MARCH8 inhibits influenza A virus infection by targeting viral M2 protein for ubiquitination-dependent degradation in lysosomes

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The membrane-associated RING-CH (MARCH) proteins are E3 ligases that regulate the stability of various cellular membrane proteins. MARCH8 has been reported to inhibit the infection of HIV-1 and a few other viruses, thus plays an important role in host antiviral defense. However, the antiviral spectrum and the underlying mechanisms of MARCH8 are incompletely defined. Here, we demonstrate that MARCH8 profoundly inhibits influenza A virus (IAV) replication both in vitro and in mice. Mechanistically, MARCH8 suppresses IAV release through redirecting viral M2 protein from the plasma membrane to lysosomes for degradation. Specifically, MARCH8 catalyzes the K63-linked polyubiquitination of M2 at lysine residue 78 (K78). A recombinant A/Puerto Rico/8/34 virus carrying the K78R M2 protein shows greater replication and more severe pathogenicity in cells and mice. More importantly, we found that the M2 protein of the H1N1 IAV has evolved to acquire non-lysine amino acids at positions 78/79 to resist MARCH8-mediated ubiquitination and degradation. Together, our data support the important role of MARCH8 in host anti-IAV intrinsic immune defense by targeting M2, and suggest the inhibitory pressure of MARCH8 on H1N1 IAV transmission in the human population.
he membrane-associated RING-CH (MARCH) family proteins are E3 ligases characterized by a highly conserved N-terminal RING-CH (C4HC3 RING) finger, which control the stability, trafficking, and function of various cellular membrane proteins. MARCH8, one of 11 members of the MARCH family, was first identified as cellular modulators of immune recognition (c-MIR) and constitutively poly-ubiquitates conserved lysine residue in the cytoplasmic tail of the MHC II chain. The ubiquitinated MHC II is recognized by the endosomal sorting complexes required for transport (ESCRT) and delivered to late endosomes and lysosomes for degradation. Non-ubiquitinated MHC II is also endocytosed, but recycles to the plasma membrane. In thymic epithelial cells, MARCH8 restricts cell surface expression of MHC II, thus modulates CD4+ T cell selection.

MARCH8 and exhibits greater virulence in mice. IAV carrying the K78R M2 protein becomes resistant to inhibits IAV replication.

**Results**

MARCH8 downregulates viral M2 protein from the cell surface. Given that MARCH8 has been shown to downregulate the expression of several viral envelope proteins, we asked whether MARCH8 also affects HA incorporation into IAV particles. The efficiency of IAV HA protein incorporation into pseudovirions was evaluated by viral transduction assay and western blot analysis. MARCH8 did not affect viral transduction efficiency of viruses carrying IAV HA/NA, or IAV HA/NA/M2 (Fig. 1a). However, MARCH8 strongly inhibited the infectivity of the reporter viruses that were pseudotyped with VSV glycoprotein, which is consistent with previous report. In agreement with the infectivity data, MARCH8 profoundly reduced the level of VSV-G protein both in whole-cell lysate and in virions, whereas the expression of IAV HA and NA proteins was not affected (Supplementary Fig. 1a and Fig. 1b). Unexpectedly, we observed a marked decrease in the level of IAV M2 protein in cells expressing MARCH8 (Fig. 1b), which was further confirmed when only M2 and MARCH8 were expressed in HEK293T cells (Fig. 1c). This inhibition was lost for the E3 ligase-null mutant of MARCH8 (W114A) (Fig. 1d), suggesting that MARCH8 reduces M2 expression through its E3 ligase function.

We next examined whether MARCH8 also decreased M2 expression during IAV infection. HEK293T cells were transfected with the MARCH8 plasmid DNA before exposure to the infection by influenza A/WSN/33 (H1N1) virus (WSN) at MOI of 2. MARCH8 decreased M2 markedly without affecting viral proteins HA, NA, NP, PB1, and PB2 (Fig. 1e). We further examined cell-surface expression of M2 by immune-staining and flow cytometry. The mean fluorescence intensity (MFI) of M2 in MARCH8-expressing cells was twofold lower than in the control cells, whereas the W114A MARCH8 mutant did not exert a significant effect (Fig. 1f, g). This observation is supported by data of confocal imaging showing that MARCH8 prevented the localization of M2 protein to the cell periphery, but did not affect NP trafficking (Fig. 1h and Supplementary Fig. 1b). We then knocked down endogenous MARCH8 in HEK293T cells with siRNA, and found that MARCH8 deficiency supported a boost in M2 protein following IAV infection, while did not affect HA, NA, NP, PB1, PB2, and M1 accumulation (Fig. 1i). Cell-surface M2 was twofold higher in MARCH8-knockdown cells than that in the control cells, as shown by the results of flow cytometry (Fig. 1j, k and Supplementary Fig. 1c). Collectively, these results demonstrate that MARCH8 downregulates viral M2 protein from the cell surface.

