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Enhancer of Zeste Homolog 2 Is a Negative Regulator of Mitochondria-Mediated Innate Immune Responses

Shuai Chen,* Chunjie Sheng,* Dong Liu,* Chen Yao,* Shijuan Gao,* Liping Song,* Wei Jiang,* Jiandong Li,* and Wenlin Huang*†‡

The intracellular RIG-I–like receptors recognize 5’-triphosphate viral genomic RNA and initiate the production of cytokines through mitochondria adaptor VISA. The regulation of this signal pathway is largely unknown. In this study, we report that the histone methyltransferase enhancer of zeste homolog 2 (EZH2) inhibits RIG-I signal pathway in an methyltransferase-independent manner. Knockdown EZH2 expression enhances VISA-induced activation of IFN-β promoter and NF-kB signaling. Cytosolic distributed EZH2 colocalizes with VISA and binds to its caspase recruitment domain (CARD), thus blocking its association with RIG-I. During the infection of influenza A virus (IAV) strain A/WSN/33 (WSN), EZH2 translocates to RIG-I and continuously interferes the interaction between RIG-I and VISA. Both N and C termini of EZH2 interact with VISA and attenuate its downstream signaling. WSN virus infection-induced expression of TNF-α, IFN-β, and IL-8 is inhibited by EZH2 and its catalytic dead form ΔSET. EZH2 overexpression facilitates the replications of IAV strains WSN and A/Puerto Rico/8/34 influenza virus. Knockdown EZH2 expression activates infection-induced IFN-β transcription and inhibits virus replication. We further provided evidence to show that pharmacological disruption of EZH2 expression by its inhibitor 3-deazaneplanocin A activates innate immune responses and attenuates the replication of WSN virus in HeLa, MDCK, and mouse primary bone marrow–derived macrophages, but not in IFN-deficient Vero cells. Collectively, these results revealed that EZH2 binds to VISA and interferes with the interaction between VISA and RIG-I. Targeting EZH2 activates mitochondria-mediated antiviral innate immune responses, and thus represses the replication of IAV in cells.

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Abbreviations used in this article: BMM, bone marrow–derived macrophage; CARD, caspase recruitment domain; CC3, median cytotoxic concentration; DZNep, 3-deazaneplanocin A; EZH2, enhancer of zeste homolog 2; HA, hemagglutinin; H3K27me3, histone H3 K27 trimethylation; IAV, influenza A virus; ID, immunoblot; IRF, IFN regulatory factor; MTase, methyltransferase; PR8, A/Puerto Rico/8/34; PRC2, polycomb repressive complex 2; PR8-GFP, genetically modified A/Puerto Rico/8/34 influenza virus that expresses GFP; qRT-PCR, quantitative real-time PCR; siRNA, small interfering RNA; TM, transmembrane domain; vRNA, viral genomic RNA; WSN, A/WSN/33.

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kines. Disruption of EZH2 expression by its inhibitor 3-deaza-7-azaneplanocin A (DZNep) activates infection-induced IFN-β transcrip-
tion and inhibits the replication of IAV. All these data suggest that EZH2 is a negative regulator of antiviral innate immune responses and a new target for the development of antiviral agents.

Materials and Methods

Plasmins and influenza virus

Plasmins encoding RIG-I, MDA5, VISA, TNFR-associated factor 6, TBK1, and IRF3 were provided by Prof. Xin Ye at Institute of Microbiology, Chinese Academy of Sciences (Beijing, China). The IKKα expression plasmid was shared by Prof. Hongbin Shu at Wuhan University (Wuhan, China). The ZBP1 encoding plasmid was provided by Prof. Victor DeFilippis at Oregon Health and Science University (Beaverton, OR). The expression vectors encoding myc-EZH2 and ΔSET mutant were de-
scribed before (14). The Δ12 and Δ34 mutants of EZH2 were generated by subcloning the corresponding sequences into pcDNA4-myc/his plasmid (Invitrogen). The expression vectors for GST-fused EZH2 fragments (EZ1, EZ2, EZ3, and EZ4) were generated by subcloning cDNA sequences encoding indicated regions of EZH2 (aa 1–173, 174–340, 341–560, 560–751) into the pGEX4T-1 plasmid (GE Health). Hemagglutinin (HA)-tagged VISA (VISA ΔN) was kindly provided by Prof. Zhijian Chen (Northwestern Medical Center, Dallas, TX). In brief, the Δ transmembrane do-
main (∆TM) truncated form of VISA contains aa 1–510; the ΔProATM mutant contains an additional deletion of amino acids from 103–153; the ∆CARD/∆TM mutant contains an additional deletion of amino acids from 1–100 (5). The reverse genetic system of A/WSN/33 (WSN) virus was used into cells using Lipofectamine 2000 reagent according to the manufac-
turer’s instructions (Invitrogen).

