesh, Matloob Husain
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ABSTRACT
Viruses dysregulate the host factors that inhibit virus infection. Here, we demonstrate that human enzyme, histone deacetylase 1 (HDAC1) is a new class of host factor that inhibits influenza A virus (IAV) infection, and IAV dysregulates HDAC1 to efficiently replicate in epithelial cells. A time-dependent decrease in HDAC1 polypeptide level was observed in IAV-infected cells, reducing to <50% by 24 h of infection. A further depletion (97%) of HDAC1 expression by RNA interference increased the IAV growth kinetics, increasing it by >3-fold by 24 h and by >6-fold by 48 h of infection. Conversely, overexpression of HDAC1 decreased the IAV infection by >2-fold. Likewise, a time-dependent decrease in HDAC1 activity, albeit with slightly different kinetics to HDAC1 polypeptide reduction, was observed in infected cells. Nevertheless, a further inhibition of deacetylase activity increased IAV infection in a dose-dependent manner. HDAC1 is an important host deacetylase and, in addition to its role as a transcription repressor, HDAC1 has been lately described as a coactivator of type I interferon response. Consistent with this property, we found that inhibition of deacetylase activity either decreased or abolished the phosphorylation of signal transducer and activator of transcription 1 (STAT1) and expression of interferon-stimulated genes, IFIT3, 15S15, and viperin in IAV-infected cells. Furthermore, the knockdown of HDAC1 expression in infected cells decreased viperin expression by 58% and, conversely, the over-expression of HDAC1 increased it by 55%, indicating that HDAC1 is a component of IAV-induced host type I interferon antiviral response.

IMPORTANCE
Influenza A virus (IAV) continues to significantly impact global public health by causing regular seasonal epidemics, occasional pandemics, and zoonotic outbreaks. IAV is among the successful human viral pathogens that has evolved various strategies to evade host defenses, prevent the development of a universal vaccine, and acquire antiviral drug resistance. A comprehensive knowledge of IAV-host interactions is needed to develop a novel and alternative anti-IAV strategy. Host produces a variety of factors that are able to fight IAV infection by employing various mechanisms. However, the full repertoire of anti-IAV host factors and their antiviral mechanisms has yet to be identified. We have identified here a new host factor, histone deacetylase 1 (HDAC1) that inhibits IAV infection. We demonstrate that HDAC1 is a component of host innate antiviral response against IAV, and IAV undermines HDAC1 to limit its role in antiviral response.

Influenza A virus (IAV), a prototypic member of family Orthomyxoviridae, has been a successful human respiratory pathogen. IAV has prevented the development of a universal vaccine so far and rendered the currently approved anti-influenza virus drugs almost ineffective. A unique genetic make-up comprising of a segmented RNA genome and a broad host range of humans, birds, pigs, dogs, cats, horses, seals, and bats allows the regular emergence of novel and drug-resistant IAV strains, causing regular seasonal epidemics, irregular pandemics, and zoonotic outbreaks (1, 2). The worldwide annual influenza vaccination program alternating in the northern and southern hemispheres is the major tool to prevent or control seasonal influenza epidemics. Despite this effort, according to the World Health Organization estimate, influenza manages to cause approximately 1 billion cases of illu, 3 to 5 million cases of severe illness, and 300,000 to 500,000 deaths worldwide annually (http://www.who.int/immunization/topics/influenza/en/). In addition, seasonal influenza epidemics result in significant productivity and economic losses due to work and school absenteeism, doctor visits, and hospitalizations. A new IAV pandemic further exacerbates these problems by many fold. Furthermore, frequent zoonotic outbreaks of deadly avian IAV infections in humans highlight the constant threat of the emergence of a new pandemic IAV strain (1, 2).

Therefore, there is a need to develop novel, alternative, and long-lasting anti-influenza strategies. One strategy is to strengthen host defenses by targeting the antiviral host factors. Host cells produce variety of factors that target various steps of virus life cycle to inhibit virus infection and multiplication (3). However, viruses have evolved their own strategies to antagonize those factors (3). Recently, we discovered that the human enzyme histone deacetylase 6 (HDAC6) has an anti-IAV function (4) and that IAV downregulates HDAC6 activity and induces a caspase-mediated cleavage of HDAC6 polypeptide to antagonize its antiviral function (5, 6). These findings led to the hypothesis that other human HDACs potentially have a similar role in IAV infection.
HDACs are a family of enzymes that catalyze the deacetylation of acetylated proteins (7). Acetylation is a posttranslational modification of proteins that has been discovered and studied extensively in histones to understand the chromatin structure and gene transcription (8, 9). Now, acetylation is also known to occur in a variety of nonhistone proteins. A proteomic study has identified at least 3,600 acetylation sites in 1,750 nuclear and nonnuclear proteins, indicating a broader role of acetylation/deacetylation in nuclear and cytoplasmic functions of the cell (10). Acetylation/deacetylation is a reversible process that is regulated by the competition of histone acetyltransferases (HATs) and HDACs, consequently influencing diverse cellular processes, such as the cell cycle, chromatin remodeling, RNA splicing, gene expression, cell signaling, and protein stability and transport (7, 11). HDACs are expressed in all eukaryotic cells. Mammalian HDACs are a family of at least 18 members, which have been classified into four classes based on their homology to yeast HDACs (12, 13). The class I, II, and IV HDACs are considered classical HDACs and are zinc dependent. The HDACs in each class vary in structure, enzymatic activity, intracellular localization, and expression pattern. The class I HDACs (HDAC-1, -2, -3, and -8) are expressed in all tissues and are mainly localized to the nucleus. Class II HDACs, which are further subdivided into class IIa (HDAC-4, -5, -7, and -9) and class IIb (HDAC-6 and -10), shuttle between the nucleus and the cytoplasm (7, 12, 13). Class III HDACs, commonly known as sirtuins, comprise seven members (SIRT1 to -7). Each sirtuin has a unique subcellular localization and distinct function (14). The sirtuins are different from classical HDACs and require NAD⁺ for enzymatic activity. Lastly, class IV HDACs (HDAC 11), which shares homology with both class I and class II HDACs (12). Previously, we found that HDAC6, a class II HDAC, possesses an anti-IAV property and IAV undermines HDAC6 activity and integrity to limit its antiviral function (4-6). Likewise, we demonstrate here that HDAC1, a class I HDAC, also possesses anti-IAV properties and is an integral part of host type I interferon (IFN)–mediated response against IAV. In turn, IAV downregulates HDAC1 expression and deacetylation activity to antagonize its antiviral function and efficiently replicate in epithelial cells.

