Among the hundreds of thousands, and occasionally millions of cell types that make up a person's body, it is the immune cells that we rely on most for surveillance and protection against pathogens. The immune system is a complex array of cells, organs, and molecules that work together to identify and destroy foreign invaders, which is the primary function of the adaptive immune system. This process involves the recognition of specific foreign molecules, usually proteins or carbohydrates, by the immune system, which then mounts a coordinated attack against the invader. The immune system is also responsible for maintaining the body's internal balance, or homeostasis, by detecting and responding to changes in the body's environment. This balance is essential for maintaining the body's normal functioning and for preventing the development of diseases or infections.

Immune System Functions

The immune system has several key functions, including:

1. **Recognition and Response**: The immune system recognizes and responds to foreign substances, such as bacteria, viruses, and other pathogens. This recognition is achieved through the actions of specific immune cells, which can include B cells, T cells, natural killer (NK) cells, and macrophages.

2. **Elimination of Pathogens**: Once the immune system recognizes a pathogen, it mounts a coordinated attack against the invader. This attack can involve the release of substances that kill the pathogen, as well as the direct destruction of infected cells by immune cells.

3. **Maintenance of Homeostasis**: The immune system plays a crucial role in maintaining the body's internal balance, or homeostasis. This balance is essential for preventing the development of diseases or infections.

4. **Memory and Longevity**: The immune system remembers pathogens that it has encountered in the past, allowing it to respond more quickly and effectively to the same pathogen in the future. This memory is essential for preventing the development of chronic infections and for ensuring the longevity of the immune system.

5. **Regulation and Modulation**: The immune system also plays a role in regulating and modulating the body's response to various stimuli. This modulation is achieved through the actions of specific immune cells, which can include regulatory T cells (Tregs), which suppress the immune response, and regulatory B cells (Bregs), which inhibit the activation of immune cells.

The immune system is composed of numerous cell types and molecules that work together to protect the body from infection and disease. These components include:

- **White Blood Cells**: These cells are responsible for the body's immune response. They include B cells, T cells, natural killer (NK) cells, and macrophages.

- **Antibodies**: These are proteins produced by B cells that help the immune system recognize and neutralize foreign substances.

- **Complement System**: This is a group of proteins that work together to help the immune system eliminate pathogens.

- **Inflammation**: This is a natural response of the immune system to injury or infection. It involves the release of chemicals that cause the blood vessels to dilate, making them more permeable, and the recruitment of immune cells to the area of injury.

The immune system is a complex and highly regulated system that performs a critical role in protecting the body from infection and disease. It is essential for maintaining the body's internal balance, or homeostasis, and for ensuring the longevity of the immune system.
Anti-influenza mechanism and antagonism of host HDAC4

So far, 18 HDACs have been identified in the human HDAC family and classified into four main classes based on sequence similarities to yeast proteins (13). The class I comprises four members, HDAC1, -2, -3, and -8. The class II has been subclassified into class IIa and class IIb with four members (HDAC4, -5, -7, -9) and two members (HDAC6 and -10), respectively. The class III possesses seven members, which are commonly known as sirtuins (SIRT 1–7). Finally, class IV is comprised of only one member, HDAC11. We and others have demonstrated that at least one member of each class/subclass (i.e., HDAC1, -2, -6, -11, and SIRT 1–7) possesses anti-IAV properties (9–12, 26). We have also demonstrated that, in turn, IAV antagonizes the antiviral function of identified HDACs by employing various strategies, such as down-regulation of their expression at mRNA level (e.g., HDAC11 (12)) and facilitating their degradation by host proteasome machinery (e.g., HDAC1 and HDAC2, Ref. 10, 11). However, a role of class IIa members in IAV infection has not been investigated. Here we demonstrate that HDAC4 (a class IIa member) possesses anti-IAV properties and, in turn, IAV antagonizes the antiviral function of HDAC4, rather strongly, by down-regulating its mRNA level via viral protein PA-X as well as facilitating the proteolytic degradation of its polypeptide by host caspases.

Results

Human HDAC4 possesses the anti-IAV properties

To investigate a role for HDAC4 in IAV infection, we employed the HDAC4 depletion and overexpression strategies. For depletion of HDAC4 expression, we employed RNAi and obtained a predesigned small interfering RNA (siRNA) oligonucleotide targeting human HDAC4. The A549 cells were transiently transfected with different nanomolar (nM) quantities of HDAC4-targeting siRNA and a nontargeting control siRNA. After 72 h, the depletion of HDAC4 polypeptide was analyzed and quantified by Western blotting. To quantify the depletion efficiency, the intensity of HDAC4 and protein-disulfide isomerase (PDI, as loading control) polypeptide bands on blots was quantified using Image Studio Lite software (version 5.0, LI-COR). Then, the amount of HDAC4 was normalized with the corresponding PDI amount. We found that 1 nM quantity of HDAC4 siRNA was sufficient to deplete HDAC4 polypeptide expression by >98% (Fig. 1, A, UNI/H4D4 lane, and B), without significantly influencing the cell viability (Fig. 1C). For further experiments, 1 nM concentration of HDAC4, as well as control siRNA, was used to deplete HDAC4 expression. Next, A549 cells, transfected with HDAC4 siRNA or control siRNA were either infected with influenza virus A/PR/8/34(H1N1) strain (henceforth referred to as PR8) or influenza A/California/07/2009(H1N1) strain (henceforth referred to as CA09) at a multiplicity of infection (m.o.i.) of 1.0. After 24 h, the culture medium and the infected cells were harvested separately. The culture medium was either titrated by microplate assay on MDCK cells or analyzed by Western blotting detection of viral hemagglutinin (HA) and nucleoprotein (NP). The former was employed to quantify the amounts of released infectious viral progeny, whereas the latter was employed to measure the released total viral progeny. On the other hand, the infected cells were processed either for Western blot to detect the intracellular level of HDAC4, viral NP, or PDI polypeptides or for quantitative real-time PCR (qPCR) to measure the intracellular level of viral NP and M mRNAs. By employing these methods, we found that the growth of IAV was higher in HDAC4-depleted cells compared with control cells (Fig. 1, D–H), indicating an antiviral function of host HDAC4. Specifically, there was a significant 2.6- (p = 0.0123) and 2.7-fold (p = 0.0088) increase in the release of PR8 and CA09 infectious progeny, respectively, from HDAC4-depleted cells than the control cells (Fig. 1D). Similar results were obtained when PR8 progeny release was measured from the infected cells treated with HDAC inhibitors, trichostatin A and SAHA (Fig. S1). Furthermore, the ratio of viral NP and HA levels detected by Western blotting (Fig. 1E) in culture medium harvested from the control cells and HDAC4-depleted cells was 1:2.5 (n = 3, p = 0.006) and 1:1.7 (n = 3, p = 0.001), respectively. Furthermore, there was a significant 1.9- (p = 0.0087) and 3.8-fold (p = 0.0158) increase in the intracellular level of PR8 NP and M mRNAs, respectively, in HDAC4-depleted cells compared with control cells (Fig. 1F). Likewise, the intracellular level of CA09 NP and M mRNAs was also increased by significant 1.8- (p = 0.0004) and 3.0-fold (p = 0.048), respectively, in HDAC4-depleted cells compared with control cells (Fig. 1F). Finally, there was a significant 1.9-fold (p = 0.027) increase in the intracellular NP polypeptide level in HDAC4-depleted cells compared with control cells (Fig. 1G).