MARCH8 inhibits IAV replication in respiratory epithelial cells. We then investigated whether MARCH8 affects IAV replication in respiratory epithelial cells. A549 cell clone was generated to stably express exogenous MARCH8 (Fig. 2a). The control and MARCH8-overexpressing A549 cells were challenged with IAV. Production of IAV from the infected A549 cells was monitored over different time intervals. The results showed that MARCH8 significantly reduced progeny virus titer at each time point tested with either high MOI = 1.0 or low MOI = 0.002 of IAV (Fig. 2b, c). Next, we used siRNA to deplete the endogenous MARCH8 in A549 cells (Fig. 2d). We found that cells lacking MARCH8 allowed greater IAV replication than control cells (Fig. 2e, f). We also generated knockout cell lines using CRISPR/Cas9, which was verified by western blot and further genotyped with PCR (Fig. 2g and Supplementary Fig. 2a). IAV replication significantly increased in MARCH8-depleted A549 cells (Fig. 2h, i). The replication of IAV, as determined by flow cytometry (Fig. 2j) and Supplementary Fig. 2b) and immune fluorescence staining of viral NP-positive cells (Fig. 2k), was increased in MARCH8-knockout cells than in control cells. These results suggest that endogenous MARCH8 in respiratory epithelial cells substantially inhibits IAV replication.

MARCH8 inhibits IAV replication in vivo. To investigate the role of MARCH8 in IAV infection in vivo, we used peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs) to deplete MARCH8 in mouse lungs and then evaluated the effect on IAV infection and viral pathogenesis. PBs, the control (PPMO-NC), or MARCH8-targeting PPMOs (PPMO-M8) were administered intranasally in mice for 2 days, before mice were either sacrificed as the baseline or challenged with 200 PFU of PR8 virus on day 0 (Fig. 3a). Depletion of MARCH8 expression in mouse lungs was observed on day 0 (before infection)
Following infection, the PPMO-M8 treated mice lost more weight compared with the PBS or PPMO-NC treated mice (Fig. 3b). Concurrently, much greater titers of IAV were detected in the lungs of MARCH8-knockdown mice on day 3 and day 6 post infection, compared to PBS or PPMO-NC treated mice (Fig. 3c). Histological analysis of lung slices showed PPMO treatment itself did not induce any marked damage to the lung epithelium prior to infection (Fig. 3d, day 0). PPMO-M8 treated mice showed obvious inflammatory cell infiltration at day 3 after IAV infection and showed bronchiolitis reducing the alveolar airspace, and leukocyte infiltration at day 5 after infection. In contrast, PBS or PPMO-NC treated mice showed mild pathogenic changes at day 3 after IAV infection and the extent of bronchiolitis was markedly reduced compared with PPMO-M8 treated mice at day 5 after infection (Fig. 3d, day 3, and day 5). Overall, these results indicate an important role of MARCH8 in protecting mice from IAV infection and in mitigating IAV pathogenicity in mice.