Abs and reagents

The mouse anti-Flag (F3165) Ab was purchased from Sigma. The mouse anti-HA (sc-7392), anti-myc (sc-40), anti-NP (sc-101352), anti-MAVS (sc-68881), anti-β-actin (sc-47788) Abs, and rabbit anti-RIG-I (sc-98911) Abs were purchased from Santa Cruz Biotechnology. The mouse anti-
EZH2 (sc#3147) Ab, rabbit anti-MAVS (sc#3993), anti-IRF3 (sc#4302), and anti–phospho-IRF-3 (Ser396, #4947) Abs were purchased from Cell Sig-
naling Technology. Rabbit monoclonal anti-CYC1 Ab was purchased from EPITOMICS (#3895-S). Rabbit anti-myc Ab was obtained from Genscript (A00172). PE-conjugated anti-mCD11b (M1011-91B) and alkalophy-
cocyanin (APC)-conjugated anti-E4/80 (M1001F1-11A) Abs were obtained from Tiantian Sungene Biotech Co. (Tianjin, China). Dual-Luciferase Reporter Assay System and the MTT assay kit were purchased from Promega. The mitochondria isolation kit was purchased from Thermo Scientific. DAPI staining kit (KGA511) was purchased from KeyGEN BioTECH (Nanjing, China). DZNep was purchased from Cayman Chemical (NSC617989; Freiburg, Germany). TNT T7 coupled reticulocyte lysate system was ob-
tained from Promega (L4601).

Cell culture and transfection

HeLa and 293T cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS (HyClone). MDCK and Vero cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (HyClone). Mouse primary bone marrow–derived macrophages (BMMs) were differentiated from freshly isolated bone marrow of BALB/c mice (purchased from Vital River Laboratories, Beijing, China) by culturing in RPMI 1640 medium supplemented with 20% FBS (HyClone), 100 μg/ml streptomycin, 100 μM L-ascorbic acid, 2 mM UltraGlutamine I, 0.1% 2-ME (Life Technologies), and 20 ng/ml recombinant mouse M-CSF (416-ML-010; R&D Systems) for 5–10 d. Transfection of cells was performed by using Lipofectamine 2000 (Invitrogen) or Entranster-H (Engreen, China) according to the manufac-
turer’s instructions. Approximately 75–90% transfection efficiencies were routinely achieved.

RNA interference

The sequences for the EZH2 small interfering RNAs (siRNAs) were designed as follows (14): si-EZH2 5'-GGCGAGGCTCGAAUUGUAATdT3', si-EZH2 5'-GGAGAAGCGAGACAAUGAAAGdT3', si-EZ3 5'-GAAUUGAAAACACCGGAAGAAGdT3', si-EZ4 5'-GAAUUGAAAACACCGGAAGAAGdT3'. The siRNA oligonucleotides were chemically synthesized in RIBOBIO (Guangzhou, China) and transfected into cells using Lipofectamine 2000 reagent according to the manufac-
turer’s instructions (Invitrogen).

Quantitative real-time PCR

Total RNA was isolated from the cells using TRNZol (TIANGEN, Beijing, China). cDNA was synthesized using the reverse transcription kit from TIANGEN. For the detection of rRNA and cRNA, RNAs from WSN vi-

us–infected cells were used to perform reverse transcription using primers specific for the amplification of NA, PB2, NP, M1, and NS genes. Quanti-
tative real-time PCR (qRT-PCR) was conducted using SYBR premix Ex Taq II (Takara, Dalian, China). The cycle conditions included an initial dena-
turation step at 95°C for 30 s followed by 40 cycles of amplification for 5 s at 95°C, 5 s at 60°C, and 30 s at 72°C. A melting curve and standard control were run to evaluate the amplification specificity and efficiency, respectively. The mRNA abundance of housekeeping gene GAPDH or Actb were measured as control. The cycle threshold values were used to calculate the relative mRNA expression levels. The primer sequences used for PCR are listed in Supplemental Table I.

Immunoprecipitation and immunoblot

Cells were harvested and lysed using cell lysis buffer (Cell Signaling Technol-

ogy). Cell lysates were centrifuged and the supernatant was incubated with indicated Abs and protein G beads (Invitrogen) at 4°C overnight. The beads were washed three times with cell lysis buffer, and the precipitated proteins were subjected to immunoblot (IB) analysis. For the IB, protein samples were prepared by lysing cells in radioimmunoprecipitation assay buffer (Cell Signaling Technology). Equal amounts of protein from cell lysates were denatured in sample buffer (Invitrogen), subjected to SDS-
PAGE, and transferred to nitrocellulose membranes (Bio-Rad). The membrane was then blocked with 5% nonfat milk in TBS for 1 h at room temperature and incubated overnight at 4°C with the primary Ab. After washing three times with TBS-T, the membrane was incubated for 2 h at room temperature with a HRP-conjugated secondary Ab (ZSBG-Bio, Beijing, China). Bands were detected using ECL (Applgen, Beijing, China).

GST pull-down assay

GST and GST-fused EZH2 fragments (EZ1, EZ2, EZ3, and EZ4) were purified from the BL21 Star (DE3) Escherichia coli strain (Invitrogen) at 30°C. The GST-fragen and GST-fused EZH2 fragments overnight at 4°C. Protein-bound GST beads were washed four times with lysis buffer and eluted in SDS-PAGE sample buffer. Eluted proteins were analyzed by IB.

Luciferase assay

Cells were transfected with a luciferase reporter and the Renilla luciferase reporter vector PRL-TK (Promega), as well as other plasmids for test. Luciferase activity was measured using the dual-luciferase reporter assay system (Promega) according to the manufacturer’s recommendations. Each set of assays was performed in triplicate.