MATERIALS AND METHODS

Cells, viruses, and plasmids. A549 and MDCK cells were grown and maintained in complete minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, and 1-glutamine (Life Technologies) at 37°C and under 5% CO₂ atmosphere. Influenza virus A/PR/8/34 (H1N1) strain, A/New Caledonia/20/1999 (H1N1) strain, and A/WSN/33 (H1N1) strain (kindly provided by Richard Webby, St Jude Children's Research Hospital) were propagated in 10-day-old embryonated chicken eggs and titrated on MDCK cells. Human HDAC1 cloned in plasmid pcDNA3.1, a gift from Eric Verdin (Addgene plasmid 13820) (15) was prepared from E. coli DH15 cells using a plasmid purification kit (Qiagen).

Infection. Cells were infected with IAV at a multiplicity of infection (MOI) of 0.1 to 5 PFU/cell. The virus inoculum was prepared in serum-free MEM and added to cells monolayers previously washed twice with serum-free MEM. For infection of MDCK cells, 1 μg of TPCK (tosyl-l-phenylalanine chloromethane ketone)-trypsin (Sigma-Aldrich)/ml was added to the virus inoculum. After 1 h of incubation at 35°C, the inoculum was removed and cells were washed once with serum-free MEM. Fresh serum-free MEM was added, and the cells were incubated back at 35°C. In some experiments, serum-free MEM was supplemented with NAC (Sigma-Aldrich), MG132 (Calbiochem), or trichostatin A (TSAs; Sigma-Aldrich). To inactivate IAV, the virus inoculum was irradiated under a 30-W UV bulb for 5 min.

Quantitative real-time PCR of HDAC1. Total RNA from the cells was isolated by using a PureLink RNA isolation kit (Life Technologies). The integrity of isolated RNA was confirmed using RNA 6000 Nano Chip on Bioanalyzer 2100 (Agilent). The RNA purity (260/280 ratio of 2.0) and quantity were measured on a NanoDrop 1000 (Thermo). Total RNA was then used as a template to synthesize the cDNA using SuperScript III first-strand synthesis system (Life Technologies). The quantitative real-time PCR of HDAC1 was performed using SYBR green select master mix (Life Technologies) and KqGqStart primers (Sigma-Aldrich) for forward primer, 5' -GGATGGAGGATCCCTACGTG-3'; reverse primer, 5' -CGT GTTCGTTAGTCTCATATGG-3' on a Viia 7 real-time PCR system (Applied Biosystems). Simultaneously, The beta-actin (forward primer, 5' -GGACGATGTTGAGAAATCTGG-3'; reverse primer, 5' -ATGATCTGG GTTACATCTC-3') was amplified as a reference gene for normalization. The fold change in the expression of HDAC1 mRNA was calculated using the 2⁻ⁿᵈⁿ value method as described elsewhere (16).

Western blotting. 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% Triton X-100, and 1% protease inhibitor cocktail [Roche]). The total amount of protein was quantitated by using a BCA kit (Thermo). Equal amounts of proteins were resolved on 10% or 15% Tris-glycine SDS-PAGE under reducing conditions and transferred onto Protran Premium nitrocellulose membrane (GE Healthcare). Membranes were probed with mouse anti-HDAC1 (1:1,000; clone 10E2; Cell Signal- ing), rabbit anti-acetyl-histone H3 (1:200; clone CB11; Cell Signal- ning), rabbit anti-histone H3 (1:1,000; clone D1H2; Cell Signaling), rabbit anti-IFITM3 (1:1,000; Abcam), rabbit anti-JASG (1:1,000; Cell Signaling), rabbit anti-viroporin (1:1,000; clone DS72X; Cell Signaling), mouse anti-STAT1 (1:1,000; clone 4G10; STAT Biologics), mouse anti- ubiquitin (1:500; clone P4D1; Santa Cruz), mouse anti-IFN-γ (1:1,000; NB-4282, obtained through BEI Resources, NIAID, NIH), goat anti-IFN-γ (1:1,000; kindly provided by Richard Webby), rabbit anti-actin (1:5,000; Abcam), or rabbit anti-protein disulfide isomerase (PDI; 1:5,000, Sigma-Aldrich) antibody, followed by horseradish peroxidase-conjugated antimouse, anti-goat, or anti-rabbit IgG antibody (1:5,000; Life Technolo- gies). Protein bands were visualized by using a chemiluminescence substrate, and images were acquired on an Odyssey Fc imaging system (Li-Cor). Images were exported as TIFF files and compiled in Adobe Photoshop CC 2015.

HDAC activity assay. An in situ fluorometric HDAC activity kit (Sigma-Aldrich, catalog no. EP003) was used to perform this assay. Briefly, cells grown in 96-well optical bottom plate (Nunc) were either mock infected or infected with IAV/PR/8/34 (H1N1) strain at an MOI of 0.5. After 20 h of infection, the cells were harvested for the assay according to the manufacturer's protocol. The fluorescence excitation/emission was read at 386/442 nm on a Varianclan Fluorimeter (Thermo).

Overexpression of HDAC1. Cells were transfected with plasmids us- ing Lipofectamine 2000 reagent (Life Technologies) following the manufacturer's guidelines. Briefly, cells (4 × 10⁵) were grown to 80—90% confluence in a 12-well culture plate (Corning). Plasmid DNA (1 μg) and Lipofectamine 2000 (3 μl) were diluted separately in Opti-MEM I medium (Life Technologies), mixed together, and incubated for 20 to 30 min at room temperature. The DNA-Lipofectamine 2000 complex was then added to the cells. The cells were grown at 37°C for 48 h before infection or further processing.

Knockdown of HDAC1 expression. Predesigned small interfering RNA (siRNA) oligonucleotides targeting the HDAC1 gene (CUUGAUCA GGACAUUGAUAGTTG(T)) and nontargeting Mission control siRNA was obtained from Sigma-Aldrich and delivered to the cells using Lipofec- tameate RNAiMAX reagent (Life Technologies) according to the manu- facturer's guidelines. Briefly, siRNA oligonucleotides (10 nM) and RNAiMAX (2 μl) (Life Technologies) were diluted separately in Opti-
MEM I medium (Life Technologies), mixed together, and incubated for 20 to 30 min at room temperature. The siRNA-RNAiMax complex was then mixed with 250 μl of cells and transferred to a 12-well culture plate. The cells were incubated at 37°C for 72 h before infection or further processing.