MARCH8 restricts IAV release. M2 is an ion channel protein, and promotes IAV replication by modulating cellular homeostasis32. To
Fig. 2 MARCH8 inhibits IAV replication in vitro. a A549—Vector and A549—MARCH8 cells were constructed and MARCH8 expression determined by western blotting. b, c A549—Vector and A549—MARCH8 cells were infected with WSN virus for indicated times. Viral titers of supernatants were quantified by plaque assay. d A549 cells were transfected with siRNA targeting MARCH8. MARCH8 expression was examined by western blotting. e, f A549 cells transfected with siNC or siMARCH8 were infected with WSN virus. Viral titers of supernatants were quantified by plaque assay. g The endogenous MARCH8 in A549 cells was knocked out through the use of lentiviral CRISPR-Cas9. MARCH8 expression was determined by western blotting. h, i Control (Ctrl) or MARCH8-knockout (KO MARCH8) A549 cells were infected with WSN virus. Supernatants were harvested and viral titers were detected at indicated times. b, e, h MOI = 1. c, f, i MOI = 0.002. j, k Ctrl and KO MARCH8 A549 cells were infected with WSN virus. At 24 h post infection, viral NP protein expression was measured by flow cytometry (j) and confocal microscopy (k). Scale bar, 200 μm. Data represent averages of independent biological replicates and are presented as means ± SD (b, e, and h, n = 4, c, f, i, and j, n = 3). **P < 0.001, n.s., not significant, unpaired two-tailed Student t-test, without any adjustments for multiple comparisons.

dissect the role of MARCH8 in viral life cycle, we first examined whether MARCH8 affects virus binding or entry. Cells were incubated with WSN virus for 1 h at 4 °C to allow virus binding, followed by incubation with pre-warmed DMEM at 37 °C to allow virus entry. Quantitative RT-PCR data showed that MARCH8 did not affect virus binding or entry (Fig. 4a, b). We next investigated whether MARCH8 affects IAV RNA transcription and replication using a well-established minireplicon assay. The reporter pPolI-Luc produces a modified influenza virus RNA in which the coding region is replaced with the firefly luciferase gene. Thus, the firefly luciferase activity reports the overall transcription and replication activities of the viral RNA polymerase complex. We observed similar RdRp activity in MARCH8-knockdown or -overexpressing cells compared to the control cells (Fig. 4c, d). Taken together, these data suggest that MARCH8 does not affect the early stages of the viral replication including virus binding, virus entry, and viral RNA transcription, replication, and translation.

In IAV infection, an important function of M2 protein is to facilitate viral membrane scission, thus allowing the formation and release of progeny IAV virions. We thus examined the budding of influenza viruses in HEK293T cells by transmission electron microscopy. In control cells, the budding of IAV virions could be observed at the cell surface. The MARCH8 overexpressing cells presented many IAV virions in the process of budding but impaired in membrane scission and virus particle release (Fig. 4e). Virions on the surface of MARCH8-overexpressing cells remain attached to the plasma membrane as opposed to the completely released IAV particles in the control cells, which suggests a virus...
Results of immunofluorescence microscopy revealed that CQ treatment led to cytoplasmic sequestration of M2 and MARCH8 expression in MARCH8-expressing cells, whereas did not observe any increase in M2 expression via the lysosomal pathway. We thus used the lysosomal markers EEA1, lyso-tracker, and lysosome marker LAMP1, we were able to show that MARCH8 redistributed M2 from the plasma membrane to endosomes (Fig. 5d) and lysosomes (Fig. 5e, f). We also assessed the effect of MARCH8 overexpression on the intracellular distribution of M2 along with well-characterized markers of intracellular compartments, including Rab5-EGFP for early endosomes, Rab7-EGFP for late endosomes/MVB, in HeLa cells. In agreement with previous studies, co-localization of M2 with Rab5-EGFP or Rab7-EGFP was detected in MARCH8-expressing cells (Supplementary Fig. 4b, c). These data suggest that MARCH8 targets M2 to lysosomes for degradation.

To test whether MARCH8 causes M2 degradation by ubiquitinating M2, we first confirmed that MARCH8 associated with M2 by performing transient transfection and co-immunoprecipitation experiments (Fig. 5g). We next examined potential ubiquitination of M2 by MARCH8 in cells which were transiently transfected with M2 plasmid DNA or infected with IAV. We used CQ to slow down M2 degradation so that sufficient amount of potentially ubiquitinated M2 can be recovered for immunoprecipitation and western blot analysis. The results showed a significant increase in M2 ubiquitination when MARCH8 was overexpressed, while the W114A mutant did not affect the level of M2 ubiquitination (Fig. 5h, i). We next knocked down (KD) MARCH8 in HEK293T cells and measured the level of M2 ubiquitination. Remarkably, less ubiquitination of M2 was observed in MARCH8 KD cells (Supplementary Fig. 4d, e). Overall, these results demonstrate that MARCH8 ubiquitinates M2, which leads to M2 degradation.
MARCH8 mediates K63-linked polyubiquitination of M2 at position K78. The cytoplasmic domain of M2 contains four conserved lysine residues, K49, K56, K60, and K78, which are potential ubiquitination sites. We therefore substitute each of these four lysines for arginine (Fig. 6a), and examined the expression of these mutated M2 in MARCH8-expressing cells. MARCH8 decreased the expression of the K49R, K56R, K60R mutants to the same extent as that of the WT M2, but did not significantly affect the expression of the K78R mutant and the K/R mutants which contains only one lysine available for polyubiquitination. The results of co-immunoprecipitation and western blot showed that only K63-ubiquitin promoted polyubiquitination of M2 as efficiently as the wild-type ubiquitin (Fig. 6f).