Immunofluorescence microscopy

For the EZH2 and VISA colocalization analysis, plasmids encoding myc-
EZH2 and Flag-VISA were cotransfected into HeLa cells for 24 h. Mice anti-Flag and rabbit anti-myc Abs were used as primary Abs; FITC-
conjugated anti-mouse IgG Ab and TRITC-conjugated anti-rabbit IgG Ab were used for observation. For the EZH2 and RIG-I colocalization analysis, plasmids encoding myc-EZH2 and Flag–RIG-I were cotransfected into HeLa cells for 24 h. Mice anti-EZH2 and rabbit anti–RIG-I Abs were used as primary Abs; FITC-conjugated anti-mouse IgG Ab and TRITC-
conjugated anti-rabbit IgG Ab were used for observation. For myc–EZ2–Δ34 intracellular localization analyses, HeLa cells were transfected with each plasmid for 24 h. Mouse anti-myc Ab was used as primary Ab, and FITC-conjugated anti-mouse IgG Ab was used for the staining. DAPI was used to stain the nucleus.

Generation of IAV and infection

The generation of IAV particles and infection were performed as previously described (15). In brief, 293T cells were plated in a 100-mm dish, and the 12-plasmid IA V system (2

was transfected into 293T cells using Lipofectamine 2000 (Invitrogen). After 72 h, the influenza virus was harvested and propagated in MDCK cells. In infection assays, the recipient cells were washed with PBS three times and infected with the virus in infection medium (culture medium lacking FCS and containing 2.5 μg/ml trypsin). In each assay, the
viruses were added into the infection medium, which was mixed thoroughly and separated to plates or dishes for equal infection. After incubation for 1 h, the cells were washed three times with PBS and then infection medium was added to the cells. The infected cells were cultured at 35˚C in 5% CO2. The supernatant of the culture was used for IB detecting the NP protein of the virus and for the measure of HA value. The selective index was calculated by dividing the median cytotoxic concentration (CC50) of DZNep on MDCK cells with IC50 of DZNep on the replication of WSN virus in MDCK cells (CC50/IC50).

**Flow cytometry analysis**

For PR8-GFP, 293T cells were infected with the virus in infection medium for 20 h, and the cells were harvested and washed twice using PBS. The GFP+ cells were counted by FACSCalibur (BD, Heidelberg, Germany). The suspended cells were directly introduced to flow cytometer equipped with an argon laser exciting at 488 nm. For each sample, 20,000 events were collected. CellQuest software (BD) was used for the analyses. Determination of GFP+ events was performed by a standard gating technique. In brief, the control sample was displayed as a dot plot of GFP signals. The gate was drawn along a line of maximum detected GFP intensity for control samples. The percentage of positive events was calculated as the events within the gate divided by total number of events, then subtracting percentage of control samples. For the analyses of differentiated BMMs, PE-conjugated anti-mCD11b and allophycocyanin-conjugated anti-F4/80 Abs were used for the staining, and positive cells were counted with methods described earlier using corresponding wavelength.

**Statistics**

Experiments were carried out with three or more replicates. Statistical analyses were performed by two-tailed Student t test. A p value <0.05 was considered statistically significant.

**Results**

**EZH2 is a negative regulator of RIG-I signaling**

We performed a screening assay to identify candidate regulators of RIG-I signaling. We transfected individual full-length cDNA encoding human protein together with a luciferase reporter containing IFN-β promoter into 293T cells, a cell line that does

**FIGURE 1.** EZH2 inhibits RIG-I signaling. (A) A model for the screening of IFN-β regulators. (B) Effects of myc-tagged EZH2 and the ΔSET mutant on the activation of IFN-β promoter induced by individually transfected components of RIG-I signaling pathway. (C) Effects of myc-tagged EZH2 and the ΔSET mutant on the activation of NF-κB reporter induced by the transfection of Flag-tagged RIG-I, MDA5, and VISA. (D) Myc-EZH2 inhibits pIC transfection–stimulated IFN-β transcription in 293T cells. (E) Myc-EZH2 inhibits Flag-MyD88 transfection–induced activation of NF-κB reporter in 293T cells. (F) Myc-EZH2 attenuates TNF-α treatment (30 ng/ml, 4 h)–stimulated activation of NF-κB reporter in 293T cells. (G) Effects of myc-EZH2 on VISA or ZBP1 transfection–induced activation of IFN-β promoter in 293T cells. Error bars indicate SD. **p < 0.01. pIC, Polynosinic-polycytidylic acid.