The immunofluorescent staining of viral NP and host cell nuclei after 12 h of infection followed by cell counting was used to ascertain that a similar percentage of HDAC4 siRNA-transfected cells (71%, n = 4, p = <0.0001) and control siRNA-transfected cells (69%, n = 4, p = <0.0001) were infected with PR8. The depletion of HDAC4 at the polypeptide level in infected cells was confirmed by Western blotting (Fig. 1A, INF/H4D4 lane). However, we noticed that, after 24 h of infection, the level of HDAC4 polypeptide in infected cells (Fig. 1A, INF/CT lane) was much lower than its level in uninfected cells (Fig. 1A, UNI/CT lane) (see Fig. S1). This indicated that, like HDAC1 (10) and HDAC2 (11), IAV is also inducing the down-regulation of HDAC4 polypeptide in infected cells to antagonize its antiviral function. Such HDAC4 polypeptide down-regulation is potentially the reason we observed a modest increase in IAV growth characteristics in HDAC4-depleted infected cells when compared with control infected cells above. IAV on its own was able to deplete the HDAC4 level in control cells hence skewing the real comparison with HDAC4-depleted cells. Before further investigating the HDAC4 down-regulation and its mechanism in response to IAV infection, we analyzed the IAV growth kinetics in HDAC4-depleted cells infected with a lower m.o.i. to minimize the extent of HDAC4 antagonism in control cells and aid the comparison. For this, A549 cells, transfected with HDAC4 or control siRNA as above, were infected with PR8 at a m.o.i. of 0.1. Subsequently, the release of viral progeny in culture medium was quantified by microplate assay after 6, 12, and 24 h of infection. Consistent with above data, IAV exhibited faster growth kinetics in HDAC4-depleted cells compared with control cells (Fig. 1H). But, here the mag-
Figure 1. The knockdown of HDAC4 expression promotes IAV infection. A–F, A549 cells were transfected with 1 nM control siRNA or HDAC4 siRNA for 72 h. The cells were then infected with IAV PR8 or CA09 strains at a m.o.i. of 1.0. After 24 h, the cells and the medium was harvested separately. A, total lysates of uninfected (UNI) and PR8-infected (INF) cells transfected with control siRNA (CT) or HDAC4 siRNA (HD4) were prepared, and the HDAC4 (140 kDa), PDI (57 kDa), and viral NP (56 kDa) polypeptides were detected by Western blotting. Note: the HDAC4 blot was reused to probe for PDI and NP, and the MW, UNI/CT, and UNI/INF lanes were combined with INF/CT and INF/HD4 lanes after cropping out the less relevant middle lanes of same blot. B, the HDAC4 and PDI bands in panel A were quantified using the Image Studio Lite software (LI-COR). The level of HDAC4 polypeptide in each sample was then normalized with corresponding PDI levels. Finally, the normalized level of HDAC4 polypeptide in control siRNA-transfected cells was considered 100% to compare its level in HDAC4 siRNA-transfected cells. C, A549 cells were transfected with either Lipofectamine (LF) alone or in complex with control siRNA or HDAC4 siRNA. After 72 h, the viability of cells was determined by MTT assay. Then, the viability of Lipofectamine only transfected cells was considered 100% to compare the viability of the cells transfected with control siRNA and HDAC4 siRNA. D, the culture medium from the cells transfected with control siRNA or HDAC4 siRNA and infected with IAV PR8 or CA09 strains was titrated on MDCK cells by microplaque assay. Then, the amount of viral progeny released from control siRNA-transfected cells was considered 1-fold to compare the amount of viral progeny released from HDAC4 siRNA-transfected cells. E, the total culture medium from the cells transfected with control siRNA or HDAC4 siRNA and infected with PR8 was concentrated by TCA precipitation, and the levels of viral HA (68 kDa) and viral NP were detected by Western blotting. Note: the HA blot was reused to probe for NP, and the MW lane was combined with CT and HD4 lanes after cropping out the less relevant middle lanes of same blot. F, total RNA from the cells transfected with control siRNA or HDAC4 siRNA and infected with PR8 or CA09 was isolated, and the levels of viral NP, viral M, and actin mRNAs were detected by qPCR. Then, the levels of NP and M mRNAs were normalized with the level of corresponding actin mRNA. G, the NP polypeptide level in infected cells in panel A was quantified and normalized as in panel B. Then, the normalized level of NP in control siRNA-transfected cells was considered 1-fold to compare its level in HDAC4 siRNA-transfected cells. H, A549 cells, transfected with control siRNA or HDAC4 siRNA for 72 h, were infected with PR8 at a m.o.i. of 0.1. After 6, 12, and 24 h of infection, the culture medium was titrated by microplaque assay to determine the titers of released viral progeny. Error bars represent the mean ± S.E. of three independent experiments (B, D, F, and G), biological replicates (H), or technical replicates (C). The asterisks represent p values mentioned in the text calculated by unpaired t test (B, F, and G) or ANOVA (C, D, and H), and indicate the significant differences in means. MW, molecular weight; CT, control siRNA; HD4, HDAC4 siRNA.

To further validate that host HDAC4 possesses anti-IAV properties, we analyzed IAV growth characteristics in A549 cells overexpressing human HDAC4 from a plasmid. However, like endogenously-expressed HDAC4 polypeptide, the level of plasmid-expressed HDAC4 polypeptide was also significantly decreased in A549 (and MDCK) cells after IAV infection. Therefore, we switched to HEK-293T cells, and transfected them with HDAC4 plasmid or an empty plasmid and subsequently infected them with PR8 at a m.o.i. of 1.0. After 24 and 48 h of infection, the culture medium was titrated by microplaque assay to quantify released infectious PR8 progeny and the cell lysates were analyzed by Western blotting to confirm HDAC4 overexpression. In HEK-293T cells, the level of ectopically-expressed HDAC4 polypeptide under the uninfected and infected conditions were comparable although there was sign of its degradation under the latter (Fig. 2A). Consistent with the results obtained with HDAC4 depletion, overexpression of HDAC4 inhibited the IAV infection. Specifically, compared with control cells, there was a significant 41.6 (p = 0.004) and 46.5% (p = 0.002) decrease in the release of PR8 progeny from HDAC4-overexpressing cells after 24 and 48 h of infection, respectively (Fig. 2B). Furthermore, compared with control cells, there
Anti-influenza mechanism and antagonism of host HDAC4