The K78R mutant is more virulent than the wild-type IAV while infecting mice. To demonstrate how mutation of K78 in M2 affects IAV replication, we generated recombinant WSN and PR8 that bear the K78R M2 mutant and infected the control or MARCH8-expressing A549 cells with the wild-type or the K78R viruses. Indeed, the K78R viruses showed complete resistance to MARCH8 inhibition (Supplementary Fig. 6a). And levels of the K78R M2 protein were not decreased by MARCH8 (Supplementary Fig. 6b). These results suggest that the K78 amino acid in M2 renders IAV sensitivity to MARCH8 inhibition.

We next examined the effect of the K78R mutation in M2 on the virulence of IAV in the mouse model (Fig. 7a). Different doses of IAV, from 20 to 1600 PFU, were used to challenge mice. The median lethal dose (MLD50) was fourfold lower for K78R virus which contains only one lysine available for polyubiquitination. The results of co-immunoprecipitation and western blot showed that only K63-ubiquitin promoted polyubiquitination of M2 as efficiently as the wild-type ubiquitin (Fig. 6f).
(47.7 PFU) than for WT virus (204.1 PFU) (Supplementary Table 1), indicating a marked increase of virulence due to the K78R mutation in M2. At the dose of 200 PFU, K78R IAV-infected mice lost weight more quickly than the wild-type IAV (Fig. 7b), and all of the K78R IAV-infected mice had to be euthanized by day 8, whereas only 40% of the WT virus-infected mice succumbed (Fig. 7c). Associated with the high pathogenicity, much higher titers of K78R IAV were detected in the lungs of the infected mice compared to those of the wild-type IAV (Fig. 7d). Histopathological assessment of lungs from the infected mice showed that K78R IAV caused more serious bronchiolitis and bronchitis and lumen debris accumulation by 3 days post infection. By day 6, mice infected with K78R virus all
progressed to bronchopneumonia, as opposed to mice infected with WT virus which showed moderate lung inflammation, and only 3 out of 5 mice displayed evidence of bronchopneumonia (Fig. 7e). These results suggest that K78 in M2 is a determinant of IAV virulence.

**Fig. 6 MARCH8 mediates K63-linked polyubiquitination of M2 at position K78.** a) Illustration of IAV M2 domain organization and the cytoplasmic tail amino-acid sequence of WSN virus M2 and the indicated mutants. Lysine residues (K) are marked as red, and the K > R mutants are marked as blue. b, c) HEK293T cells were transfected with M2 mutants and vector or MARCH8. Surface M2 was determined by flow cytometry (b). Quantitation of M2 positive cells was shown in (c). Data shown are the means ± SD (n = 3 biological replicates). **P < 0.001; n.s., nonsignificant, unpaired two-tailed Student t-test, without any adjustments for multiple comparisons. d) Ubiquitination of M2 mutants by MARCH8. HEK293T cells were transfected with M2 mutants and vector or MARCH8. Cell lysates were subject to IP with anti-M2 antibody, and the IP and input were analyzed by western blotting with antibodies against the indicated targets. e) HEK293T cells were transfected with M2 and vector or MARCH8. Cell lysates were subject to IP with anti-M2 antibody, and the IP and input were analyzed by western blot with antibodies against the indicated targets.