**FIGURE 2.** Knockdown EZH2 expression activates VISA-induced activation of IFN-β promoter. (A and B) Evaluation of EZH2 knockdown efficiency at mRNA level in 293T cells (A) and protein level in HeLa cells (B) 48 h after the transfection of individual siRNA. (C and D) Effects of EZH2 knockdown on the activity of IFN-β promoter (C) and NF-κB reporter (D) induced by VISA. Error bars indicate SD. *p < 0.05, **p < 0.01.
not express TLRs. The IFN-β promoter was activated by cotransfection of VISA, a key player of RIG-I signal pathway (Fig. 1A). This screen assay identified several new regulators of RIG-I signaling including histone MTase EZH2 (Fig. 1B). Interestingly, transfection of ΔSET, a SET domain truncated form of EZH2 that lacks the MTase activity (16), also inhibited the activation of IFN-β (Fig. 1B). Parallel experiments were performed by transfecting components of RIG-I signal pathway (RIG-I, TBK1, and IRF3) individually into 293T cells to activate IFN-β luciferase reporter with or without cotransfection of EZH2 or ΔSET (Fig. 1B). We found that ectopic expression of these constructs inhibits IFN-β promoter activation induced by RIG-I, but not TBK1 or IRF3 (Fig. 1B). In addition to IFN-β, VISA also activates NF-κB to initiate the transcription of proinflammatory cytokines (4). We transfected EZH2 or ΔSET together with NF-κB luciferase reporter into 293T cells, and the NF-κB reporter was activated by cotransfection of VISA or its upstream molecules RIG-I or MDA5 (Fig. 1C). As predicted, both constructs significantly inhibit NF-κB activation (Fig. 1C). qRT-PCR assays suggested that ectopic-expressed EZH2 significantly repressed polyinosinic-polycytidylic acid transfection–induced IFN-β transcription in 293T cells (Fig. 1D). These results suggest that EZH2 negatively regulates RIG-I signaling upstream of VISA in an MTase-independent manner.

We next evaluated the regulation of EZH2 on innate immunity pathways other than RIG-I signaling. EZH2 overexpression significantly represses MyD88 transfection or TNF-α treatment–induced activation of NF-kB reporter in 293T cells (Fig. 1E, 1F), indicating that EZH2 also acts as a negative regulator of TLR and TNFR signals. It has been reported that DNA sensor ZBP1 is a cytosolic DNA sensor that activates innate immune responses through TBK1 (17). Our results showed that EZH2 does not have obvious effects on ZBP1-stimulated activation of IFN-β promoter (Fig. 1G). As a control, EZH2 overexpression inhibits the activity of VISA (Fig. 1G).

Subsequently, two independent siRNAs against EZH2 (si-EZ3 and si-EZ4) were synthesized as previously described (14). We first evaluated the knockdown efficiency of these siRNAs. Forty-eight hours after transfection, both of these siRNAs significantly decreased the expression of endogenous EZH2 mRNA in 293T cells (Fig. 2A) and EZH2 protein in HeLa cells (Fig. 2B). Then we detected the effects of these siRNAs on VISA-induced activation of IFN-β promoter (Fig. 2C) and NF-κB reporter (Fig. 2D). EZH2

**FIGURE 3.** EZH2 attenuates IAV infection–induced cytokine expression. (A) Control vector or myc-tagged EZH2 or ΔSET plasmids were transfected into HeLa cells for 24 h, and the cells were infected with WSN virus. The expressions of IFN-β, TNF-α, and IL-8 were evaluated by qRT-PCR. (B) IB detecting the expression of myc-EZH2, myc-ΔSET, and NP protein using parallel samples of (A). (L), Light exposure; (S), strong exposure. (C and D) HeLa cells were infected with WSN virus for 18 and 36 h. The expressions of EZH2 and IL-8 (C), as well as vRNAs (D), were evaluated by qRT-PCR. (E) IB detecting the expression of EZH2 during WSN virus infection in HeLa (left panel) and mouse primary BMMs (right panel). (F–I) EZH2 knockdown increases WSN virus infection–induced cytokine expression. Control siRNA or siRNAs targeting EZH2 (si-EZ3 and si-EZ4) were transfected into 293T cells for 48 h followed by the infection of WSN virus for an additional 16 h. The expressions of IFN-β (F), TNF-α (G), EZH2 (H), and vRNA (I) were evaluated by qRT-PCR. Error bars indicate SD. *p < 0.05, **p < 0.01.
knockdown increased the activity of VISA in both assays, and the expression of VISA was not interfered with (Fig. 2C, 2D).

**EZH2 inhibits IAV infection–induced cytokine expression**

The innate immune responses stimulated by intracellular vRNA triggers the expression of a series of cytokines. The negative regulation of RIG-I signal pathway by EZH2 suggests it may repress IAV infection–induced cytokine expression. We overexpressed EZH2 and ΔSET in HeLa cells and infected the cells with reverse genetically produced WSN virus. The expression of IFN-β, TNF-α, and IL-8 were evaluated by qRT-PCR (Fig. 3A). Viral protein NP and ectopic-expressed EZH2 and ΔSET were detected by IB (Fig. 3B). WSN virus infection stimulated the transcription of these cytokines, which were significantly repressed by ectopic-expressed EZH2 and ΔSET (Fig. 3C). WSN virus infection does not affect the expression of EZH2 at mRNA level (Fig. 3C) or at protein level (Fig. 3E, left panel) in HeLa cells. WSN virus infection also has no effects on the abundance of EZH2 in mouse primary BMMs (Fig. 3E, right panel). As a positive control, WSN virus infection significantly induced the transcription of IL-8 (Fig. 3C), and the infection was confirmed by detecting the expression of vRNAs (NA, PB2, NP, M1, and PA; Fig. 3D) or viral proteins (NS1 or NP; Fig. 3E).