Cell viability assay. An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide) assay was performed to ascertain the viability of the cells. The MTT assay is based on the conversion of MTT into formazan crystals by living cells and determines the mitochondrial activity, which for most cell populations is related to the number of viable cells (17). Cells that had undergone various treatments were washed twice with phosphate-buffered saline. One milliliter of MTT reagent (Sigma-Aldrich) was added to the cells, and the cells were incubated at 37°C for 1 h. Subsequently, 1 ml of dimethyl sulfoxide (Calbiochem) was added, and the cells were further incubated for 15 to 20 min at room temperature, with shaking. Finally, the absorbance was measured at 595 nm on Multiskan Ascent plate reader (LabSystems).

Virus release assays. The culture medium from infected cells was harvested, the cell debris was cleared off by low-speed centrifugation, and the remainder was divided into two parts. One part was subjected to protein precipitation by trichloroacetic acid (TCA; Calbiochem), whereas the other part was mixed with 0.3% RNA and treated on MDCK cells, followed by a microplate assay. For protein precipitation, ice-cold TCA was mixed with culture medium at a final concentration of 20%, followed by incubation on ice for 30 min. The mixture was then centrifuged at 20,000 × g and 4°C for 30 min. The supernatant was removed carefully, and the pellet was washed twice with ice-cold acetone. The pellet was air dried and directly suspended in SDS-PAGE sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 30% glycerol, 5% 2-mercaptoethanol, and 0.04% bromophenol blue). Proteins were resolved by SDS-PAGE, and viral nucleoprotein (NP) was detected by Western blotting. For microplate assay, confluent monolayers of MDCK cells were infected with 10-fold serial dilutions of the culture medium. The viral inoculum was removed, and the cells were overlaid with serum-free MEM containing 1 μg of TPCK-trypsin/ml and 0.8% Avicol (RC-581; FMC Biopolymer). After 18 to 20 h of incubation, the overlay was removed, and the cells were fixed with 4% formalin (Sigma-Aldrich) and subsequently permeabilized with 0.3% Triton X-100 in 200 mM glycine solution. The cells were then stained with mouse anti-NP antibody (1:1,000), followed by horseshadish peroxidase-conjugated anti-mouse IgG antibody (1:1,000). Plaques were developed by adding a substrate solution containing AEC (3-amino-9-ethylcarbazole; Sigma-Aldrich) at 0.4 mg/ml in 0.05 M sodium acetate buffer (pH 5.5) and 0.03% hydrogen peroxide (Calbiochem).

Statistical analysis. All statistical analyses were performed using Prism 6 (GraphPad). The P values were calculated by using unpaired t tests for pairwise data comparisons, one-way analysis of variance (ANOVA), or two-way ANOVA for multiple data set comparisons. A P value of <0.05 was considered significant.

RESULTS
IAV downregulates the expression of HDAC1. We selected HDAC1 for the present study to test our hypothesis that like HDACs, a class II HDAC, class I HDACs also possess anti-IAV properties. HDAC1 is a prototypic member of class I HDACs (13). It is well established that viruses subvert host cell transcription and translational machinery to express their own polypeptides and decrease or shut down the expression of host proteins involved in antiviral response (18,19). Therefore, we first analyzed the expression of host HDAC1 in response to IAV infection. Human lung epithelial A549 cells were infected with influenza virus A/PRI/8/34 (H1N1) strain (here referred as PR8) at an MOI of 0.5 PFU per cell, and the culture medium and the infected cells were harvested separately. The cells were processed to measure the level of HDAC1 mRNA by quantitative real-time PCR. We found that IAV infection caused a moderate 23% but statistically significant (P = 0.005) reduction in HDAC1 mRNA level in A549 cells (Fig. 1A). Similar results were obtained when cells were infected with PR8 at an MOI of 5.0 PFU per cell (data not shown). The infection of A549 cells was confirmed by detecting the presence of released IAV progeny in the culture media by plaque assay (data not shown).

After detecting a moderate, but statistically significant reduction in HDAC1 transcript level, we analyzed the level of HDAC1 polypeptide in A549 cells in response to IAV infection. Cells were infected with PR8 as above and harvested after 2, 6, 12, and 24 h of infection. The HDAC1 polypeptide was detected in total cell lysates by Western blotting (WB), and the intensities of protein bands were quantified. We found that IAV infection reduced the level of HDAC1 polypeptide (62 kDa) in A549 cells in a time-dependent manner (Fig. 1B). There were significant 44% (P = 0.015), 63% (P = 0.001), and 67% (P = 0.001) decreases in HDAC1 polypeptide level after 6, 12, and 24 h of infection, respectively, compared to the 2-h postinfection time point (Fig. 1C). Further, there was a significant 57% (P = 0.002) decrease in HDAC1 polypeptide level after 24 h of infection compared to the uninfected cells harvested at the same time. A similar trend in HDAC1 polypeptide level was observed when A549 cells were infected with PR8 at an MOI of 5.0 PFU per cell for 24 h (data not shown). Furthermore, the reduction in HDAC1 polypeptide level was even more profound and dose dependent when A549 cells were infected with IAV/WSN/33 (H1N1) strain for 24 h (Fig. 1D). Compared to the uninfected cells, approximately 80% and 95% decreases in the level of HDAC1 polypeptide were observed in cells infected with the WSN strain at MOIs of 3.0 and 5.0 PFU per cell, respectively (data not shown). Finally, by using a UV-irradiated PR8 inoculum, we confirmed that a replication-competent IAV was required to reduce the HDAC1 polypeptide levels in A549 cells (Fig. 1E). Furthermore, almost identical results were obtained when Fig. 1E experiment was repeated using the influenza virus A/New Caledonia/20/1999 (H1N1) strain, a more clinically relevant strain of IAV (Fig. 1F).