IAV down-regulates the HDAC4 polypeptide level in mammalian cells

As mentioned above, the levels of both endogenously- and ectopically-expressed HDAC4 polypeptides were down-regulated in A549 cells in response to IAV infection. We next endeavored to understand the kinetics as well as the mechanism of this phenomenon. To understand the former, A549 or MDCK cells were infected with PR8 at a m.o.i. of 0.5 or 5.0. After 24 h, the HDAC4 polypeptide levels in total cell lysates were analyzed by Western blotting. We found that, indeed, IAV down-regulated the HDAC4 polypeptide level in both MDCK (Fig. 3A) and A549 (Fig. 3B) cells in a dose-dependent manner. To quantify the extent of HDAC4 polypeptide down-regulation, the intensity of HDAC4 and actin (loading control) bands was quantified as above. Then, the amount of HDAC4 was normalized with a corresponding actin amount. Finally, the normalized amount of HDAC4 in uninfected cells was considered 100% to compare its amount in infected cells. We found that, in MDCK cells, there was a significant dose-dependent 53.8 (p = 0.03) and 70.5% (p = 0.02) decrease in HDAC4 polypeptide level in response to infection with PR8 at m.o.i. of 0.5 and 5.0, respectively (Fig. 3F). Likewise, in A549 cells, there was a significant dose-dependent 40 (p = 0.04) and 63.7% (p = 0.03) decrease in HDAC4 polypeptide level in response to infection with PR8 at m.o.i. of 0.5 and 5.0, respectively (Fig. 3F). Next, to determine whether the down-regulation of HDAC4 polypeptide in response to IAV infection was strain-independent, MDCK cells were infected with IAV CA09 or WSN strains at a m.o.i. of 0.5 or 5.0. Subsequently, the level of HDAC4 polypeptide was detected and quantified as above. Consistent with above results, both CA09 (Fig. 3C) and WSN (Fig. 3D) strains down-regulated the HDAC4 polypeptide level in a dose-dependent manner. Specifically, there was 55.3 (n = 1) and 86.3% (n = 1) reduction in the HDAC4 polypeptide level in response to infection with CA09 at m.o.i. of 0.5 and 5.0, respectively. Similarly, the level of HDAC4 polypeptide was reduced by 56.7 (n = 1) and 78.8% (n = 1) in the cells infected with WSN at m.o.i. of 0.5 and 5.0, respectively. Finally, by using an UV-irradiated PR8 inoculum, we confirmed that a replication-competent IAV was required to down-regulate the HDAC4 polypeptide level in infected cells (Fig. 3F).

Next, to determine the kinetics of HDAC4 polypeptide down-regulation, MDCK cells were infected with PR8 at a m.o.i. of 0.5, and subsequently harvested after 2, 6, 12, and 24 h after infection. The level of HDAC4 polypeptide at each time point was then analyzed and quantified as above. Consistent with the above results, both CA09 (Fig. 3C) and WSN (Fig. 3D) strains down-regulated the HDAC4 polypeptide level in a dose-dependent manner. Specifically, there was 55.3 (n = 1) and 86.3% (n = 1) reduction in the HDAC4 polypeptide level in response to infection with CA09 at m.o.i. of 0.5 and 5.0, respectively. Similarly, the level of HDAC4 polypeptide was reduced by 56.7 (n = 1) and 78.8% (n = 1) in the cells infected with WSN at m.o.i. of 0.5 and 5.0, respectively. Finally, by using an UV-irradiated PR8 inoculum, we confirmed that a replication-competent IAV was required to down-regulate the HDAC4 polypeptide level in infected cells (Fig. 3F).

The caspase 3 cleaves HDAC4 polypeptide in infected cells

Next, we endeavored to further understand the mode of proteolytic degradation of HDAC4 polypeptide in IAV-infected...
Figure 3. IAV down-regulates the level of HDAC4 polypeptide in epithelial cells. MDCK (A and C) or A549 (B and D) cells were infected with IAV PR8 (A and B), CA09 (C), or WSN (D) strains at a m.o.i. of 0.5 or 5.0. After 24 h, the uninfected (UNI) and infected (0.5, 5.0) cells were harvested, total cell lysates were prepared, and the HDAC4, actin (42 kDa), and viral NP polypeptides were detected by Western blotting. E, MDCK cells were infected with live (INF) or UV-irradiated (INFUV) PR8 at an m.o.i. of 0.5. After 24 h, total cell lysates were prepared, and the HDAC4, actin, and NP polypeptides were detected by Western blotting. Note: the HDAC4 blots were reused to probe for actin. F, the level of HDAC4 polypeptide in panels A and B blots were quantified and normalized as described in the legend to Fig. 1B. Then, the normalized level of HDAC4 polypeptide in uninfected cells (UNI) was considered 100% to compare its level in cells infected (INF) with 0.5 and 5.0 m.o.i. G, MDCK cells were infected with PR8 at a m.o.i. of 0.5, and harvested after 2, 6, 12, and 24 h of infection. The total cell lysates were resolved on 7–12% gradient SDS-PAGE, and the HDAC4, PDI, and NP polypeptides were detected by Western blotting. Arrows indicate the HDAC4 polypeptide cleavage products. Note: the HDAC4 blot was reused to probe for PDI, and the MW lane was combined with rest of the lanes after cropping out the inconsistent second lane of same blot. H, the level of full-length HDAC4 polypeptide in panel G was quantified and normalized as described in the legend to Fig. 1B. Then, the normalized level of HDAC4 polypeptide at 2 h infection was considered 100% to compare its levels at 6, 12, and 24 h infection. I, MDCK cells were infected with PR8 and harvested at the times indicated in panel G. Then, the full-length caspase 3 (Cas3-FL) and cleaved caspase 3 (Cas3-CL) polypeptides were detected in total cell lysates by Western blotting. Note: the MW lane was combined with rest of the lanes after cropping out the less relevant middle lanes of same blot. Error bars represent the mean ± S.E. of three independent experiments. The asterisks represent p values mentioned in the text calculated by unpaired t test (F) or ANOVA (H), and indicate the significant differences in means. MW, molecular weight.
cells. Primarily, lysosome-mediated and proteasome-mediated pathways facilitate the degradation of polypeptides in mammalian cells. To identify the pathway leading to the degradation of HDAC4 polypeptide in IAV-infected cells, we perturbed these two pathways. For this, MDCK cells were infected with PR8 as above and subsequently treated with lysosome inhibitor, NH4Cl, or proteasome inhibitor, MG132. In addition, one set of infected cells was also treated with a caspase 3 inhibitor (Cas3-I). After 24 h, the HDAC4 polypeptide levels were analyzed and quantified as described above. We found that the treatments with NH4Cl and Cas3-I rescued the HDAC4 polypeptide level in infected cells. Specifically, compared with 81% \((p < 0.0001)\) reduction in the HDAC4 polypeptide level in mock-treated infected cells, there was only 54 and 46% reduction in HDAC4 polypeptide levels in NH4Cl–treated and Cas3–I–treated infected cells, respectively (Fig. 4B). In other words, the level of HDAC4 polypeptide in infected cells recovered from 19 to 46% (statistically nonsignificant) and a significant 54% \((p = 0.02)\) after the treatment with NH4Cl and Cas3–I, respectively (Fig. 4B). Such recovery in the HDAC4 polypeptide level in Cas3–I–treated infected cells also coincided with the inhibition of proteolytic activation of caspase 3 in infected cells (Fig. 4A, last panel, Cas3–I/INF lane). Furthermore, the proteolytic activation of caspase 3 was also slightly reduced in NH4Cl–treated infected cells (Fig. 4A, last panel, compare Mock/INF and NH4Cl/INF lanes). This indicated that the proteolytic activation of caspase 3 was associated with lysosomal activity. On the contrary, treatment with MG132 worsened the degradation of HDAC4 polypeptide in infected cells (Fig. 4A, MG132/INF lane). Specifically, compared with 81% \((p < 0.0001)\) reduction in the HDAC4 polypeptide level in mock-treated infected cells, there was a significant 94.4% \((p = 0.0039)\) reduction in the HDAC4 polypeptide level in MG132–treated infected cells (Fig. 4B). The MG132 treatment even induced the HDAC4 polypeptide degradation in uninfected cells (Fig. 4A, MG132/UNI lane). This effect of MG132 treatment on the HDAC4 polypeptide level, both in uninfected and infected cells, further indicated that the HDAC4 polypeptide is degraded by caspases. It is known that MG132 treatment induces the apoptosis, hence the activation of caspases in mammalian cells (27). Consistent with this, we observed a rather profound proteolytic activation of caspase 3 both in uninfected and infected cells treated with MG132 (Fig. 4A, last panel, MG132/UNI and MG132/INF lanes). Interestingly, MG132 treatment of infected cells also increased the Western blot detection limit and revealed that the HDAC4 polypeptide was actually being cleaved into two fragments, resolving around the 36-kDa marker (indicated by arrows a and b in Fig. 4A) instead of one observed in mock–treated infected cells. To further confirm the role of caspase 3 in HDAC4 polypeptide degradation in response to IAV infection, we knocked down the expression of caspase 3 in A549 cells by RNAi. Then, A549 cells were infected with PR8 and the level of HDAC4 polypeptide was analyzed and quantified by Western blotting as described above. Consistent with the above observations, the knockdown of caspase 3 expression (>90%, confirmed by qPCR, Fig. 4E) rescued the level of HDAC4 polypeptide in infected cells (Fig. 4C). Specifically, compared with 77% \((p < 0.0001)\) reduction in the HDAC4 polypeptide level in infected cells transfected with control siRNA, there was only 28% \((p = 0.004)\) reduction in the HDAC4 polypeptide level in infected cells transfected with caspase 3 siRNA (Fig. 4D). In other words, the level of HDAC4 polypeptide in infected cells recovered from 22.7% to a significant 71.7% \((p = 0.0002)\) after the depletion of caspase 3 expression (Fig. 4D).