We further determined the effects of EZH2 knockdown on infection-induced cytokine expression. Results of qRT-PCR showed that WSN virus infection–induced transcription of IFN-β and TNF-α were significantly increased in response to siRNA-mediated EZH2 knockdown (Fig. 3F, 3G). In these samples, the abundance of EZH2 is significantly reduced upon the transfection of both siRNAs (Fig. 3H), and EZH2 knockdown decreased the level of intracellular vRNAs detected (Fig. 3I), although these cells were equally infected with WSN virus. These experiments indicated that the disruption of EZH2 activates RIG-I signaling and inhibits IAV replication.

**EZH2 interacts with the CARD domain of VISA**

The functional connection between EZH2 and VISA prompted us to detect the physical interaction between these two proteins. Expression vector encoding Flag-VISA was transfected into 293T cells, and the cell lysates were used for coimmunoprecipitation assays with anti-Flag or control mouse IgG (Fig. 4A). IB results indicated that endogenous EZH2 is complex with Flag-VISA (Fig. 4A). Reciprocally, Flag-VISA was identified interacts with EZH2 (Fig. 4B). We also produced Flag-VISA and myc-EZH2 proteins by in vitro translation using reticulocyte lysate system (Fig. 4C). Coimmunoprecipitation assays using anti-Flag Ab or anti-EZH2 Ab were used for immunoprecipitation; anti-EZH2 and anti-VISA Abs were used for IB. (D) Mitochondria of 293T cells (left panel) or mouse primary BMMs (right panel) were isolated, and the fractions of cytoplasm (Cyto) and mitochondria (Mito) were used for IB with anti-EZH2 and anti-CYC1 Abs. (E) The localization of EZH2 on mitochondria during the infection of WSN virus was evaluated by anti-EZH2 Ab. (F) Top panel is schematic diagram of EZH2 and its GST-fused truncations. In bottom panel, GST pull-down assays were carried out using E. coli–expressed GST and GST-EZH2 fragments and lysates of 293T cells transfected with Flag-VISA. Anti-Flag Ab was used for IB. (G) Top panel is schematic diagram of the domain composition of VISA and its three HA-tagged truncations. In bottom panel, 293T cells were transfected with indicated plasmids and the cell lysates were used for coimmunoprecipitation assays with mouse IgG or anti-HA Ab. Anti-myc and anti-HA Abs were used to detect myc-EZH2 and HA-tagged VISA truncations, respectively. (H) EZH2 interacts with MDA5, RIG-I, and VISA. Flag-tagged MDA5, RIG-I, or VISA was cotransfected into 293T cells with myc-EZH2. The cell lysates were carried out for coimmunoprecipitation assays. (I and J) Immunofluorescence microscopy observing the colocalization between myc-EZH2 and Flag-VISA (I) or myc-EZH2 and Flag-RIG-I (J) in HeLa cells (see Materials and Methods for detailed description). Scale bars, 10 μm. CBS, Coomassie blue staining.
control IgG followed by IB with anti-EZH2, and anti-VISA Abs showed a direct interaction between these two proteins (Fig. 4C). The distribution of EZH2 on mitochondria was detected in 293T cells and mouse primary BMMs (Fig. 4D). The amount of mitochondria located EZH2 decreased in a time-dependent manner during the infection of WSN virus (Fig. 4E).

Subsequently, we determined regions in EZH2 interact with VISA. We expressed and purified GST-fused EZH2 fragments (termed EZ1, EZ2, EZ3, and EZ4; encoding regions of EZH2 from aa 1–173, 174–340, 341–559, and 560–751, respectively) from E. coli, and GST pull-down assays were performed by incubating these GST-fused proteins with lysates from Flag-VISA–transfected 293T cells (Fig. 4F, top). IB with anti-Flag Ab showed that all four fragments of EZH2, but not the control protein GST, interact with Flag-VISA (Fig. 4F, bottom). We then thought to identify regions required for EZH2-mediated VISA inhibition. We constructed vectors expressing myc-tagged EZH2 truncations with deletion of deletion 1 and 2 (EZ–Δ12, which does not contain SET domain) or fragments 3 and 4 (EZ–Δ34, which does not contain nuclear localization signal) and expressed them in cells (Supplemental Fig. 1A and 1B). The intracellular localizations of these two proteins were also observed (Supplemental Fig. 1C). Interestingly, both of these two truncations failed to repress the transcription of well-defined EZH2 target DAB2IP (Supplemental Fig. 1D), but significantly inhibit VISA-induced NF-kB activation (Supplemental Fig. 1E). These results indicated that EZH2 inhibits RIG-I signaling independent of its MTase activity.

Next, we determined the region in VISA that recruits EZH2. HA-tagged TM truncated VISA (ΔTM), ΔTM mutant with further deletion of the Proline-rich region (ΔProΔTM), or ΔTM mutant with additional deletion of the CARD domain (ΔCARDΔTM) (5) was cotransfected with myc-EZH2 into 293T cells (Fig. 4G, top). Coimmunoprecipitation assays were performed with anti-HA Ab, and the binding of myc-EZH2 to these VISA mutants were detected with anti-myc Ab. EZH2 binds to ΔTM and ΔProΔTM, but not ΔCARDΔTM (Fig. 4G, bottom), indicating that the CARD domain of VISA is responsible for EZH2 recruitment. Because there are several CARD domain–containing proteins in RIG-I signaling pathway including RIG-I, MDA5, and VISA (2), we compared the interaction between EZH2 and these proteins using coimmunoprecipitation assays. Flag-tagged MDA5, RIG-I, or VISA was cotransfected into 293T cells together with myc-EZH2, and the cell lysates were used for coimmunoprecipitation with anti-Flag Ab or control IgG (Fig. 4H). IB using anti-myc and anti-Flag Abs showed that EZH2 interacts with all three proteins, and stronger binding to MDA5 and VISA was detected (Fig. 4H). Immunofluorescence microscope analyses also identified the colocalization of cytosolic distributed EZH2 with VISA (Fig. 4I) and RIG-I (Fig. 4J) in HeLa cells.