**Strain-specific degradation of M2 by MARCH8.** When we examined the sequences of M2 cytoplasmic domain from four IAV strains, laboratory-adapted IAV strains WSN and PR8, a 2009 pandemic H1N1 IAV strain (pdm09), and a seasonal H3N2 IAV strain (Supplementary Fig. 7a), we noticed that residue K78
is conserved in M2 proteins of WSN, PR8, and H3N2, but not in the pdm09 in which K78 has been changed to Glutamine. Since the M2 protein of pdm09 cannot be detected with M2 monoclonal antibody (C7), we attached the flag tag to the C-terminus of M2 to facilitate detection by western blot (Fig. 8a). When the M2 DNA from each of the above IAV strains was transfected into HEK293T cells together with the MARCH8 DNA, the M2 proteins of WSN, PR8, and H3N2 were ubiquitinated and degraded by MARCH8, while M2 of the pdm09 was resistant to MARCH8 (Fig. 8b, c). We next examined the effect of MARCH8 on the replication of different IAV strains. Replication of the seasonal H3N2 and the two laboratory-adapted IAV strains was markedly inhibited by MARCH8, while the pdm09 strain was not affected (Fig. 8d). These data suggest that different IAV strains have different sensitivity to MARCH8 inhibition.

Since the 1918 H1N1 flu pandemic, the causative H1N1 IAV drifted and reassorted with influenza viruses of other species over time, and has caused several flu pandemics and epidemics in history. We aligned M2 sequences of the IAV epidemics from 1918 until the most recent 2019. Clearly, from 1918 to 1957, human H1N1 IAV strains tended to have K78 in M2, although IAV strains containing non-lysine residues at position 78 slowly emerged over time. This evolving trend became clear in 1979 and 1980 when position 78 in M2 was completely occupied by non-lysine amino acids. While MARCH8 target sites (K78) evolved in M2 DNA from each of the above IAV strains was transfected into cultured cells and in vivo studies suggest that the amphipathic helix region (residues 45–62) of M2 plays pivotal roles in this function. A member of the Ca$^{2+}$-dependent membrane-binding proteins, annexin A6, has been shown to interact with the M2 C-terminal region and interfere with the M2-mediated IAV budding, indicating the presence of cellular mechanisms antagonizing M2 protein. Our study further shows that IAV M2 protein is a substrate of the MARCH8 E3 ligase, and the polyubiquitinated M2 is targeted to lysosome for degradation. Through this mechanism, MARCH8 profoundly inhibits IAV infection both in cultured cells and in mice. MARCH8 has been reported to inhibit VSV, HIV-1, and other retroviruses by ubiquitinating viral envelope proteins and targeting these key viral proteins for degradation. Interestingly, we did not observe any marked effect of MARCH8 on the levels of IAV HA and NA proteins in both co-transfection and viral infection.

**Discussion**

In contrast to many other enveloped viruses, the viral membrane scission and virion budding of IAV are independent of cellular ESCRT, rather are mediated by viral protein M2. Both in vivo and in vitro studies suggest that the amphipathic helix region (residues 45–62) of M2 plays pivotal roles in this function. A member of the Ca$^{2+}$-dependent membrane-binding proteins, annexin A6, has been shown to interact with the M2 C-terminal region and interfere with the M2-mediated IAV budding, indicating the presence of cellular mechanisms antagonizing M2 protein. Our study further shows that IAV M2 protein is a substrate of the MARCH8 E3 ligase, and the polyubiquitinated M2 is targeted to lysosome for degradation. Through this mechanism, MARCH8 profoundly inhibits IAV infection both in cultured cells and in mice.

MARCH8 has been reported to inhibit VSV, HIV-1, and other retroviruses by ubiquitinating viral envelope proteins and targeting these key viral proteins for degradation. Interestingly, we did not observe any marked effect of MARCH8 on the levels of IAV HA and NA proteins in both co-transfection and viral infection.
experiments. Rather, MARCH8 decreased the expression of viral M2 protein. HA, NA, and M2 are all membrane-bound viral proteins, incorporated into IAV particles, perform different yet essential functions for IAV infection. While HA and NA proteins associate with lipid raft microdomains and initiate viral budding, M2 protein localizes at the neck of the incompletely formed virus particle and mediates membrane scission28,36. During IAV infection, M2 has been shown to localize in Golgi during early infection, then be incorporated into the apical vesicles and travels to the plasma membrane31,39. Our results suggest that in addition to viral envelope glycoproteins, MARCH8 is also able to target other viral membrane-bound proteins for ubiquitination and degradation, and as a result, restrict viral infection.