### The IAV protein PA-X is involved in down-regulation of HDAC4 in infected cells

To further understand the mechanism of HDAC4 antagonism, we next endeavored to identify the viral gene(s) IAV is employing to down-regulate the HDAC4 expression in infected cells. To accomplish this, we employed RNAi to knockdown the expression of 6 internal IAV genes: M, NP, NS, PA, PB1, and PB2, by using the previously described siRNAs (28). However, the M and NS siRNAs did not work in our hands and potency of PB2 siRNA could not be confirmed. Nevertheless, we delivered control, NP, NS (as negative control), PA, and PB1 siRNAs to A549 cells and subsequently infected them with PR8 at a m.o.i. of 1.0. Then, the levels of HDAC4 polypeptide in each sample were analyzed and quantified by Western blotting as described above. Of the viral genes that were targeted, only the knockdown of PA gene expression rescued the level of HDAC4 polypeptide in infected cells (Fig. 5A). Specifically, compared with 73.6% \((p < 0.0001)\) reduction in the HDAC4 polypeptide level in infected cells transfected with control siRNA, there was only 36% \((p = 0.0001)\) reduction in the HDAC4 polypeptide level in infected cells transfected with PA siRNA (Fig. 5B). In other words, the level of HDAC4 polypeptide in infected cells recovered from 26.4% to a significant 63.8% \((p = 0.0001)\) after the knockdown of PA expression (Fig. 5B). The knockdown in NP, PA, and PB1 mRNA expression by a significant 83.7, 96.7, and 87% \((p < 0.0001)\), respectively, was confirmed by qPCR (Fig. 5C). The depletion of NP polypeptide was also confirmed by Western blotting (Fig. 5A). Although knockdown of PA and PB1 expression at the mRNA level above reduced the viral gene expression (as indicated by reduced NP polypeptide level detected in Fig. 5A) and the yield of released viral progeny (not shown), it did not fatally affect the viral growth. Therefore, this and nonworking NS siRNA helped to conclude that the rescue of HDAC4 polypeptide in PA mRNA knockdown cells was specific. Next, we wondered whether IAV PA-X was involved in the down-regulation of HDAC4 expression. The PA-X is a second product of PA mRNA resulting from the ribosomal frameshifting during translation (29), and is implicated in shutoff of host gene expression (30) and consequently dampening of host innate anti-IAV response. We expressed PA-X in A549 cells from a plasmid (31) and subsequently analyzed and quantified the HDAC4 polypeptide level by Western blotting as described above. Indeed, like IAV infection, the expression of PA-X alone down-regulated the HDAC4 polypeptide level in A549 cells (Fig. 5D). Specifically, when compared with the cells transfected with empty plasmid, there was a significant 66% \((p = 0.0002)\) reduction in the HDAC4 polypeptide level in cells transfected with PA-X plasmid (Fig. 5E). The expression of PA-X in A549 cells was confirmed by absolute qPCR (Fig. 5F).

Interestingly, like in infected cells, we could not detect a cleavage product of HDAC4 polypeptide in the cells expressing
PA-X alone (compare HDAC4 blots in Fig. 5, A and D). Furthermore, PA-X–mediated down-regulation of full-length HDAC4 polypeptide could not be rescued by Cas3-I treatment (Fig. S2). This indicated that, in addition to down-regulating the HDAC4 expression at the polypeptide level via caspase 3, IAV is also down-regulating HDAC4 expression at the mRNA level; and IAV is accomplishing this by employing PA-X, a RNA endonuclease (29). To investigate this further, A549 cells were transfected with control siRNA or PA-targeting siRNA, and subsequently infected with PR8 at a m.o.i. of 1.0. After 24 h infection, cells were processed to measure the levels of HDAC4 mRNA and actin mRNA and 18S rRNA (18S RNA) as reference by qPCR. Indeed, we discovered that the level of HDAC4 mRNA in A549 cells was reduced in response to IAV infection (Fig. 6A).