The lack of a linear correlation between the reduction of HDAC1 mRNA and polypeptide levels in PR8-infected cells prompted us to investigate whether IAV promotes the degradation of HDAC1 polypeptide in infected cells. Two main pathways, one mediated by proteasome and other mediated by lysosome, govern the degradation of proteins in eukaryotic cells. To identify the pathway leading to the degradation of HDAC1 polypeptide in IAV-infected cells, PR8-infected cells were treated with proteasome inhibitor MG132 (20 μM) or lysosome inhibitor NH4Cl (20 mM); at these concentrations, MG132 and NH4Cl have been shown to inhibit proteasomal and lysosomal activity, respectively (20,21). The levels of HDAC1 polypeptide were then analyzed and quantified as described above. We found that MG132 treatment reversed the effect of IAV infection on HDAC1 polypeptide level and rescued its level in infected cells almost to the level of uninfected cells, whereas NH4Cl treatment did not reverse the effect of IAV infection on HDAC1 polypeptide (Fig. 1G). Consistent with above data, there was a significant 54% (P = 0.001) and 60% (P = 0.0004) decrease in HDAC1 polypeptide level in mock-treated and NH4Cl-treated infected cells, respectively, compared to their uninfected controls (Fig. 1H). In contrast, there was an insignificant 3% decrease in HDAC1 polypeptide level in MG132-treated infected cells compared to the uninfected con-
control (Fig. 1H), indicating that IAV promoted the degradation of HDAC1 polypeptide by the proteasome pathway. An increase in the amount of ubiquitinated proteins in MG132-treated cells confirmed the potency of MG132 (Fig. 1I).

Endogenous HDAC1 possesses an anti-IAV property. The downregulation of HDAC1 expression in infected cells indicated a potential anti-IAV function for HDAC1. To test this, we analyzed the growth kinetics of IAV in AS49 cells depleted of HDAC1 expression by RNA interference. First, various concentrations of a human HDAC1-targeting siRNA (10 to 100 nM) and a nontargeting control siRNA (50 nM) were delivered to AS49 cells to obtain a significant depletion of HDAC1 expression. A 10 nM siRNA concentration was sufficient to knock down HDAC1 expression by 97% in AS49 cells (Fig. 2A), with negligible cytotoxicity compared to control siRNA (Fig. 2F). Therefore, in the next and subsequent experiments, AS49 cells were transfected with 10 nM HDAC1-targeting siRNA, as well as nontargeting control siRNA.

The cells were then infected with PR8 at an MOI of 0.5, and the culture medium and the infected cells were harvested separately after 2, 6, 12, and 24 h of infection. The virus yield in the culture medium was measured by WB of NP (B) and by microplaque assay (C). The data presented are means ± the standard errors of the means of three independent experiments; the P value was calculated by using two-way ANOVA. (D) Total lysates of the cells were prepared, and HDAC1, PDI, and NP were detected by WB. (E) AS49 cells transfected with CT or HD1 siRNAs, as described above, were infected with PR8 at an MOI of 0.1 in the presence of 0.1 µg of trypsin/µl, and the virus yield in the culture medium was measured by microplaque assay after 24 and 48 h. The virus yield from respective CT siRNA samples was considered to be 1-fold for comparisons to HD1 siRNA samples. The data presented are means ± the standard errors of the means of three independent experiments; the P values were calculated using one-way ANOVA. (F) AS49 cells were transfected with no siRNA, CT siRNA, or HD1 siRNA for 72 h. Cell viability was determined using an MTT assay. M, molecular weight; ns, not significant.
FIG 1 IAV downregulates the expression of HDAC1. (A) A549 cells (8 × 10^5) were infected with PR8 at an MOI of 0.5 for 24 h. The uninfected (UNI) and infected (INF) cells were harvested and processed, and HDAC1 mRNAs and beta-actin mRNAs were detected by quantitative real-time PCR. The level of HDAC1 mRNA was normalized to beta-actin mRNA. The normalized value of HDAC1 mRNA in UNI sample was considered 100% for comparison to the INF sample. Error bars represent the means ± the standard errors of the means of three independent experiments; the P value was calculated using a t-test. (B) A549 cells were infected as described above and harvested at the indicated times. Total cell lysates were prepared, and HDAC1 (62 kDa), PDI (57 kDa), and viral NP (56 kDa) were detected in uninfected and infected (2, 6, 12, and 24 h) cell lysates by WB. PDI was detected as the loading control, and NP was detected as the infection marker. (C) The HDAC1 and PDI protein bands were quantified using Image Studio Lite V4.0 software (LI-COR), and the amount of HDAC1 was normalized to PDI. The normalized amount of HDAC1 in the UNI sample or the 2-h sample was considered 100% for comparisons to the 6-, 12-, and 24-h samples. Data presented are means ± the standard errors of the means of three independent experiments; the P value was calculated using one-way ANOVA. (D) A549 cells were infected with IAV WSN strain at an MOI of 0.5, 3.0, and 5.0 for 24 h. Total cell lysates were prepared, and HDAC1, PDI, and NP were detected in UNI and infected (0.5, 3.0, and 5.0) cell lysates by WB. (E) A549 cells were infected with live or UV-irradiated PR8 at an MOI of 0.5 for 24 h. Total cell lysates were prepared, and HDAC1, actin, and NP were detected in UNI, INF, and INF-UV cell lysates by WB. (F) A549 cells were infected with live or UV-irradiated influenza virus A/New Caledonia/20/1999 (H1N1) strain at an MOI of 0.5 for 24 h. Total cell lysates were prepared, and HDAC1, actin, and NP were detected in UNI, INF, and INF-UV cell lysates by WB. (G) A549 cells were infected with PR8 as described above and subsequently treated with N1H1,3 (20 mM) or MG132 (10 μM) for 24 h. Total cell lysates were prepared, and HDAC1, actin, and NP were detected in INF and INF-UV cell lysates by WB. (H) The HDAC1 and actin protein bands were quantified as described above, and the amount of HDAC1 was normalized to actin. The normalized amount of HDAC1 in respective UNI samples was considered 100% for comparisons to INF samples. The data presented are means ± the standard errors of the means of three independent experiments; the P value was calculated using one-way ANOVA. (I) Ubiquitin (Ubq) and actin were detected in the cell lysates described above by WB. U, uninfected; I, infected. MW, molecular weight.
Pressing cells compared to empty plasmid control confirmed that the ectopically expressed HDAC1 was enzymatically active (Fig. 3E). A fluorescence microscopy image showing the transfection efficiency of A549 cells is shown in Fig. 3E; a transfection efficiency of 60 to 70% was achieved routinely.