**EZH2 blocks the interaction between VISA and RIG-I**

During the infection of RNA virus, RIG-I is activated upon conformation change and interacts with the CARD domain of VISA to initiate signal transduction (2). The binding of EZH2 to VISA’s CARD domain suggests a possible role of this molecule in the regulation of VISA–RIG-I interaction. Plasmids encoding Flag-VISA and HA–RIG-I were transfected into 293T cells, with or without cotransfection of myc-EZH2 (Fig. 5A). The cell lysates were used for coimmunoprecipitation assays with anti-Flag Ab or control IgG (Fig. 5A). IB with indicated Ab showed that enforced expression of EZH2 significantly reduced the interaction between HA–RIG-I and Flag-VISA (Fig. 5A). Reciprocal coimmunoprecipitation was also performed using anti-HA Ab or control IgG, and similar phenomenon was observed that EZH2 blocks the binding of Flag-VISA to HA–RIG-I (Fig. 5B). We also evaluated the effects of myc-EZH2 on VISA–RIG-I association using in vitro–translated proteins produced by reticulocyte lysate system, and the results confirmed the competition (Fig. 5C).

Then we thought to evaluate the effects of EZH2 on VISA–RIG-I interaction during infection. Plasmids encoding HA–RIG-I and Flag-VISA were transfected into 293T cells, with or without cotransfection of myc-EZH2. Twenty-four hours later, the cells were infected with or without WSN virus for an additional 24 h (Fig. 5D). The cell lysates were used for coimmunoprecipitation assays with anti-Flag Ab or control IgG. IB using anti-HA Ab showed that enforced expression of EZH2 significantly reduced the binding of HA–RIG-I to Flag-VISA in both groups without WSN virus infection (Fig. 5D). Coinciding with results from Fig. 4E that EZH2 dissociated from mitochondria, IB with anti-myc Ab showed that the amount of VISA-bound EZH2 decreased during WSN virus infection (Fig. 5D). We further detected the interaction between myc-EZH2 and Flag–RIG-I (Fig. 5E) using coimmunoprecipitation assays. The results showed that WSN virus infection increased EZH2’s binding to RIG-I (Fig. 5E). We also detected the interaction between EZH2 and MDA5, and results showed that the association...
between these two molecules is not impeded during WSN virus infection (Supplemental Fig. 2).

**EZH2 facilitates the replication of IAV**

The negative regulation of RIG-I signal pathway by EZH2 prompted us to detect its regulation on the replication of IAV. We transfected 293T cells with control vector or EZH2 expression vector for 24 h, and infected the cells with PR8-GFP. Sixteen hours postinfection, we counted the number of GFP+ cells by flow cytometry. In the mock-infected group, the GFP background is very low (0.93%; Fig. 6A). In the control vector transfection and PR8-GFP virus infection group, the GFP+ cells reached 29.33% (Fig. 6B). EZH2 transfection increased the GFP+ cells to 40.62% (Fig. 6C). In another HA assay, we transfected HeLa cells with control vector, wild-type, or ΔSET truncated EZH2 for 24 h and infected the cells with WSN virus. The HA values of the culture supernatants were measured 16 h postinfection. Both EZH2 and the ΔSET mutant increased viral titers (Fig. 6D). In Fig. 3B, we also observed that EZH2 and ΔSET overexpression increased NP levels in HeLa cells after the infection of WSN virus. These results suggested that EZH2 facilitates the replication of IAV in cells independent of its MTase activity.

Subsequently, we evaluated the effects of EZH2 knockdown on the replication of IAV. 293T cells were transfected with control siRNA or siRNAs (si-EZ3 and si-EZ4) targeting EZH2 for 48 h. Then the cells were infected with PR8-GFP virus for another 16 h. The replication of the virus was monitored by flow cytometry. In the control group, 32.38% of the cells were infected by PR8-GFP virus (Fig. 6E). Knockdown of EZH2 by si-EZ3 and si-EZ4 decreased the percentage of GFP+ cells to 14.58 and 15.06%, respectively (Fig. 6F, 6G). Moreover, we also observed that EZH2 knockdown significantly decreased the vRNA abundance (NA, PB2, NP, M1, and PA) of WSN virus in 293T cells (Fig. 3I). Taken together, these results indicated that disrupting the expression of EZH2 inhibits the replication of IAV.