Figure 4. The caspase 3 cleaves HDAC4 polypeptide in infected cells. A, MDCK cells were infected with PR8 at a m.o.i. of 0.5 and, after removing the virus inoculum treated with NH₄Cl (10 mM), MG132 (20 μM), or caspase 3 inhibitor (Cas3-I; 40 μM). After 24 h, the uninfected (UNI) and infected (INF) cells were harvested, total cell lysates were prepared, and the HDAC4, PDI, viral NP, and full-length caspase 3 (Cas3-FL) and cleaved caspase 3 (Cas3-CL) polypeptides were detected by Western blotting. Note: the HDAC4 blot was reused to probe for PDI and NP. Arrows indicate the HDAC4 polypeptide cleavage products. B, the full-length HDAC4 polypeptide level in panel A was quantified and normalized as described in the legend to Fig. 1. Then, the normalized level of HDAC4 polypeptide in mock-treated uninfected (UNI) cells was considered 100% to compare its levels in mock-, NH₄Cl-, MG132-, and Cas3-I–treated infected (INF) cells. C, A549 cells were transfected in duplicate with 10 nm control siRNA (CT) or caspase 3 siRNA (Cas3) for 72 h. One set of cells was infected with PR8 at a m.o.i. of 1.0. After 24 h, cells were harvested and the HDAC4, NP, and actin polypeptides were detected in total cell lysates by Western blotting. Note: the HDAC4 blot was reused to probe for actin and NP. Arrows indicate the HDAC4 polypeptide cleavage products in the higher exposure of the dotted rectangle. D, the full-length HDAC4 polypeptide level in panel C was quantified and normalized as described in the legend to Fig. 1. Then, the normalized level of HDAC4 polypeptide in uninfected/control siRNA-transfected cells (UNI/CT) was considered 100% to compare its levels in infected/control siRNA-transfected cells (INF/CT) and infected/caspase 3 siRNA-transfected cells (INF/Cas3). E, the second set of cells from panel C was processed to detect the levels of caspase 3 and actin mRNAs by qPCR. Then, the levels of caspase 3 mRNA in control siRNA-transfected cells and caspase 3 siRNA-transfected cells were normalized with corresponding actin mRNA levels. Finally, the normalized level of caspase 3 mRNA in control siRNA-transfected cells (CT) was considered 100% to compare its level in caspase 3 siRNA-transfected cells (Cas3). Error bars represent the mean ± S.E. of three independent experiments. The asterisks represent p values mentioned in the text calculated by ANOVA (B and D) or unpaired t test (E), and indicate the significant differences in means. MW, molecular weight.
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tion in the HDAC4 mRNA level in infected cells (Fig. 6A). Similarly, when normalized to the 18S RNA level and compared with uninfected cells, there was a significant 92% ($p = 0.0001$) decrease in the HDAC4 mRNA level in infected cells (Fig. 6A). However, in the infected cells that were transfected with PA siRNA, such reduction in the HDAC4 mRNA level was only 41.6 ($p = 0.0005$) and 30.6% ($p = 0.004$) when normalized to actin mRNA and 18S RNA levels, respectively (Fig. 6A). In other words, the level of HDAC4 mRNA in infected cells recovered from 8.7 and 7.2% to a significant 58.3 ($p < 0.0001$) and 69.4% ($p < 0.0001$) when normalized to actin mRNA and 18S RNA levels, respectively, after the knockdown of PA mRNA expres-
PA-X mRNA copy numbers in PA-X plasmid-transfected cells. Parallel, the number of PA-X cDNA copies in 10-fold serial dilutions were calculated using the known plasmid DNA concentration and plasmid molecular weights. For this, PA-X cDNA from PA-X plasmid-transfected cells and 10-fold serial dilutions of PA-X plasmid was amplified by qPCR. In parallel, the number of PA-X cDNA copies in 10-fold serial dilutions were calculated using the known plasmid DNA concentration and plasmid molecular weight. Then, a standard curve of cycle threshold (CT) value from qPCR versus cDNA copy numbers was generated and was used as a reference to calculate the PA-X mRNA copy numbers in PA-X plasmid-transfected cells. Error bars represent the mean ± S.E. of three independent experiments. The asterisks represent p values mentioned in the text calculated by ANOVA, and indicate the significant differences in means. MW, molecular weight.

HDAC4 is involved in IAV-induced host innate antiviral response

The data presented above demonstrate anti-IAV properties of HDAC4 and, in turn, a strong antagonism of HDAC4, both at mRNA and polypeptide levels, by IAV. Next, we investigated the anti-IAV mechanism of HDAC4. Previously, we have demonstrated that HDAC1, HDAC2, and HDAC11 exert their anti-IAV function through their involvement in IAV-induced host innate antiviral response (10–12). Hence, to get insight into the anti-IAV mechanism of HDAC4, we examined the role of HDAC4 in IAV-induced signaling of signal transducer and activator of transcription 1 (STAT1), one of the main inducers of innate antiviral cascade and subsequent expression of interferon-stimulated genes (ISGs) in IAV-infected cells (32–34). We compared the kinetics of STAT1 phosphorylation and subsequently ISG expression in IAV-infected cells with the normal or depleted expression of HDAC4. For this, A549 cells, transfected with the control siRNA or HDAC4 siRNA, were infected with PR8 at a m.o.i. of 1.0. After 0, 6, 12, and 24 h of infection, cells were processed, and the level of phosphorylated STAT1 (pSTAT1) was analyzed and quantified by Western blotting as described above. We found that there was a noticeable decrease in the pSTAT1 level in HDAC4-depleted cells, particularly after 12 and 24 h of infection (Fig. 7A). Specifically, compared with normal cells, the level of pSTAT1 in HDAC4-depleted cells was reduced by a significant 62.6 (p = 0.018) and 66% (p = 0.013) after 12 and 24 h of infection, respectively (Fig. 7B). This indicated a reduced induction of host innate antiviral response against IAV in the absence of HDAC4. To investigate this further, we compared the expression kinetics of ISGs: interferon-induced transmembrane protein 3 (IFITM3), ISG15, and viperin (also known as RSAD2) polypeptides in control and HDAC4-depleted cells in response to IAV infection. These three ISGs have been directly implicated in the inhibition of IAV infection (35–37). To do this, A549 cells were subjected to quantified the HDAC4 mRNA level by qPCR as above. Indeed, like IAV infection, the expression of PA-X alone down-regulated the HDAC4 mRNA level in A549 cells (Fig. 6B). Compared with the cells transfected with empty plasmid, there was a significant 43.8 (p = 0.0001) and 57.6% (p = 0.0001) decrease in the HDAC4 mRNA level in the cells transfected with PA-X plasmid when normalized to actin mRNA and 18S RNA levels, respectively (Fig. 6B).

![Graph A](https://via.placeholder.com/150)

**Figure 6.** IAV protein PA-X down-regulates HDAC4 at mRNA level. A, A549 cells were transfected with the control siRNA or viral PA siRNA and subsequently infected with PR8 at a m.o.i. of 1.0 for 24 h. Then, the levels of HDAC4 mRNA, actin mRNA, and 18S RNA were detected by qPCR. The levels of HDAC4 mRNA in all samples were normalized with corresponding actin mRNA or 18S RNA levels. Finally, the normalized level of HDAC4 mRNA in control siRNA-transfected/uninfected (CT/UNI) cells were considered 100% to compare its levels in the control siRNA-transfected/infected (CT/INF) cells and PA siRNA-transfected/infected (PA/INF) cells. B, A549 cells were transfected with empty plasmid pcDNA3 (PD) or PA-X plasmid (PX) as above, and the levels of HDAC4 mRNA, actin mRNA, and 18S RNA were detected by qPCR. Then, the levels of HDAC4 mRNA in all samples were normalized with corresponding actin mRNA or 18S RNA levels. Finally, the normalized level of HDAC4 mRNA in pcDNA3-transfected cells (PD) were considered 100% to compare its level in the PA-X plasmid-transfected cells (PX). Error bars represent the mean ± S.E. of three independent experiments. The asterisks represent p values mentioned in the text calculated by ANOVA, and indicate the significant differences in means. MW, molecular weight.