**IAV downregulates HDAC1 activity.** After discovering that HDAC1 polypeptide has an anti-IAV property and IAV downregulates HDAC1, we next examined whether HDAC1 enzymatic activity is also downregulated in infected cells. To assess this, we compared the levels of HDAC1 substrate, acetylated-histone H3 in infected cell lysates with uninfected cell lysates by WB. An increase in acetylated-histone H3 level would mean that HDAC1 activity is downregulated and vice versa. To detect acetylated-histone H3, we selected an antibody (CSB11; Cell Signaling) that detects acetylated-histone H3 (Lys9), i.e., histone H3 acetylated on lysine residue at position 9. Previously, acetylated-histone H3 (Lys9) has been shown to be a specific substrate for HDAC1 (24, 25), and our HDAC1 overexpression data presented above (Fig. 3D) have confirmed it as an HDAC1 substrate. A time course experiment revealed that IAV downregulates the HDAC1 activity, and it does this mainly between 6 and 12 h of infection (Fig. 4A), which is the growth period of IAV in cultured cells (Fig. 2C). There were significant 3.0-fold \((P = 0.02)\) and 3.38-fold \((P = 0.009)\) increases in the levels of acetylated-histone H3 (Lys9) in infected cells after 6 and 12 h of infection, respectively, compared to uninfected cells (Fig. 4B). Interestingly, after 24 h of infection, the HDAC1 activity in infected cells (1.74-fold) came back up almost to the level of uninfected cells (Fig. 4B).

The deacetylase activity of class I and class II HDACs has anti-IAV properties. The downregulation of HDAC1 activity by IAV indicated an anti-IAV role for HDAC1. Its antiviral function, one way to further investigate this is by using a selective inhibitor of HDAC1 enzymatic activity. However, to our knowledge, a selective HDAC1 inhibitor is not commercially available; most of the available HDAC inhibitors cross-inhibit other class I and class II HDACs (26). So, we determined the cell death activity of class I and II HDACs (12) in response to IAV infection. To accomplish this, we used an in situ fluorometric HDAC activity assay kit, which essentially measures the activity of class I and class II HDACs. This kit utilizes a cell-permeable HDAC substrate containing an acetylated lysine side chain, which is deacetylated by intracellular HDACs. A developer then cleaves the deacetylated substrate and releases a fluorophore, which can be quantified. Using this kit, we measured an ~35% reduction in HDAC activity in infected cells compared to uninfected cells (not shown). Next, we sought to determine whether class I and class II HDAC activity has an anti-IAV function. To accomplish this, we used trichostatin A (TSA), an antifungal antibiotic that is widely used as an inhibitor of class I and class II HDACs, including HDAC1 (27). We found that, like HDAC1 depletion, TSA treatment increased the release of infectious IAV progeny and in a dose-dependent manner (Fig. 5). Compared to mock-treated infected cells, treatment of infected cells with 1 and 5 \(\mu\)M TSA resulted in significant 3.1-fold \((P = 0.03)\) and 5.3-fold \((P = 0.001)\) increases, respectively, in the release of infectious IAV progeny (Fig. 5A) and a negligible effect on infected cell viability (Fig. 5B). A corresponding increase in the levels of acetylated-histone H3 (Lys9), but not total histone H3 in TSA-treated cells indicated the inhibition of class I (including HDAC1) and class II HDAC activity (Fig. 5C).

**FIG 4 IAV downregulates the activity of HDAC1.** (A) MDCK cells \((8 \times 10^4)\) were infected with PR8 at an MOI of 0.5 and harvested after 2, 6, 12, and 24 h of infection. Total cell lysates were prepared, and acetylated-histone H3 (Lys9) (Acet H3), total histone H3 (Total H3), PDI, HDAC1, actin, and NP were detected in uninfected (UNI) and infected (2, 6, 12, and 24 h) cell lysates by WB. Acet H3, an HDAC1 substrate, was detected as a marker of HDAC1 activity, and total H3, PDI, and actin were detected as loading controls. (B) Acet H3 and total H3 protein bands were quantified as Fig. 1C, and the amount of Acet H3 was normalized to the total H3. The normalized amount of Acet H3 in UNI sample was considered 1-fold for comparisons to 2-, 6-, 12-, and 24-h samples. The data presented are means \(\pm\) standard errors of the means of three independent experiments; the \(P\) value was calculated by using one-way ANOVA. MW, molecular weight.

The deacetylase activity of class I and class II HDACs is important for IAV-induced host type I IFN-mediated response. An increase in the release of infectious IAV progeny from TSA-treated cells confirmed that the enzymatic activity of class I and class II HDACs has anti-IAV properties. We next endeavored to determine the anti-IAV mechanism of class I and class II HDAC activity. In addition to its role in host gene transcription, the deacetylase activity of class I and class II HDACs has been shown to be a coactivator of type I interferon (IFN)-mediated host innate antiviral response and the subsequent expression of IFN-stimulated genes (ISGs) (28–32). However, a precise role of class I and class II HDACs in IAV-induced host innate antiviral response is yet to be
**FIG 3** The overexpression of HDAC1 inhibits IAV infection. (A, B, and D) A549 cells (8 × 10^6) were transfected with empty plasmid pCDNA3( pc) or pCDNA3 containing HDAC1 (HD1) for 48 h. Cells were then infected with PR8 at an MOI of 0.5, and the culture medium and the cells were harvested separately after 24 h. The virus yield in the culture medium was measured by WB of NP (A) and by microplate assay (B). The data presented are means ± standard errors of the means of three independent experiments; the P value was calculated using a t test. (C) A549 cells transfected with pc or HD1 plasmids, as described above, were infected with PR8 at an MOI of 0.1 in the presence of 0.1 μg of trypsin/ml, and the virus yield in the culture medium was measured by microplate assay after 24, 48, and 72 h. The virus yield from “pc” samples was considered 100% for comparisons to HD1 samples. The data presented are means ± standard errors of the means of three independent experiments; the P value was calculated by using one-way ANOVA. (D) Total lysates of the cells were prepared, and HDAC1, acetylated-histone H3 (Lys9) (Acet H3; 17 kDa), total histone H3 (Total H3; 17 kDa), and NP were detected by WB. Acet H3 was detected as the HDAC1 substrate, and total H3 was detected as the loading control. (E) Transfection efficiency of A549 cells. Cells were transfected with a plasmid expressing green fluorescent protein as a control. The cells were used, and an image was acquired on an inverted fluorescence microscope (Olympus) under a magnification of ×10. MW, molecular weight.