**DZNep activates antiviral innate immunity and represses IAV replication**

Several low m.w. inhibitors of EZH2 have been developed including 3-deazaneplanocin A (DZNep), which promotes EZH2 degradation without affecting its mRNA abundance (18). The negative regulation of IFN-β transcription and positive regulation of IAV replication by EZH2 prompted us to detect the effects of DZNep on antiviral innate immune responses. We first evaluated the effects of DZNep on EZH2 abundance. As shown in Fig. 7A, 1 μM DZNep treatment significantly repressed the expression of EZH2. At the same concentration, DZNep treatment increased VISA-induced activation of IFN-β promoter (Fig. 7B). We then pretreated HeLa cells with 1 μM DZNep for 6 h and infected the cells with WSN virus. Results of qRT-PCR showed that DZNep treatment significantly increased virus infection–induced transcription of IFN-β (Fig. 7C).

Next, we evaluated the effects of DZNep on the replication of WSN virus. We pretreated HeLa cells with 1 μM DZNep for 6 h and infected the cells with WSN virus. The viral titers were monitored by IB detecting the NP protein in the supernatant of cell cultures. Once infected with the WSN virus, the amount of NP increased, which was completely reduced by the treatment of 1 μM DZNep (Fig. 7D). HA values in the supernatant were also measured. The Log2(HA value) increased to 5 upon infection of WSN virus, which was reduced to zero with DZNep treatment (Fig. 7D). In another assay, we pretreated MDCK cells with DZNep at different concentration (0, 1, 10, 100, 1000, or 10000 nM) for 6 h and infected the cells with WSN virus. The viral titers

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**FIGURE 6.** EZH2 facilitates the replication of IAV. (A–C) Control vectors (A, B) or EZH2 expression vectors (C) were transfected into 293T cells for 24 h, and the cells were mock infected (A) or infected with PR8-GFP virus (B, C). Twenty-four hours after the infection, the percentage of GFP+ cells was monitored by flow cytometry. (D) The effects of EZH2 and ΔSET overexpression on the replication of WSN virus in HeLa cells detected by HA assay. (E–G) 293T cells were transfected with si-Control (E), si-EZ3 (F), or si-EZ4 (G) for 48 h, and the cells were further infected with PR8-GFP virus. The GFP+ cells were counted by flow cytometry.
were measured by HA assays 20 h postinfection (Fig. 7E). The results showed that DZNep treatment significantly reduced viral titers, and the IC$_{50}$ is 6.28 nM (Fig. 7E). Next, we compared the effects of DZNep on virus replication in MDCK and IFN-deficient Vero cells. We pretreated the cells with DZNep at different concentrations (0, 5, 50, 500, or 5000 nM) for 6 h and infected the cells with WSN virus, and the viral titers were tested by HA assays 24 h postinfection (Fig. 7F). The results showed that DZNep treatment significantly repressed viral replication in MDCK cells, and the IC$_{50}$ is calculated to 2.5 nM in this assay (Fig. 7F). Interestingly, we found that DZNep has no effect on WSN replication in Vero cells (Fig. 7F). These findings suggest that the antiviral effect of DZNep is mediated by IFN. To monitor the cytotoxicity of DZNep, a different concentration of this compound (0, 1, 10, 100, 1000, or 10,000 nM) was added to the culture medium of MDCK cells for 26 h, and the cytotoxicity was evaluated by MTT assay. The CC$_{50}$ of DZNep on MDCK cells is 10,000 nM, and the selective index is 1000 (Fig. 7G). These results suggested that DZNep is a selective inhibitor of WSN virus, although a minor level of cytotoxicity was observed (Fig. 7G).

Furthermore, we evaluated the effects of DZNep in mouse primary BMMs. Flow cytometry analyses using mCD11b and F4/80 Abs showed that the BMMs were well induced (Fig. 8A). We treated BMMs with or without 5 µM DZNep for 12 h, and subsequently infected the cells with or without WSN virus for 24 h. DZNep treatment significantly reduced EZH2 expression and increased infection-induced IRF3 phosphorylation in BMMs (Fig. 8B). Results from qRT-PCR showed that WSN virus infection significantly induced the expression of cytokines including Ifn-b, Ifn-a4, Tnf-a, and Il-6, which were further stimulated by DZNep (Fig. 8C). The replication of WSN virus in BMMs was also monitored at the same time. DZNep treatment significantly reduced the HA value in the supernatant of culture medium (Fig. 8B, bottom) and the NP level in BMMs (Fig. 8B). qRT-PCR experiment showed that in addition to inducing cytokine expression (Fig. 8C), DZNep also significantly suppresses the amount of vRNA (Fig. 8D) and cRNA (Fig. 8E) in BMMs. These results suggested that DZNep treatment activates antiviral innate immune responses and inhibits virus replication in primary macrophages.

**Discussion**

EZH2 is the core subunit of PRC2 and catalyzes H3K27me3 (7). EZH2 regulates the expression of differentiation-associated genes and is important for physiological processes such as hematopoietic stem cell pluripotency (19) and early B cell development (20). EZH2 and H3K27me3 eraser JMJD3 were also reported to play important roles in the regulation of NF-κB (21–24). Small molecular inhibitors targeting JMJD3 represses LPS-induced expression of proinflammatory cytokines (25). However, the regulation of these H3K27me3 modifiers on RIG-I signal pathway remains a mystery.