![Graph B](https://via.placeholder.com/150)

**Figure 5.** The IAV protein PA-X is involved in down-regulation of HDAC4 polypeptide. A–C, A549 cells were transfected in duplicate with 50 nM control siRNA (CT) or viral NP, NS, PA, or PB1 siRNA for 24 h. Cells were then infected with PR8 at a m.o.i. of 1.0 for further 24 h. A, one set of cells was harvested and the HDAC4, PDI, and NP polypeptides were detected in total cell lysates by Western blotting. Note: the HDAC4 blot was reused to probe for PDI and NP. B, the full-length HDAC4 polypeptide levels in UNI/CT, INF/CT, and INF/PA lanes of panel A were quantified and normalized as described in the legend to Fig. 18. Then, the normalized level of the HDAC4 polypeptide in uninfected/control siRNA-transfected (UNI/CT) cells was considered 100% to compare its levels in infected/control siRNA-transfected (INF/CT) cells and the PA siRNA-transfected/infected (INF/PA) cells. C, the second set of cells was processed to detect the levels of NP, NS, PA, PDI, and actin mRNAs by qPCR. Then, the levels of viral gene mRNAs in control siRNA-transfected and viral gene siRNA-transfected cells were normalized with corresponding actin mRNA levels. Finally, the normalized levels of viral mRNAs in control siRNA-transfected cells were considered 100% to compare their levels in viral gene siRNA-transfected cells. D–F, A549 cells were transfected in duplicate with no DNA (ND), empty plasmid pcDNA3 (PD), or PA-X plasmid (PX) for 48 h. D, one set of cells was harvested and the HDAC4 and PDI polypeptides were detected in total cell lysates by Western blotting. Note: the HDAC4 blot was reused to probe for PDI and E, the full-length HDAC4 polypeptide level in panel D was quantified and normalized as described in the legend to Fig. 18. Then, the normalized level of HDAC4 polypeptide in pcDNA3-transfected cells (PD) was considered 100% to compare its level in the PA-X plasmid-transfected cells (PX). F, the second set of cells was processed to determine the PA-X mRNA copy number by absolute qPCR using the PA-X plasmid as a calibrator. For this, PA-X cDNA from PA-X plasmid-transfected cells and 10-fold serial dilutions of PA-X plasmid was amplified by qPCR. In parallel, the number of PA-X cDNA copies in 10-fold serial dilutions were calculated using the known plasmid DNA concentration and plasmid molecular weight. Then, a standard curve of cycle threshold (CT) value from qPCR versus cDNA copy numbers was generated and was used as a reference to calculate the PA-X mRNA copy numbers in PA-X plasmid-transfected cells. Error bars represent the mean ± S.E. of three independent experiments. The asterisks represent p values mentioned in the text calculated by ANOVA (B and C) or unpaired t test (E), and indicate the significant differences in means. MW, molecular weight.
the same conditions as used to detect the pSTAT1 level above. Under such conditions, the IAV-induced expression of IFITM3, ISG15, and viperin polypeptides in control cells was first detected after 6 h of infection that reached a significant level after 24 h of infection (Fig. 7A). However, in HDAC4-depleted cells, the IAV-induced expression of all three polypeptides was reduced at the corresponding time points (Fig. 7A). Particularly, their level in HDAC4-depleted cells were barely detectable after 12 h of infection. We quantified these levels and found that, after 12 h of infection, there was a significant 52 (p =...
Figure 8. The IAV-induced expression of ISG15 and viperin is augmented in HDAC4-supplemented cells. HEK-293T cells were transfected in duplicate with either empty plasmid pcDNA3 (pc) or HDAC4 (HD4) plasmid for 24 h. A, one set of cells was harvested, total cell lysates were prepared, and the HDAC4 and PDI polypeptides were detected by Western blotting. Note: the HDAC4 blot was reused to probe for PDI. B, the other set of cells was infected with PR8 at a m.o.i. of 1.0 for 6 h. Then, the mRNA levels of the indicated ISGs and actin were detected by qPCR. The levels of each ISG in both samples were normalized with corresponding actin mRNA levels. Finally, the normalized mRNA levels of each ISG in pcDNA3-transfected cells were considered 1-fold to compare their levels in HDAC4-transfected cells. Error bars represent the mean ± S.E. of three biological replicates. The asterisks represent *p values mentioned in the text calculated by ANOVA, and indicate significant differences in means. MW, molecular weight.

0.002), 73 (p = 0.0001), and 59.6% (p = 0.006) reduction in IAV-induced expression of IFITM3 (Fig. 7C), viperin (Fig. 7D), and ISG15 (Fig. 7E) polypeptides, respectively, in HDAC4-depleted cells. Similarly, after 24 h of infection, there was a significant 70 (p = 0.0005) and 79.6% (p = 0.0001) reduction in IAV-induced expression of IFITM3 (Fig. 7C) and viperin (Fig. 7D) polypeptides, respectively, in HDAC4-depleted cells. Intriguingly, after 24 h of infection, the level of ISG15 polypeptide in HDAC4-depleted cells recovered to its level as in control cells (Fig. 7E).

Next, we compared the IAV-induced expression of ISGs in normal and HDAC4-overexpressing cells to conversely examine the involvement of HDAC4 in host innate anti-IAV response. In this experiment, besides IFITM3, ISG15, and viperin, we also examined additional ISGs: cholesterol-25-hydroxylase (CH25H), IFITM1 and -2, myxovirus-resistance protein-1 (MX-1), and tripartite motif protein 22 (TRIM22). These ISGs also have been implicated in IAV infection (26, 27). For this, HEK-293T cells were transfected with either empty plasmid or HDAC4-expressing plasmid. After 24 h, one set of cells was processed to confirm the HDAC4 overexpression by Western blotting (Fig. 8A), whereas the other set of cells was infected with PR8 at a m.o.i. of 1.0. After 6 h of infection, cells were processed, and the levels of mRNA of above ISGs and actin and 18S RNA were detected by qPCR. Consistent with the above findings, when normalized to the actin mRNA level and compared with control cells, there was a significant 8.2- fold (p = 0.0001) and 5.0-fold (p = 0.007) increase in the mRNA levels of ISG15 and viperin, respectively, in HDAC4-overexpressing cells (Fig. 8B). Similar results were obtained when ISG15 and viperin mRNA levels were normalized to the 18S RNA level (data not shown). Intriguingly, the mRNA level of IFITM3 increased only by 1.5-fold (statistically nonsignificant) in HDAC4-overexpressing cells (Fig. 8B). No noticeable change was observed in the mRNA levels of other ISGs (except TRIM22, by 1.5-fold) in HDAC4-overexpressing cells (Fig. 8B).