siRNA (Fig. 2B). Similarly, cells transfected with HDAC1-targeting siRNA released 2.4- and 3.2-fold (P = 0.002) more infectious virions after 12 and 24 h of infection, respectively, than the cells transfected with control siRNA at the corresponding times (Fig. 2C). The WB analysis of infected cell lysates confirmed the depletion of HDAC1 expression at each time point (Fig. 2D). Immunofluorescent staining of NP, followed by confocal microscopy, was used to ascertain that a similar percentage of HDAC1-targeting siRNA (92.4 ± 1.2, n = 5) and control siRNA (93.2 ± 2.2, n = 5)-transfected cells were infected with PR8 after 12 h. Likewise, when infected with PR8 at an MOI of 5.0, HDAC1-depleted A549 cells released 2.3-fold more infectious virions than the control cells after 24 h (data not shown). To further substantiate the higher IAV growth kinetics in the absence of endogenous HDAC1, HDAC1-depleted cells were infected with PR8 after 12 h. Likewise, when infected with PR8 at an MOI of 0.1 in the presence of trypsin, the release of infectious IAV progeny was measured after 24 and 48 h, as described above. Consistent with above data, cells transfected with HDAC1-targeting siRNA released a 2.15-fold (P = 0.0001) more infectious virions than the cells transfected with control siRNA after 24 h of infection (Fig. 2E). After the 48 h of infection, the release of infectious IAV progeny from HDAC1-depleted cells was further increased to 6.4-fold (P = 0.005) compared to control cells (Fig. 2E). Similarly, when infected with PR8 at an MOI of 0.1 in the presence of trypsin, cells transfected with HDAC1-targeting siRNA released 1.6- and 6.8-fold more infectious virions than the cells transfected with control siRNA after 24 and 48 h of infection, respectively (not shown).

**Ectopically expressed HDAC1 inhibits IAV infection.** The data presented above demonstrated that endogenously expressed HDAC1 has an anti-IAV property and that IAV grows to a higher titer in HDAC1-depleted A549 cells. Next, we wanted to find out whether ectopically expressed HDAC1 would have the opposite effect. A549 cells were transfected with an HDAC1-expressing plasmid or an empty plasmid and subsequently infected with PR8 at an MOI of 0.5 for 24 h. The culture medium and the infected cells were harvested separately. The culture medium was analyzed by WB and plaque assay as in Fig. 2B and C, respectively. The cell lysates were subjected to WB to analyze the HDAC1 overexpression and subsequent effect of overexpressing HDAC1 on its substrate acetylated-histone H3 (Lys9) (22–25). Indeed, in contrast to the results obtained with HDAC1 depletion, the overexpression of HDAC1 caused a reduction in IAV infection (Fig. 3). The HDAC1-overexpressing cells released less total viral progeny (Fig. 3A) and a significant 2.3-fold (P = 0.01) fewer infectious virions (Fig. 3B) in the culture medium than did the cells transfected with empty plasmid. Likewise, when infected with PR8 at an MOI of 0.1 in the presence of trypsin, the HDAC1-overexpressing cells released 42% (P < 0.01), 47% (P < 0.01), and 57% (P < 0.01) less infectious viral progeny after 24, 48, and 72 h of infection, respectively, than did the cells transfected with empty plasmid (Fig. 3C). Similar results were obtained when cells were infected with PR8 at an MOI of 5.0 for 24 h (data not shown). The overexpression of HDAC1 in cells was confirmed by WB (Fig. 3D). A visually noticeable reduction in the acetylated-histone H3 (Lys9) level and a negligible change in total histone H3 level in HDAC1-overexp-
proteasome-mediated degradation of HDAC1 polypeptide is plausible, because HDAC1 has been previously reported to be ubiquitinated by Mdm2 E3 ubiquitin ligase (39, 40). It remains to be determined whether HDAC1 is also ubiquitinated in IAV-infected cells. A previous study reported an increase in IAV entry in HDAC1-depleted cells using a microscopy-based assay, but it did not monitor productive IAV replication cycles measuring the release of IAV progeny from HDAC1-depleted cells and did not envisage an anti-IAV role for HDAC1 (41). The outcomes of the HDAC1 knockdown and overexpression experiments described here showing an increase and decrease in IAV progeny release, respectively, confirmed an anti-IAV role of host HDAC1. However, such increase or decrease did not result in a noticeable increase or decrease in intracellular NP levels in HDAC1-depleted or HDAC1-overexpressing cells, respectively. A plausible explanation for this is that NP is one of the most abundant IAV proteins in infected cells, and a significant, but modest 3-fold increase in NP levels that may alter the intracellular abundance of NP in HDAC1-depleted or HDAC1-overexpressing cells, which may not be discernible by WB. Further, a significant, but modest 3-fold increase in the growth characteristics of IAV in HDAC1-depleted cells after 24 h could be partly attributed to the compensatory role of other class I HDACs in the absence of HDAC1. HDAC1 shares at least 86% similarity in nucleotide sequence with HDAC2 and about 63% similarity with HDAC3 (13, 25). It is believed that HDAC1 and HDAC2 genes are a duplicated copy of one gene derived from a common ancestor (13). Evidently, depletion of HDAC1 expression results in the enhanced expression of HDAC2 and HDAC3 (23, 25; P. T. Nagesh and M. Husain, unpublished data.). A further investigation is under way to determine the role of HDAC2 and HDAC3 alone, as well as in conjunction with HDAC1 in IAV infection.

The data showed that deacetylase activity also has an anti-IAV function. Therefore, in addition to expression, IAV also downregulated the deacetylase activity of HDAC1, as determined by detecting the increase in the levels of specific HDAC1 substrate, acetylated-histone H3 (Lys9) (24, 25) in infected cells. One could argue that the increase in the levels of acetylated-histone H3 (Lys9) in IAV-infected cells is due to the decrease in the levels of HDAC1 polypeptide. However, time course experiments revealed a slightly different kinetics for decrease in the HDAC1 polypeptide levels and increase in the acetylated-histone H3 (Lys9) levels. For instance, HDAC1 polypeptide level continued to decrease after 12 h of infection, whereas the HDAC1 deacetylase activity started to come back up to the basal level after 12 h of infection. Nevertheless, a further investigation is needed to delineate the independent roles of HDAC1 catalytic domain and the rest of the HDAC1 polypeptide in its anti-IAV function.