In this study, we reported that EZH2 interacts with VISA and inhibits its downstream signaling. Coimmunoprecipitation experiments identified that EZH2 binds to VISA’s N-terminal CARD domain (Fig. 4G), which plays important roles in signal transduction (26–29), including mediating the interaction between RIG-I and VISA (27). We found that EZH2 blocks the association between VISA and RIG-I in conditions with or without WSN virus infection (Fig. 5A–D). We generated two truncated forms of EZH2 (D$_{12}$ and D$_{34}$) and evaluated their effects on VISA-induced signal transduction. EZH2 inhibits innate immune responses and inhibits virus replication in primary macrophages.
EZ-Δ34 does not harbor nuclear localization signal sequence (Supplemental Fig. 1A) and is distributed in cytoplasm where it could not affect chromatin modification (Supplemental Fig. 1C). EZ-Δ12 and EZ-Δ34 do not inhibit the transcription of well-defined EZH2 downstream gene DAB2IP (Supplemental Fig. 1D), but both of them inhibit VISA-induced activation of NF-κB signaling (Supplemental Fig. 1E). In addition to repressing VISA-induced activation of IFN-β promoter (Fig. 1B) and NF-κB reporter (Fig. 1C), ΔSET also attenuates WSN virus infection–induced cytokine expression (Fig. 3A). We concluded that EZH2 negatively regulates mitochondria-mediated innate immunity independent of its MTase activity.

As a histone modifier, it is an interesting phenomenon to find that EZH2 regulates the activity of mitochondrial protein VISA. A previous report showed that a small part of EZH2 does accumulate in the cytoplasm and regulates nonhistone substrates (13). Our data showed that EZH2 can be detected on mitochondria in 293T cells and mouse primary BMMs (Fig. 4D), where its blocks the interaction between RIG-I and VISA (Fig. 5A–D). WSN virus infection decreases the accumulation of EZH2 on mitochondria (Fig. 4E) and promotes the dissociation of EZH2 from VISA (Fig. 5D). WSN virus infection also increased the interaction between EZH2 and RIG-I (Fig. 5E), where EZH2 keeps interfering with the association between RIG-I and VISA in the context of infection (Fig. 5D). The colocalization of cytosolic distributed EZH2 with VISA and RIG-I was observed by immunofluorescence analyses (Fig. 4I, 4J). The mechanism of WSN virus infection–induced translocation of EZH2 from VISA to RIG-I remains to be identified.

Influenza viruses belong to the family of Orthomyxoviridae, which comprises enveloped viruses with segmented RNA genomes of negative polarity (30). Because of mutation and antigenic drift, influenza virus strains resistant to traditional drugs are emerging, and new agents need to be developed (31). DZNep is a member of S-adenosylhomocysteine hydrolase inhibitors. DZNep induces degradation of EZH2 in a proteasome-dependent manner without changing its mRNA abundance (18, 32). It has been reported that DZNep and its derivatives are broad-spectrum antiviral agents (33–36); however, the mechanism is unknown. We found that overexpression of EZH2 attenuates infection-induced transcription of antiviral cytokine IFN-β (Fig. 3A) and facilitates the replication of IAV (Figs. 3B, 6A–D). Conversely, knockdown of EZH2 expression increased infection-induced transcription of IFN-β (Fig. 3F) and inhibits the replication of IAV (Figs. 3L, 6E–6G). These results predicted an IFN-dependent antiviral effect of DZNep. We found that DZNep decreases EZH2 expression (Figs. 7A, 8B) and increases WSN virus infection–induced IFN-β transcription in HeLa cells (Fig. 7C) and mouse primary BMMs (Fig. 8C). DZNep

FIGURE 8. DZNep treatment activates antiviral innate immune responses and inhibits WSN virus replication in mouse primary BMMs. (A) Flow cytometry analyses of induced mouse primary BMMs by immunostaining of macrophage markers CD11b and F4/80. (B) Mouse primary BMMs were treated with 5 μM DZNep for 12 h, and were infected with WSN virus for an additional 24 h. The levels of EZH2, NP, phosphorylated IRF3 (IRF3-p), total IRF3, and β-actin (Actb) were detected by IB using indicated Abs. (C) Parallel samples demonstrated in (B) were used for qRT-PCR detecting the expression of Ifn-β, Ifn-α4, Tnf-α, and Il-6. (D and E) qRT-PCR analyzing the expression of vRNA (D) and cRNA (E) of WSN virus by detecting NA, PB2, NP, M1, and PA in samples described in (B) and (C). *p < 0.05, **p < 0.01.
treatment inhibits the replication of WSN virus replication in HeLa cells (Fig. 7D), MDCK cells (Fig. 7E, 7F), and mouse primary BMIs (Fig. 8D, 8E), but DZNep does not affect virus replication in Vero cells (Fig. 7F). Because Vero is a cell line deficient of IFN, these results provide evidence that DZNep inhibits influenza virus replication in an IFN-dependent manner.

Collectively, the findings in this report provided proof of a principle that EZH2 attenuates mitochondria-mediated innate immune responses by regulating the interaction between RIG-I and VISA. In uninfected cells, the cytosolic localized EZH2 binds to VISA and prevents its interaction with RIG-I and turns off IFN transcription. Once the cells are infected with influenza virus, EZH2 translocates from VISA to RIG-I where it keeps interfering with the interaction between RIG-I and VISA. DZNep treatment promotes the degradation of EZH2 and turns on the signaling cascade to initiate IFN transcription and inhibits the replication of the virus.

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Disclosures
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References

EZH2 INHIBITS INNATE IMMUNITY

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