Discussion

Acetylation is one of the most ubiquitous post-translation modifications of proteins as it is now known to exist in all three domains of life and control diverse cellular processes (38, 39). Unsurprisingly, a clear role of acetylation in the infection of viruses that cause both acute and persistent infections is also emerging (40). Hence, HDACs, the enzymes central to regulating the acetylation are bound to play a crucial role, either pro-viral or antiviral, in the virus infections. So far, we and others have demonstrated that at least one member of each class/subclass of HDAC family possesses an anti-IAV property (9–12, 26). On a quest to investigate the interplay of individual HDAC with IAV, we have demonstrated herein the anti-IAV properties of class Ila HDAC, HDAC4, and a rather remarkably strong antagonism of HDAC4 by the IAV. We were able to determine that, compared with control cells, IAV replicated more efficiently in HDAC4-depleted cells and less efficiently in HDAC4-supplemented cells. We analyzed several parameters of IAV multiplication, viz. extracellular (total and infectious) virion release, intracellular viral mRNA and protein levels, and utilized two different IAV H1N1 strains to show that the IAV growth was 2–3–fold higher in HDAC4-depleted cells when infected at m.o.i. of 1.0. One could argue that although statistically significant, this is a modest increase in virus growth. However, one obvious and simple explanation for this could be the profound down-regulation of HDAC4 expression in infected cells. Before the HDAC4 was able to effectively assert its antiviral effect, its expression in infected control cells came down to a level that was only subtly higher to its level in infected cells depleted with HDAC4 (Fig. 1A, compare INF/CT and INF/H4 lanes). Hence, the resulting difference observed on virus growth could not be as profound. This argument is well-supported by 4.4-fold more virus released from HDAC4-depleted cells when they were infected with a low m.o.i. of 0.1 (Fig. 1H). Further-
Anti-influenza mechanism and antagonism of host HDAC4

more, this argument also finds support from a recent finding where the ectopic expression of HDAC4 protected human cells from vesicular stomatitis virus-induced death, but only when infection was at a lower m.o.i. of 0.1 (41).

IAV antagonized the HDAC4 by down-regulating its expression at both mRNA and polypeptide levels. Although the degradation of HDAC4 in IAV-infected cells is consistent with the degradation of HDAC1 and HDAC2, the recently identified anti-IAV HDACs, in IAV-infected cells (10, 11) their mechanisms of degradation are distinct. Although the HDAC4 is degraded by caspasas as demonstrated herein, HDAC1 and HDAC2 are degraded by proteasome as both polypeptides are known to undergo ubiquitination (10, 11). This indicates that IAV is antagonizing various HDACs based upon the inherent characteristics of their polypeptides. Evidently, an earlier report revealed that HDAC4 is highly unstable in several mammalian cell lines, with its mRNA half-life less than 4 h and polypeptide half-life less than 8 h (42). Furthermore, the same report showed that the HDAC4 polypeptide degradation could be further induced by nonviral induction of apoptosis in those cell lines. Hence, IAV, which is known to induce apoptosis in mammalian cells, was able to create a favorable environment for the degradation of the HDAC4 polypeptide in infected cells. This is evident from the degradation kinetics of HDAC4 polypeptide coinciding with the proteolytic activation of caspase 3, one of the executor proteases of apoptotic pathway, in infected cells. Such HDAC4 degradation and caspase 3 activation, and the infection-mediated cell death (Fig. 3), were significantly reversed by a caspase 3 inhibitor. Furthermore, the HDAC4 degradation was also significantly reversed by RNAi-mediated depletion of caspase 3 in infected cells. However, unlike HDAC4 polypeptide cleavage product “a,” neither the inhibition nor the depletion of caspase 3 made the HDAC4 polypeptide cleavage product “b” completely disappear (Fig. 4, A and C). Instead, after these manipulations, an additional HDAC4 polypeptide fragment (indicated by arrow “c”) lagging fragment b appeared in infected cells (Fig. 4, A and C). This indicated that either different caspasas or other host proteases, independently or in conjunction with caspases, are also causing the HDAC4 polypeptide degradation in IAV-infected cells. Among other host proteases, the obvious candidates are lysosomal proteases, cathepsins, because here a lysosomal inhibitor did rescue, albeit partially, the IAV-induced HDAC4 polypeptide degradation (Fig. 4, A and B). Consistent with this observation, the HDAC4 polypeptide has recently been shown to undergo cleavage by cathepsin H in a heterologous system (43). Interestingly, the nature of the degradation of HDAC4 polypeptide in IAV-infected cells discovered here is very similar to the lysosome-associated caspase-mediated degradation of host cortactin polypeptide in IAV-infected cells reported by us recently (44). Therefore, we believe that the lysosomes are closely associated with the apoptotic pathway in IAV-infected cells. Evidently, lysosomes have been described to be involved in the induction of both intrinsic and extrinsic apoptotic pathways in heterologous systems (45–48). A further detailed investigation is needed to determine the precise role of lysosomal proteases and their linkages with intrinsic or extrinsic apoptotic pathways in the degradation of HDAC4 polypeptide in IAV-infected cells. In addition to polypeptide, the HDAC4 mRNA level was also significantly down-regulated in IAV-infected cells. Using RNAi and overexpression strategies, we identified that IAV is employing its protein PA-X to accomplish this. PA-X is a newly identified IAV protein, and is highly conserved in IAV genome, including PR8 and CA09 strains used here (29, 49). PA-X is a RNA endonuclease and selectively degrade the host mRNAs (30), which results in shutoff of host gene expression and allows IAV to escape host innate antiviral response (29, 30, 49). Potentially, the combined HDAC4 characteristics, anti-IAV activity through involvement in host antiviral response and reduced mRNA half-life, made the HDAC4 mRNA an ideal target for PA-X endonuclease-mediated degradation. The selectivity of PA-targeting siRNA rescuing the HDAC4 expression in infected cells and complementarity of PA-X expression alone reducing the HDAC4 expression in cells confirmed the antagonism of HDAC4 by IAV via PA-X.