The HDAC1, a mammalian homolog of the yeast pleiotropic transcriptional regulator Rpd3, was the first protein to be shown to have deacetylase activity (42). HDAC3 is primarily localized to the nucleus and prominently deacetylates acetylated lysine residues present at the N-terminal tails of histone H3 and H4 (25). Further, HDAC1 is believed to be the main driver of overall cellular deacetylase activity (23). The catalytic activity of HDAC1 and HDAC2 is largely dependent on its association with multiprotein complexes, which include Sin3, NuRD, CoREST, and NODE complexes (13). It remains to be investigated whether IAV destabilizes or prevents the formation of these complexes to downregulate the HDAC1 activity. Historically, HDAC1 has been described
FIG 5. The inhibition of HDAC1 activity promotes IAV infection. (A to C) A549 cells (8 × 10⁵) were infected with PR8 at an MOI of 0.5 and subsequently treated with DMSO (DMSO) or the indicated concentrations of TSA (in DMSO) for 24 h. (A) The culture medium and the cells were harvested separately, and the virus yield in the culture medium was measured by microplate assay. The data presented are means ± the standard errors of the means of three independent experiments; the P value was calculated by using one-way ANOVA. (B) A549 cells were infected and treated with DMSO and TSA for 24 h, and the cell viability was determined by an MTT assay. The viabilities of DMSO-treated infected cells was considered 100% for comparison to TSA-treated infected cells. (C) Total cell lysates were prepared, and Acet H3, total H3, and NP were detected by WB as the markers of TSA potency, loading control, and infection, respectively, MW, molecular weight.

understood. It has been well established that host cells produce type I IFNs upon IAV infection (33). Type I IFNs then engage host cell type I IFN receptor in an autocrine and paracrine manner and activate the cytoplasmic Janus kinases (JAKs) that in turn, phosphorylate the cytoplasmic signal transducer and activator of transcription 1 (STAT1). The phosphorylated STAT1 (pSTAT1) then translocates to the nucleus and forms a transcription complex called ISG factor 3 (ISGF3). ISGF3 then binds to the IFN-stimulated response element of ISGs in a sequence-specific manner and induces the expression of over 300 ISGs, including IFITM3, ISG15, and viperin, which inhibit IAV infection by targeting various steps of the virus life cycle (33–36). To induce the expression of ISGs, ISGF3 specifically interacts with several coactivators, which include class I and class II HDACs, particularly HDAC1 (28–32). Therefore, to determine the role of class I and class II HDAC activity in type I IFN-mediated host response against IAV, we treated the PR8-infected cells with TSA (1 and 5 μM) and analyzed the levels of pSTAT1 and ISGs, i.e., IFITM3, ISG15, and viperin, by WB. A decrease in the levels of these proteins in TSA-treated infected cells compared to untreated infected cells would indicate the involvement of class I and class II HDAC activity in type I IFN-mediated host antiviral response against IAV. As expected, IAV infection induced the phosphorylation of STAT1 and expression of IFITM3, ISG15, and viperin in A549 cells (Fig. 6). Consistent with this hypothesis, TSA treatment reduced the level of pSTAT1 (Fig. 6A), as well as the levels of IFITM3 (Fig. 6B), ISG15 (Fig. 6C), and viperin (Fig. 6D), in infected cells. TSA seems to have a dose-dependent effect on the phosphorylation of STAT1 (Fig. 6A) and the expression of ISG15 (Fig. 6C), whereas it has more profound effect on the expression of IFITM3 (Fig. 6B) and viperin (Fig. 6D), since they were barely detectable in infected cells treated with TSA.

HDAC1 is involved in IAV-induced viperin expression. It has been demonstrated that HDAC1 specifically coactivates the promyelocytic leukemia zinc finger and promyelocytic leukemia protein that are involved in type I IFN-mediated innate immune response and subsequent expression of ISGs like viperin (32, 37). The above data demonstrated that class I and class II HDAC activity is required for the IAV-induced host type I IFN-mediated antiviral response and the expression of ISGs, including viperin. Therefore, we next sought to determine whether HDAC1 plays a specific role in the expression of viperin in IAV-infected cells. To accomplish this, HDAC1 expression was either depleted by RNA interference (as in Fig. 2) or increased from a plasmid (as in Fig. 3) in A549 cells before infecting them with PR8 and subsequently measuring the viperin levels by WB (as in Fig. 6D). Consistent with the data presented in Fig. 6, viperin expression was decreased in HDAC1-depleted infected cells (Fig. 7A) and increased in HDAC1-overexpressing infected cells (Fig. 7C). About 58% less (P = 0.0001) viperin was detected in infected cells transfected with HDAC1-targeting siRNA than in infected cells transfected with nontargeting control siRNA (Fig. 7B). Conversely, about 55% more (P = 0.0001) viperin was detected in infected cells transfected with HDAC1-expressing plasmid than in infected cells transfected with empty plasmid (Fig. 7D). Interestingly, a noticeable increase in the basal level of viperin was also observed in uninfected cells transfected with HDAC1-expressing plasmid (Fig. 7C).

DISCUSSION

The data presented here demonstrate that HDAC1 is a component of host antiviral response against IAV and that, in turn, IAV dysregulates HDAC1 to undermine its role in host antiviral response. IAV downregulates HDAC1 expression at both the mRNA and the polyprotein level. The reduction in HDAC1 mRNA level in IAV-infected cells observed here is consistent with a recent microarray-based gene expression profiling study (38). By using mainly a luciferase reporter assay and quantitative real-time PCR, Buggele et al. (38) demonstrated that IAV induces host microRNA, miR-449b that targets the 3′ untranslated region of HDAC1 mRNA, and when added exogenously, miR-449b further reduced HDAC1 mRNA level in infected cells. However, they did not determine HDAC1 polyprotein level in response to the direct IAV infection and could not establish a direct anti-IAV role of miR-449b or HDAC1 since the depletion or overexpression of miR-449b did not have an effect on IAV infection (38). Here, we demonstrated that IAV also reduces the HDAC1 polyprotein level in infected cells by promoting its degradation by a proteasomal pathway. A
as the regulator of chromatin structure and repressor of gene transcription (13). However, lately HDAC1 has also been implicated in nontranscriptional functions such as DNA repair, splicing, and cell division and has been described as both a positive and a negative regulator of gene expression (13, 24, 42). Some of the genes that are positively regulated by HDAC1 are type I IFNs and ISGs, such as ISG15, ISG54, IFITM1, IFITM2, and viperin (24, 32, 37, 44). Similarly, cellular deacetylase activity also promotes the type I IFN-stimulated expression of ISGs (28–30). In other words, the expression of type I IFNs and ISGs is downregulated in HDAC1-deficient cells and in cells treated with HDAC inhibitors. Therefore, a noticeable decrease in pSTAT1 and IFTM3, ISG15, and viperin levels in TSA-treated IAV-infected cells shown here indicated that class I and class II HDAC activity is an important component of canonical type I IFN-mediated host antiviral responses against IAV. Specifically, the decrease or increase in the IAV-induced expression of viperin in HDAC1-depleted or HDAC1-overexpressing cells, respectively, indicated that HDAC1 is a co-factor for the expression of viperin in IAV-infected cells. However, it is unlikely that viperin is the only ISG through which HDAC1 is exerting its anti-IAV function. A direct role of HDAC1 in the expression of other ISGs that inhibit IAV infection remains to be investigated. Further, HDAC1 has been reported to inhibit IAV entry by interfering with host microtubule-mediated endosomal transport, but a direct link between exclusively nuclear HDAC1 and exclusively cytoplasmic microtubule network remains to be elucidated (41).