M2 protein plays several key roles in IAV replication. First, it is located at the assembly site of IAV and facilitates the scission of viral membrane and subsequent release of the newly formed IAV particles. Indeed, we observed drastic reduction of IAV production in MARCH8-expressing cells, and a marked increase in IAV production when MARCH8 was depleted with siRNA or CRISPR/Cas9. Second, M2 protein can form an ion channel in viral membrane and primes the infectious entry of viral RNA/nucleocapsid complex into the cytoplasm. In addition, when present in cellular membranes, M2 interferes with the pH levels in early endosomes and trans-Golgi network of the secretory pathways40, thus affecting apical membrane protein trafficking33, which is believed to be one of the mechanisms leading to rhinorhea and lung edema, causing exacerbation of respiratory pathology41. By restricting the levels of M2 protein in IAV producing cells, MARCH8 may help to mitigate and prevent the cellular pathology associated with M2. Therefore, MARCH8 protects mice from IAV infection not only through direct reduction of IAV replication but also through mitigation of M2-caused damage to cells and tissues.

Each E3 ubiquitin ligase has its specific group of substrate proteins, which often underlies the function of the E3 ligase in cells including antiviral defense. Our study revealed IAV protein M2 as a viral protein substrate of MARCH8, thus rendering MARCH8 the role of restricting IAV infection and pathogenesis. We also determined the K78, not the other lysine residues in M2, as the main acceptor of polyubiquitin catalyzed by MARCH8. We also determined the K78, not the other lysine residues in the M2-K78R mutation effect on IAV multiple cycle replication still needs further study.

In our study, the M2-K78R mutation in the PR8 background promotes viral replication in cultured cells and in mice. We further showed that the M2-K78R mutant virus increases M2 accumulation at the cell surface, which was believed to enhance IAV replication based on the previous study reporting that M2 interacts with autophagy factor LC3 at cell surface and augments IAV budding and release44,45. We noted that our results are not in agreement with the deleterious effect of M2-K78R mutation on IAV (WSN strain) replication reported by another group46. How M2-K78R mutation effect on IAV multiple cycle replication still needs further study.
A number of innate restriction mechanisms have been discovered that protect mice from lethal IAV infection. One of the first findings in this regard is the interferon-induced myxovirus resistance (Mx) genes that are often defective in mice which are commonly used in laboratories and succumb to IAV infection. In addition to Mx proteins that target IAV nucleocapsid and inhibit viral RNA transcription, IFITM proteins provide another layer of defense against IAV infection by deterring viral entry. MARCH genes are not induced by interferon, whereas Mx and IFITM genes are interferon-stimulated genes. Results of our study demonstrated that deletion of MARCH8 renders mice to succumb to otherwise non-lethal dose of IAV infection, thus uncovering a distinct key intrinsin immune mechanism protecting mice from lethal IAV infection.

In summary, our data support the potent restriction of IAV infection by MARCH8 both in tissue culture and in the mouse model. In contrast to other known anti-IAV innate immune factors, MARCH8 acts by targeting IAV M2 protein for ubiquitination-mediated degradation in lysosomes, thus profoundly inhibiting IAV release and production. The inhibitory pressure of MARCH8 on IAV is further supported by evasion of IAV from MARCH8 over the prolonged period of circulation in humans.

**Methods**

**Plasmids and reagents.** Human MARCH8 expression plasmids pQCXIP-MARCH8 and its RING-CF domain mutant pQCXIP-MARCH8-W114A were constructed by inserting the MARCH8 cDNA into the Not I/ BamHI Site I in the pQCXIP retroviral vector (Clontech). The pcDNA3.1 vectors encoding HA, NA, M, and M2 of A/WSN/33(H1N1) were generated as previously described. MARCH8 genes are not induced by interferon, whereas Mx and IFITM genes are interferon-stimulated genes. pcDNA3.1 vectors encoding HA, NA, M, and M2 of A/WSN/33(H1N1) were generated as previously described. MARCH8 genes are not induced by interferon, whereas Mx and IFITM genes are interferon-stimulated genes. Results of our study demonstrated that deletion of MARCH8 renders mice to succumb to otherwise non-lethal dose of IAV infection, thus uncovering a distinct key intrinsin immune mechanism protecting mice from lethal IAV infection.