Like HDAC1, HDAC2, and HDAC11 that exhibit anti-IAV function, HDAC4 is part of the host innate anti-IAV response. In the absence of HDAC4 expression, the extent of IAV-induced phosphorylation of STAT1 and subsequent signaling cascade was reduced in A549 cells. Consequently, the IAV-induced expression of ISGs, IFITM3, ISG15, and viperin, which have been previously implicated to control IAV infection, was down-regulated in HDAC4-depleted cells. Conversely, the IAV-induced expression of these ISGs was up-regulated in HDAC4-supplemented cells. Nevertheless, such involvement of HDAC4 in the IAV-induced host innate antiviral response as well as in IAV-induced apoptosis in host cells needs to be investigated in more detail. Furthermore, it remains to be determined where and how many times on HDAC4 polypeptide the proteolytic cleavage occurs. One likely cleavage position is the aspartic acid residue at position 289, which has been described previously to be targeted by caspase 3 in heterologous systems (42, 50, 51). The cleavage at this position generates an N-terminal fragment that is quite similar to the size of fragments resolving around the 36-kDa m marker detected here by HDAC4 antibody recognizing an N-terminal epitope (42, 50, 51) (Fig. 3G). It is also critical to understand whether proteolytic cleavage of the HDAC4 polypeptide during IAV infection occurs just to antagonize its anti-IAV function or the HDAC4 cleavage products, particularly the above-mentioned N-terminal fragment, also play some significant roles in IAV replication. It has been reported that this N-terminal fragment of the HDAC4 polypeptide is quite stable and translocate to the nucleus, where it binds to the MEF2 (myocyte enhancer factor) transcription factor and represses its activity (52). Furthermore, this HDAC4 N-terminal fragment also induces the intrinsic apoptotic pathway in mammalian cells under heterologous conditions (50, 51). Evidently, both the repressed host transcription and the apoptosis are favorable environments for IAV to proliferate (53). Further investigations will delineate that IAV manipulates host HDAC4 not just to antagonize its antiviral function but also as a means to promote its own replication. In addition, it will also be useful to investigate the role of HDAC4 in IAV-induced respiratory tract tissue damage during flu.

In summary, we have provided here evidence that a class IIa HDAC (HDAC4) joins the list of HDACs to exhibit anti-IAV
properties. This report also reinforces the notion that the HDACs are a family of anti-IAV factors. Next, the understanding of the intermolecular interactions between various HDACs, their host partners, and viral proteins during IAV infection will help understand the mechanisms of their antiviral function as well as their antagonism. Consequently, the resulting knowledge will help to assess the potential of HDACs as the targets for host-directed anti-influenza virus therapies.

**Experimental procedures**

**Cells, viruses, and plasmids**

A549, MDCK, and HEK293 cells were grown and maintained in complete minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine (Life Technologies) at 37 °C and under 5% CO2 atmosphere. Influenza virus strains A/Puerto Rico/8/1934(H1N1), A/California/07/2009(H1N1), and A/WSN/1933(H1N1) (WSN) were propagated in 10-day-old embryonated chicken eggs, and titrated on MDCK cell monolayers. Influenza A virus PA-X (WSN) plasmid (gifted by Yoshihiro Kawaoka, The University of Tokyo, Japan) and human HDAC4 plasmid (gifted by Eric Verdin, Addgene plasmid number 13821) were amplified in *Escherichia coli* DH5α strain and extracted using a plasmid purification kit (Qiagen).

**Infection**

The virus inoculum was prepared in serum-free MEM and added to the cell monolayers, which had been washed twice with serum-free MEM. For infection of MDCK cells, 1 μg/ml of TPCCK (tosylvan (Sigma-Aldrich), SAHA (Sigma-Aldrich), NH4Cl (Sigma-Aldrich), MG132 (Calbiochem), or caspase 3 inhibitor: Z-DEVD-fmk (Calbiochem). In some experiments, the virus was incubated 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 trichostatin A (Sigma-Aldrich), SAHA (Sigma-Aldrich), NH4Cl (Sigma-Aldrich), MG132 (Calbiochem), or caspase 3 inhibitor: Z-DEVD-fmk (Calbiochem). In some experiments, the virus inoculum was irradiated with a 30 W ultraviolet (UV) bulb for 30 min before adding to the cells and proceeding with the infection.

**Quantitative real-time PCR**

Total RNA from the cells was isolated using a Nucleospin RNA isolation kit (Macherey-Nagel) and the cDNA was synthesized using PrimeScript RT reagent kit (Takara). The predesigned siRNA targeting the human HDAC4 gene (ID: SASI_Hs02_00340778; 5’-CAGCAAGCUUCUGCA-GCA-3’) and caspase 3 gene (ID: SASI_Hs01_00139105; 5’-CAGCUUCAUGAUAAGCA-3’), the siRNAs targeting the individual influenza gene segments (sequence mentioned in Ref. 28), and a nontargeting siRNA (MISSION universal negative control #1) were obtained from Sigma-Aldrich. First, different nanomolar quantities of siRNAs and 2 μl of Lipo-fectamine RNAiMAX reagent (Life Technologies) were diluted separately in OptiMEM I reduced serum medium (Life Technologies). Then both solutions were mixed together, and incubated for 30 min at room temperature for siRNA-RNAiMAX complex to form. This complex was then mixed with the cell suspensions, and cells were seeded in a cell culture plate. The
cells were then incubated at 37 °C under 5% CO₂ atmosphere for 24–72 h before infection or further processing.

Overexpression

Cells were grown to 80–90% confluence in a cell culture plate, and transfected with the plasmids using Lipofectamine 2000 reagent (Life Technologies). First, 1 μg of plasmid DNA and 3 μl of Lipofectamine 2000 were diluted separately in OptiMEM. Then both solutions were mixed together, and incubated for 30 min at room temperature for a DNA-Lipofectamine complex to form. The DNA-Lipofectamine complex was then added to the cells, and cells were incubated at 37 °C for 24–48 h before infection or further processing.

Cell viability assay

The viability of cells was evaluated by MTT assay. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (Sigma-Aldrich) was added to the cells, which were then incubated at 37 °C under 5% CO₂ atmosphere for 1 h. Subsequently, 1 milliliter of dimethyl sulfoxide (DMSO; Calbiochem) was added to the cells and cells were rocked at room temperature for 15 min. Finally, the absorbance was measured at 570 nanometer wavelength using a 680 microplate reader (Bio-Rad).

Virus release assay

The culture medium from infected cells was collected and cleared off of cell debris by centrifugation at 17,000 × g for 1 min. Such cleared medium was then divided into two parts. One part was subjected to the protein precipitation by trichloroacetic acid (TCA) (Calbiochem) and the other part was mixed with 0.3% BSA (Sigma-Aldrich) and titrated by microplaque assay. For protein precipitation, the medium was mixed the ice-cold TCA to a final concentration of 20% and incubated 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 by centrifugation as above. The pellet was then 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% bromphenol blue) and proteins were resolved on SDS-PAGE. The viral HA and NP was then detected by Western blotting. For microplaque assay, the confluent monolayers of MDCK cells were infected with 10-fold serial dilutions of the culture medium as described above. The inoculum was then removed and the cells were fixed with 4% formalin (Sigma-Aldrich) and permeabilized with 0.5% Triton X-100 prepared in 20 mM glycine. Cells were then stained with mouse anti-NP (1:1,000) followed by horseradish peroxidase-conjugated anti-mouse IgG antibody (1:1,000). The plaques were visualized by using TrueBlue substrate (KPL Biosciences).

Statistical analysis

The statistical analyses were performed using Prism 6 or 8 (GraphPad). The p values were calculated using unpaired t tests for pairwise data comparisons and one-way analysis of variance (ANOVA) or two-way ANOVA for multiple data set comparisons. A p value of ≤0.05 was considered significant.

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