Previously, a role of HDAC1 (and HDAC2 and -3) has also been studied in the infection of other viruses, especially herpesviruses and HIV-1 that cause both latent and lytic infections (45-46). Paradoxically, these HDACs play opposing roles during latent and lytic viral infections (46). Because of their role in promoting the compact chromatin organization and repression of transcription, these HDACs promote latency of herpesviruses and HIV-1 and have been the target of HDAC inhibitors to reactivate these viruses for getting rid of the infection (47–50). In contrast, during herpesvirus infections, HDAC1 (and HDAC2) plays an antiviral role by repressing the viral gene transcription and inducing the type I IFN response (44, 49, 51). In gammaherpesvirus-infected primary microphages, HDAC1 (and HDAC2) has been shown to be involved at early steps of the induction of type I IFN response and required for the phosphorylation of interferon regulatory factor 3 (IRF3), the accumulation of phosphorylated IRF3 at the IFN-β promoter, and the subsequent production of type I IFNs (44). The data we have presented here indicate that HDAC1 and deacetylase activity are involved in JAK/STAT pathway induced in IAV-infected cells. However, our data do not demonstrate that HDAC3 or deacetylase activity work upstream and are involved in the production of type I IFNs in IAV-infected cells. It will be interesting to examine the phosphorylation of IRF3 and subsequent production of type I IFNs in HDAC1-depleted and HDAC1-overexpressing cells. The complex IFN signaling pathways represent the first line of host antiviral defense against invading viruses, including IAV. However, like other viruses, IAV has evolved various strategies to subvert IFN signaling to multiply and cause respiratory disease. It has yet to be determined whether HDAC1 plays more of a canonical role or a unique role in the IAV-induced host antiviral response.

With this report, it is clear that at least one member of three main classes of HDACs possesses an anti-IAV function, and HDACs are potentially a novel family of anti-IAV host factors. Recently, we reported that HDACs, a class II member, inhibits IAV infection (4). Likewise, Koyuncu et al. recently demonstrated that class III HDACs (sirtuins) inhibit IAV infection, too (52). Because of their role in multiple biological processes, HDACs have been the subjects of therapeutic targets for treatment of various human diseases, such as different types of cancers, neurodegen-
erative diseases, and inflammatory disorders (33). The regular emergence of novel, drug-resistant, and zoontic IAV strains in humans emphasizes the need for the development of an alternative, next-generation anti-IAV strategy. A molecular understanding of IAV and HDACs interplay may lead to the development of such a strategy.

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The funders had no role in experimental design, data acquisition and interpretation, or the decision to submit the data for publication.

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Histone Deacetylase 1 is an Anti-influenza Host Factor

### Appendix III: Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>μg</td>
<td>Microgram</td>
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<tr>
<td>μl</td>
<td>Microlitre</td>
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<tr>
<td>μM</td>
<td>Micromolar</td>
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<tr>
<td>A549</td>
<td>Human lung carcinoma cell, A549</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dsRNA</td>
<td>Double stranded RNA</td>
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<tr>
<td>EDTA</td>
<td>Ethylenedinitrilotetraacetic acid</td>
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<tr>
<td>eIF</td>
<td>Eukaryotic translation initiation factor</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>HA</td>
<td>Hemagglutinin</td>
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<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
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<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HEK 293T</td>
<td>Human embryonic kidney cell, 293T</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HeLa</td>
<td>Human cervical cancer cell</td>
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<tr>
<td>HI</td>
<td>Hemagglutination inhibition</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>IP</td>
<td>Immunoprecipitation</td>
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<td>K</td>
<td>Lysine</td>
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<tr>
<td>L</td>
<td>Litre</td>
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<tr>
<td>IFITM</td>
<td>Interferon inducible transmembrane protein</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<td>Interferon alpha</td>
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<tr>
<td>IFNβ</td>
<td>Interferon beta</td>
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<tr>
<td>IKK</td>
<td>Inhibitor of nuclear factor kappa-B kinase</td>
</tr>
<tr>
<td>IKKα</td>
<td>Inhibitor of nuclear factor kappa-B kinase alpha</td>
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<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
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<tr>
<td>IRF3</td>
<td>Interferon regulatory factor 3</td>
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<td>Interferon stimulated gene 15</td>
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<td>ISREs</td>
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<td>JAK</td>
<td>Janus-activated kinase</td>
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<tr>
<td>KD</td>
<td>Knockdown</td>
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<td>M1</td>
<td>Matrix protein 1</td>
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<tr>
<td>M2</td>
<td>Matrix protein 2</td>
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<td>MAVS</td>
<td>Mitochondrial antiviral signaling</td>
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<tr>
<td>MDA5</td>
<td>Melanoma differentiation-associated protein 5</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
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<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>Mx1</td>
<td>Myxovirus resistance 1</td>
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<td>Myeloid differentiation primary response gene 88</td>
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<td>NEP</td>
<td>Nuclear export protein</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
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<td>NLS</td>
<td>Nuclear localization signal</td>
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<td>NP</td>
<td>Nucleoprotein</td>
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<td>Non Structural protein</td>
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<td>PA</td>
<td>Polymerase Acid</td>
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<td>Polymerase Basic 1</td>
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<td>Abbreviation</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PFU</td>
<td>Plaque forming unit</td>
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<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<td>PKR</td>
<td>Protein kinase R</td>
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<td>Polymerase</td>
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<td>PRR</td>
<td>Pathogen recognition receptor</td>
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<td>p.i.</td>
<td>Post infection</td>
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<td>qPCR</td>
<td>Quantitative real-time PCR</td>
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<td>RNA</td>
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<td>Ribonucleoprotein</td>
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<td>rpm</td>
<td>Revolutions per minute</td>
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<td>Sialic acid</td>
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<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
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<td>Ultraviolet</td>
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