Real-time quantitative PCR (qPCR) analysis. Total cellular RNA was extracted using TRIzol PreLink RNA Extraction Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. cDNA was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa). A VP RNA specific primer was used for reverse transcription of VP RNA of NP. An oligo(dT)20 primer was used for reverse transcription of cellular mRNA. β-Actin mRNA was used as an internal control. The sequences of primers used in qPCR reactions were provided in Supplementary Table 1. Real-time qPCR assays were further analyzed by Bio-Rad CFX Manager.

Electron microscopy. For thin-section electron microscopy, WSN IAV-infected HEK293T cells were scrapped off the plates. Cells were then centrifuged at 1000 x g for 10 min to form a pellet. Cell pellets were fixed with 2% paraformaldehyde–2.5% glutaraldehyde solution for at least 4 h and then fixed with 1% osmium tetroxide for 1.5 h. Specimens were subsequently dehydrated in gradient ethanol. The cells were further embedded in epoxy resin, polymerized, and cut at 60 °C for 24 h. Eighty-nanometer-thickness sections were obtained from the resin blocks and were placed on copper grids and stained with uranyl acetate and lead citrate. The negative stained ultrathin sections were observed under the transmission electron microscope.

VLP production and purification. VLPs were produced as previously described. Briefly, cells were seeded in 10-cm dishes in complete DMEM for 24 h. Then, cells were co-transfected with indicated viral protein expression plasmids for 48 h. The culture supernatants were collected and passed through a 0.45-μm filter. VLPs were pelleted by ultracentrifugation through a 20% sucrose cushion (200,000 x g for 3 h at 4 °C, Beckman SW41Ti rotor).

NA protein ELISA. The concentration of released NA protein was measured in clarified cell culture supernatants by ELISA (Sino Biological) against standard curves of recombinant PR8 virus HA antigen, according to the manufacturer’s instructions.

Plaque assay. To determine the plaque-forming unit, viral supernatants were collected and plaque titeres were determined by the plaque assay. Briefly, monolayer MDCK cells in 12-well plates were inoculated with serial dilutions (10 times) of viral supernatants in 0.25 ml at room temperature (RT) for 1 h with swirling every 15 min. One milliliter of 1% agarose with 0.25% fetal bovine serum was then added to the cells and left at RT until it set. Then the dishes were turned upside down and incubated at 37 °C. At 72 h post infection, the agarose layer was removed and the plaques were visualized with 0.1% crystal violet solution.

Flow cytometry. Flow cytometry analysis was performed as previously described. In brief, cells were detached and resuspended in complete growth media. Cells were
fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were then incubated for 30 min on ice with an anti-Influenza A M2 antibody (Santa Cruz; Cat# SC-23228; 1:1000), followed by staining for 30 min on ice with a rabbit anti-mouse IgG conjugated with Alexa Fluor 488 (Thermo Fisher; A21446; 1:1000). Cells were analyzed for the expression of surface M2 using BD FACSCanto II Flow Cytometer. The data were collected and analyzed with BD FACSDiva™ Software v8.0 software and Flowjo v10.

### Ethics statement

The animal experiments were performed according to the Chinese Regulations of Laboratory Animals-The Guidelines for the Care of Laboratory Animals (Ministry of Science and Technology of People’s Republic of China) and Laboratory Animal Requirements of Environment and Housing Facilities (GB 14925 ± 2010, National Laboratory Animal Standardization Technical Committee). The license number associated with this research protocol was CAU 20190601, which was approved by the Institute of Animal Use and Care Committee of the Institute of Laboratory Animal Science, Peking Union Medical College.

### Infection of mice

The mouse-adapted strain of influenza A/Puerto Rico/8/1934 (PR8) was propagated in 10-day embryonated eggs and titrated with a plaque assay. C57Bl/6 background male mice aged 6–8 weeks were purchased from Vital River Laboratory Animal Technology (Beijing). Mice were anesthetized by intraperitoneal injection of a mixture of Ketamine and Xylazine (100 and 5 μg per gram of body weight). Mice were infected intranasally with 200 pfu of viruses in 40 μL PBS and monitored daily for weight loss and clinical signs. Mice were euthanized if they had ≥30% loss of initial body weight. To analyze viral release, bronchiolar alveolar lavage fluid (BALF) was collected and processed from infected and uninfected mice by postmortem lavage with 2×300 μL ice-cold PBS as described31. For histopathologic analysis, lungs were removed on day 3 of day 6 post infection and fixed with 4% PFA. Lung samples were embedded in paraffin and cut into 5-μm-thick sections, followed by staining with haematoxylin and eosin (H&E). For the lung histology score, images were evaluated by an investigator in a blinded manner following a standardized score system as previously described31. Lung injury scoring system included five different parameters: neutrophils in alveolar airspace, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris filling the airspace, and alveolar septal thickening.

### MARCH8 knockdown by PPMOs

PPMOS targeting mouse MARCH8 gene (PPMO-M8) was designed as CATTCT- CATAACCCGAT-3′, a nontargeting PPMO (PPMO-NC) sequence (CCTCTTACCTCAGTTACAATTTATA), having little homology to mouse transcripts or in STX6. PPMOs were synthesized by IDT (CCTACTCCAGTGAATTTTATA), having little homology to influenza viral sequences, was used as control. Six to eight weeks C57Bl/6 male mice were inoculated intranasally with 10^6 PFU of PPMOs in 40 μL PBS for 2 days continuously. Lung homogenates were prepared as a FastPrep24 system (MP Biomedicals). After addition of 800 μL of PBS containing 0.3% BSA, lungs were subjected to two rounds of mechanical treatment for 10 s each at 6.5 m/s. Tissue debris was removed by low-speed centrifugation, and virus titers in supernatants were determined by plaque assay.

### Immunofluorescence microscopy

IF was performed with HeLa cells 24 h post transfection with IAV M2 and MARCH8 plasmid DNA. For lysosome labeling, Lyso-Tracker-Red99 (Invitrogen) was used to treat cells 24 h post transfection of M2 DNA and incubated for 30 min prior to IF staining. Early or late endosomes were labeled by anti-EBA1 (BD Biosciences; Cat# 610456; 1:50) or anti-LAMP-1 (BioLegend; Cat# 326802; 1:250) antibody or via transfection of a plasmid encoding Rab5-GFP or Rab7-GFP. Confocal images were collected with Leica TCS SP5 confocal microscope, and analyzed by LAS AF Lite v3.9 and ImageJ v1.44.

### Detection of ubiquitination by IP

Cells transfected with IAV M2 or infected with IAV and control cells were treated with COX (50 μM) for 4 h and lysed in a buffer containing 100 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 5 mM EDTA, 1% NP-40, 0.5% Triton X-100, DU8 inhibitors (100 μM PR619, 5 μM 1.1-phenanthroline, 5 mM NEM), and a protease inhibitor cocktail. Lysates were spun at 12,000 × g for 10 min. 300 μL reaction buffer (100 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 5 mM EDTA) was added to 300 μL lysis buffer. Anti-M2 or IgG antibodies were then added to the clarified supernatants for 2 h followed by A/G Dynabeads and 16 h incubation at 4 °C. Beads were washed with catch and release IP wash buffer (Invitrogen), and eluted in 2xSDS sample buffer. The samples were boiled and separated by SDS-PAGE, with then wet transferred to a 0.45 μm PVDF membrane (Millipore). The western blot images were collected and analyzed with LICOR Odyssey CLx with ImageStudio lite v5.2 software.

### Selection of M2 sequences for alignment

The sequences of M2 proteins from human IAVs circulating during different times were selected from the Influenza Research Database (IRD). The strain selection was reevaluated through envelopes protein translocation or degradation. Virology 518, 293–300 (2018).

### Statistics and reproducibility

For in vitro experiments, phenotypic analyses including immunofluorescence, western blot, and FACS were performed in at least three independent experiments, using biological replicates.

### Data availability

M2 sequences can be downloaded from the Influenza Research Database (https://www.fludb.org/). Source data are provided with this paper.

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Author contributions
Z.Z., S.C., C.L., J.W. and F.G. conceived the project. X.L., F.X., L.R., F.Z., Y.H., L.W., Z.F., S.M. and J.S. performed the experiments. C.W. and Y.W. performed the in vivo experiment. All authors contributed to experimental design and data analysis. X.L., C.L. and F.G. composed the manuscript. All authors reviewed the manuscript and discussed the work.

Competing interests
The authors declare no competing interests.

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